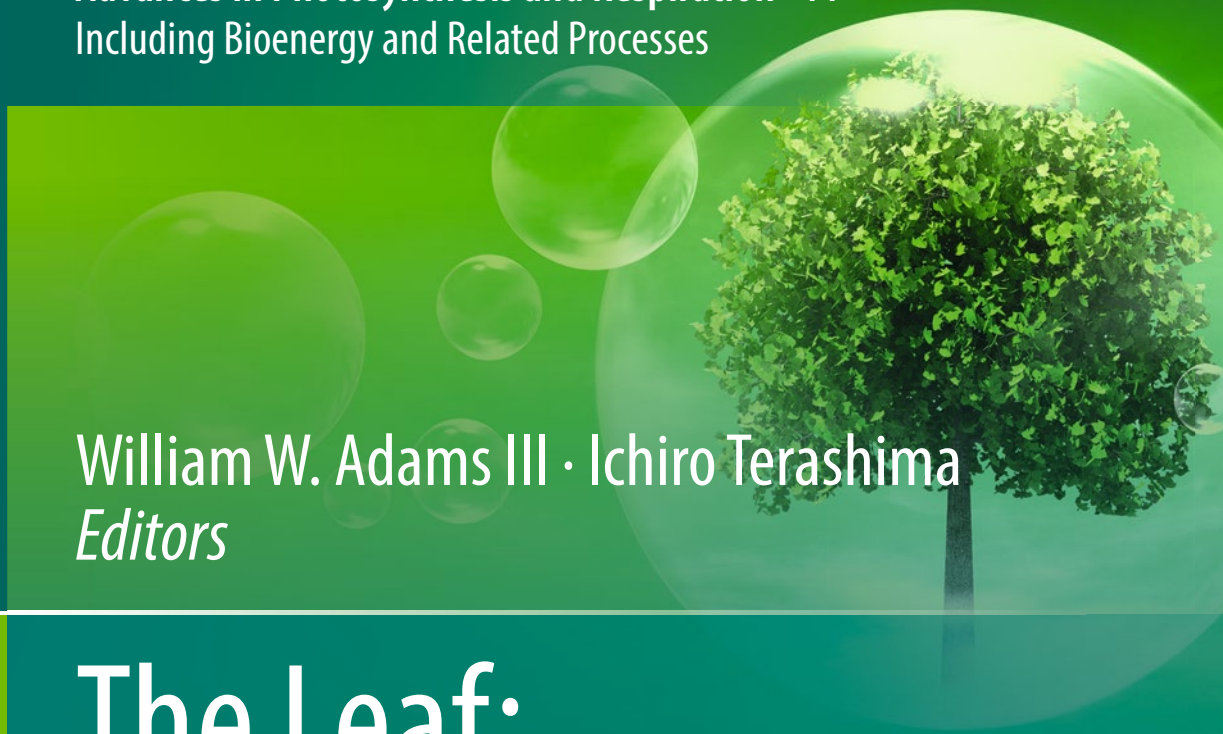
Advances in Photosynthesis and Respiration 44
Including Bioenergy and Related Processes



William W. Adams III · Ichiro Terashima
Editors

The Leaf: A Platform for Performing Photosynthesis

Springer

The Leaf: A Platform for Performing Photosynthesis

Medal (note the leaf scroll at the top) received by W. Adams at the Missouri State Science Fair. The backdrop of leaves includes those from (beginning in the upper left, moving clockwise) Gambel oak (*Quercus gambelii*), yellow twig dogwood (*Cornus sericea*), Virginia creeper (*Parthenocissus quinquefolia*), honey locust (*Gleditsia triacanthos*), Oregon grape holly (*Mahonia aquifolium*), and mullein (*Verbascum thapsus*). (Photograph by Melanie S. Adams)

Advances in Photosynthesis and Respiration
Including Bioenergy and Related Processes

VOLUME 44

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 these areas. Virtually all life on our planet Earth ultimately depends on photosynthetic energy capture and conversion to energy-rich organic molecules that are then used through respiration for fueling metabolism, growth, and reproduction. Photosynthesis is also an energy source for food, fuel, and fiber. Photosynthesis is ultimately the source of almost all Bioenergy on Earth, including fossil fuels. The fuel and energy uses of photosynthesized products and processes have become an important area of study, with competition between food and fuel leading to a 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, respiration, and related energy processes. The purpose of this series is to explore 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. Beginning in 2018, Govindjee has become emeritus, though still very interested in the success of the series. Julian Eaton-Rye has joined as co-series editor, welcome Julian.

More information about this series at http://www.springer.com/series/5599

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From the Series Editors

Advances in Photosynthesis and Respiration Including Bioenergy and Related Processes

Volume 44: The Leaf: A Platform for Performing Photosynthesis

After 43 volumes in this series, our good friend and colleague Govindjee has stepped away from his duties as series editor. This is the first volume since his retirement, but even so, volumes in this series will continue to be shaped by Govindjee's enthusiasm for all things photosynthesis and his care and dedication to this series. Julian J. Eaton-Rye will be co-series editor beginning with this volume. It is hoped that our new team of series editors will continue to identify new topics that would benefit from an edited volume and editors that will take on the task of coordinating a volume that will serve the photosynthesis community.

Our understanding of photosynthesis can be organized in many different ways. The topics of books in this series reflect this variety of approaches. Some books are focused on a type of organism, others on specific processes. In this volume, the focus is on understanding relationships between photosynthesis and leaves, the primary site of photosynthesis in most land plants. The leaf is a particularly convenient level of organization because leaves are critical for light capture, carbon uptake, carbon processing, and carbon export from the photosynthetic bacterial endosymbiont (chloroplast) to other parts of the plant used for food, fuel, and fiber. The understanding of leaf-level photosynthesis is a critical part of models that look to understand regional- and global-scale photosynthesis.

The plant leaf is like a solar panel. The ability to intercept light is dependent on only two dimensions; leaf depth plays little role in light capture. Within this two-dimensional structure, large amounts of water must be distributed throughout, and at the same time, sugars and other end products must be collected from all parts of the leaf and provided to the rest of the plant. The challenges of this plumbing have been under intensive study lately making this an opportune time for a book that tackles this important area of research.

Leaf shape and display are important for how leaves cope with their environment. Internal architecture is critical for maximizing light collection and carbon dioxide uptake. Several chapters in this book describe important leaf architecture considerations and mechanisms by which leaf architecture is determined.

This volume joins volume 42 (*Canopy Photosynthesis: From Basics to Applications*, editors Kouki Hikosaka, Ülo Niinemets, and Niels P.R. Anten) in describing the study of photosynthesis at a particular organizational scale. Together, these books provide a solid foundation for understanding how the physical environment can affect how plants develop and display their resources to maximize photosynthesis.

Authors of Volume 44

We note with great pride that the current volume is truly an international book; it has authors from the following 12 countries: Australia (4), Austria (1), Brazil (1), Canada (1), Chile (1), China (1), Czech Republic (1), France (1), Japan (19), Spain (6), UK (5), and USA (22).

There are 61 authors (including the 3 editors) who are experts in the field of leaf-level photosynthesis. Alphabetically (by last names), they are Anunción **A**badía, Javier Abadía, William W. Adams III, Evgenios Agathokleous, Fransisca C. Anozie, Brian Ayre, Anne M. **B**orland, Federica Brandizzi, Timothy J. Brodribb, Thomas N. Buckley, Marcelo L. **C**ampos, Francisco Javier Cano, Marc Carriquí, Rafael E. Coopman, Asaph B. Cousins, Barbara **D**emmig-Adams, Norikazu **E**guchi, Jaume **F**lexas, Irwin N. Forseth, Jr., Takashi Fujita, Ryo Funada, Brigitte **G**ontero, Kouki **H**ikosaka, Tadashi Hirasawa, Gregg A. Howe, Rongbin Hu, Natalia Hurtado-Castano, Kihachiro **K**ikuzawa, Sang-Jin Kim, Yong-Sig Kim, Mitsutoshi Kitao, Takayoshi Koike, Tracy **L**awson, Martin J. Lechowicz, Alistair Leverett, Stephen C. **M**aberly, Ian T. Major, Yusuke Mizokami, Fermín Morales, Erik T. **N**ilsen, Riichi **O**guchi, Yusuke Onoda, Andrej **P**avlovič, Stephanie K. Polutchko, Luciana **R**enna, Thomas D. **S**harkey, Jared J. Stewart, Mitsutaka **T**aniguchi, Ichiro Terashima, Danny Tholen, Michael F. Thomashow, Hirokazu Tsukaya, Robert Turgeon, Yin **W**ang, Makoto Watanabe, Yoko Watanabe, Sarathi M. Weraduwage, Ian J. Wright, Dongliang **X**iong, Xiaohan **Y**ang, and Yuki Yoshida. We are grateful for their efforts in making this important volume.

Our Books

We list below information on the 43 volumes that have been published thus far (see http://www.springer.com/series/5599 for the series website). Electronic access to individual chapters depends on subscription (ask your librarian), but Springer provides free downloadable front matter as well as indexes for all volumes. The available websites of the books in the series are listed below.

- **Volume 43 (2018)** ***Plant Respiration: Metabolic Fluxes and Carbon Balance***, Edited by Guillaume Tcherkez from Australia and Jaleh Ghashghaie from France. Fourteen chapters, 302 pp, Hardcover ISBN 978-3-319-68701-8, eBook ISBN 978-3-319-68703-2 [http:// http://www.springer.com/us/book/9783319687018]
- **Volume 42 (2016)** ***Canopy Photosynthesis: From Basics to Applications***, Edited by Kouki Hikosaka from Japan, Ülo Niinemets from Estonia, and Neils P.R. Anten from the Netherlands. Fifteen chapters, 423 pp, Hardcover ISBN 978-94-017-7290-7, eBook ISBN 978-94-017-7291-4 [http://www.springer.com/book/9789401772907]
- **Volume 41 (2016)** ***Cytochrome Complexes: Evolution, Structures, Energy Transduction, and Signaling***, Edited by William A. Cramer and Tovio Kallas from the USA. Thirty-five chapters, 734 pp, Hardcover ISBN 978-94-017-7479-6, eBook ISBN 978-94-017-7481-9 [http://www.springer.com/book/9789401774796]
- **Volume 40 (2014)** ***Non-Photochemical Quenching and Energy Dissipation in Plants, Algae and Cyanobacteria***, edited by Barbara Demmig-Adams, Győző Garab, William W. Adams III, and Govindjee from USA and Hungary. Twenty-eight chapters, 649 pp, Hardcover ISBN 978-94-017-9031-4, eBook ISBN 978-94-017-9032-1 [http://www.springer.com/life+sciences/plant+sciences/book/978-94-017-9031-4]
- **Volume 39 (2014)** ***The Structural Basis of Biological Energy Generation***, edited by Martin F. Hohmann-Marriott from Norway. Twenty-four chapters, 483 pp, Hardcover ISBN 978-94-017-8741-3, eBook ISBN 978-94-017-8742-0 [http://www.springer.com/life+sciences/book/978-94-017-8741-3]

- **Volume 38 (2014)** ***Microbial BioEnergy: Hydrogen Production***, edited by Davide Zannoni and Roberto De Phillipis, from Italy. Eighteen chapters, 366 pp, Hardcover ISBN 978-94-017-8553-2, eBook ISBN 978-94-017-8554-9 [http://www.springer.com/life+sciences/plant+sciences/book/978-94-017-8553-2]
- **Volume 37 (2014)** ***Photosynthesis in Bryophytes and Early Land Plants***, edited by David T. Hanson and Steven K. Rice, from USA. Eighteen chapters, approx. 342 pp, Hardcover ISBN 978-94-007-6987-8, eBook ISBN 978-94-007-6988-5 [http://www.springer.com/life+sciences/plant+sciences/book/978-94-007-6987-8]
- **Volume 36 (2013)** ***Plastid Development in Leaves during Growth and Senescence***, edited by Basanti Biswal, Karin Krupinska, and Udaya Biswal, from India and Germany. Twenty-eight chapters, 837 pp, Hardcover ISBN 978-94-007-5723-33, eBook ISBN 978-94-007-5724-0 [http://www.springer.com/life+sciences/plant+sciences/book/978-94-007-5723-3]
- **Volume 35 (2012)** ***Genomics of Chloroplasts and Mitochondria***, edited by Ralph Bock and Volker Knoop, from Germany. Nineteen chapters, 475 pp, Hardcover ISBN 978-94-007-2919-3 eBook ISBN 978-94-007-2920-9 [http://www.springer.com/life+sciences/plant+sciences/book/978-94-007-2919-3]
- **Volume 34 (2012)** ***Photosynthesis: Plastid Biology, Energy Conversion and Carbon Assimilation***, edited by Julian J. Eaton-Rye, Baishnab C. Tripathy, and Thomas D. Sharkey, from New Zealand, India, and USA. Thirty-three chapters, 854 pp, Hardcover, ISBN 978-94-007-1578-3,eBookISBN978-94-007-1579-0 [http://www.springer.com/life+sciences/plant+sciences/book/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, Hardcover ISBN 978-94-007-1532-5, Softcover ISBN 978-94-007-3832-4, eBook ISBN 978-94-007-1533-2 [http://www.springer.com/life+sciences/book/978-94-007-1532-5]
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- **Volume 31 (2010)** ***The Chloroplast: Basics and Applications***, edited by Constantin Rebeiz, Christoph Benning, Hans J. Bohnert, Henry Daniell, J. Kenneth Hoober, Hartmut K. Lichtenthaler, Archie R. Portis, and Baishnab C. Tripathy, from USA, Germany, and India. Twenty-five chapters, 451 pp, Hardcover ISBN 978-90-481-8530-6, Softcover ISBN 978-94-007-3287-2, eBook ISBN 978-90-481-8531-3 [http://www.springer.com/life+sciences/plant+sciences/book/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, Softcover ISBN 978-94-007-3073-1 eBook ISBN 978-90-481-2863-1 [http://www.springer.com/life+sciences/plant+sciences/book/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, Softcover ISBN 978-94-007-1533-2, eBook ISBN 978-1-4020-9237-4 [http://www.springer.com/life+sciences/plant+sciences/book/978-1-4020-9236-7]
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Dahl, Rüdiger Hell, David Knaff, and Thomas Leustek, from Germany and USA. Twenty-four chapters, 551 pp, Hardcover ISBN 978-4020-6862-1, Softcover ISBN 978-90-481-7742-4, eBook ISBN 978-1-4020-6863-8 [http://www.springer.com/life+sciences/plant+sciences/book/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, Softcover ISBN 978-90-481-7820-9, eBook ISBN 978-1-4020-8250-4 [http://www.springer.com/life+sciences/plant+sciences/book/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, Softcover ISBN 978-90-481-7140-8, eBook ISBN 978-1-4020-4516-5 [http://www.springer.com/life+sciences/plant+sciences/book/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, Softcover ISBN 978-90-481-7088-3, eBook ISBN 978-1-4020-4256-0 [http://www.springer.com/life+sciences/plant+sciences/book/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, Softcover ISBN 978-1-4020-6570-5, eBook ISBN 978-1-4020-4061-0 [http://www.springer.com/life+sciences/plant+sciences/book/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, eBook ISBN 978-1-4020-4254-6 [http://www.springer.com/life+sciences/plant+sciences/book/978-1-4020-4249-2]
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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, Softcover ISBN 978-94-010-3772-3, eBook ISBN 978-94-007-1038-2 [http://www.springer.com/life+sciences/plant+sciences/book/978-0-7923-6333-0]
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- **Volume 5 (1997)** ***Photosynthesis and the Environment*****,** edited by Neil R. Baker, from UK. Twenty chapters, 508 pp, Hardcover ISBN 978-07923-4316-5, Softcover ISBN 978-90-481-4768-7, eBook ISBN 978-0-306-48135-2 [http://www.springer.com/life+sciences/plant+sciences/book/978-0-7923-4316-5]
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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):

- *Photosynthesis and Climate Change* (working title) (Editors: Katie M. Becklin, Joy K. Ward, and Danielle A. Way)
- *Cyanobacteria* (Editor: Donald Bryant)
- *Photosynthesis in Algae* (Editors: Anthony Larkum and Arthur Grossman)
- *Our Photosynthetic Planet* (Editors: Mike Behrenfeld, Joe Berry, Lianhong Gu, Nancy Jiang, Anastasia Romanou, and Anthony Walker)
- *Modeling Photosynthesis and Growth* (Editors: Xin-Guang Zhu and Thomas D. Sharkey)

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

Algae and cyanobacteria: biofuel and bioenergy
Artificial photosynthesis
ATP synthase: structure and function
Bacterial respiration II
Evolution of photosynthesis
Green bacteria and heliobacteria
Interactions between photosynthesis and other metabolic processes
Limits of photosynthesis: where do we go from here?
Photosynthesis, biomass, and bioenergy
Photosynthesis under abiotic and biotic stress

If you have any interest in editing/co-editing any of the above listed books, or being an author, please send an e-mail to Tom Sharkey (tsharkey@msu.edu) and/or to Julian Eaton-Rye (julian.eaton-rye@otago.ac.nz). Suggestions for additional topics are

also welcome. Instructions for writing chapters in books in our series are available by sending e-mail requests to one or both of us.

We take this opportunity to thank and congratulate William Adams and Ichiro Terashima for their outstanding editorial work and for their highly professional dealing with the reviewing process; they have indeed done a fantastic job, not only in editing but also in organizing this book for all of us. We thank all 61 authors of this book (see the list given earlier and on the following pages); without their authoritative chapters, there would be no such volume. We give special thanks to Mr. Joseph Daniel and Mrs. Rathika Ramkumar of SPi Global, India, for directing the typesetting of this book; their expertise has been crucial in bringing this book to completion. We owe Jacco Flipsen, Andre Tournois, and Ineke Ravesloot (of Springer) thanks for their friendly working relation with us that led to the production of this book.

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Series Editors

A 2017 informal photograph of Govindjee (right) and his wife Rajni (left) in Champaign-Urbana, Illinois. (Photograph by Dilip Chhajed)

The founding series editor **Govindjee**, who uses one name only, was born on October 24, 1932, in Allahabad, India. 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, botany. and zoology) and M.Sc. (botany, plant physiology) in 1952 and 1954, from the University of Allahabad. He learned his plant physiology from Shri Ranjan, who was a student of Felix Frost Blackmann (of Cambridge, UK). Then, Govindjee studied *photosynthesis* at the UIUC, under two giants in the field, Robert Emerson (a student of Otto Warburg) and Eugene Rabinowitch (who had worked with James Franck), obtaining his Ph.D., in biophysics, in 1960.

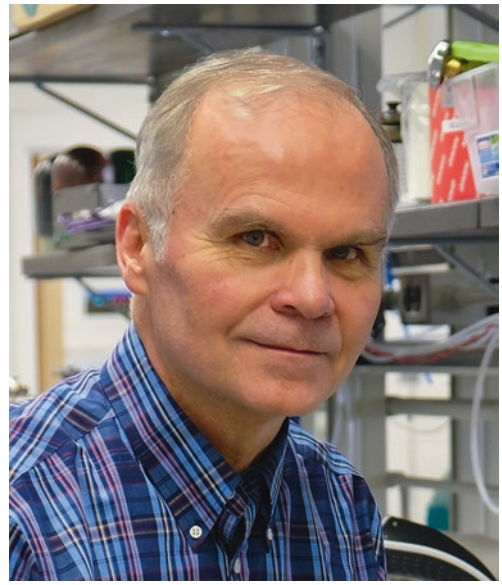
Govindjee is best known for his research on excitation energy transfer, light emission (prompt and delayed fluorescence and thermoluminescence), primary photochemistry, and electron transfer in *photosystem II* (PS II, water-plastoquinone oxidoreductase). His research, with many others, includes the discovery of a short-wavelength form of chlorophyll (Chl) *a* functioning in 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 others, include an understanding of the basic relationship between Chl *a* fluorescence and photosynthetic reactions; a unique role of bicarbonate/carbonate on the electron accep-

tor side of PS II, particularly in the protonation events involving the Q_B binding region; the theory of thermoluminescence in plants; 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 against excess light. His current focus is on the *History of Photosynthesis Research* and in *Photosynthesis Education*. He has served on the faculty of the UIUC for ~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 (1956), Fulbright senior lecturer (1997), and Fulbright specialist (2012); 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 of the Liberal Arts and Sciences Lifetime Achievement Award of the UIUC, (2008). Further, Govindjee has been honored many times: (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 J. 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, which was followed by the book *Photosynthesis: Basics and Applications* (edited by S. Itoh, P. Mohanty, and K.N. Guruprad); (3) in 2012, through *Photosynthesis: Plastid Biology, Energy Conversion and Carbon Assimilation*, edited by Julian J. Eaton-Rye, Baishnab C. Tripathy, and one of us (TDS); (4) in 2013, through special issues of Photosynthesis Research (volumes 117 and 118), edited by Suleyman Allakhverdiev, Gerald Edwards, and Jian-Ren Shen celebrating his 80th (or rather 81st) birthday; (5) in 2014, through celebration of his 81st birthday in Třeboň, the Czech Republic (O. Prasil [2014] Photosynth Res 122: 113–119); (6) in 2016, through the award of the prestigious Prof. B.M. Johri Memorial Award of the Society of Plant Research, India. In 2018, *Photosynthetica* published a special issue to celebrate his 85th birthday (editor, Julian J. Eaton-Rye; now co-series editor for this book series).

Govindjee's unique teaching of the Z-scheme of photosynthesis, where students act as different intermediates, has been published in two papers: (1) P.K. Mohapatra and N.R. Singh (2015) (Photosynth Res 123:105–114) and (2) S. Jaiswal, M. Bansal, S. Roy, A. Bharati, and B, Padhi (2017) (Photosynth Res 131: 351–359). Govindjee is a coauthor of the classic and highly popular book *Photosynthesis* (with E.I. Rabinowitch, 1969) and of the historical book *Maximum Quantum Yield of Photosynthesis: Otto Warburg and the Midwest Gang* (with K. Nickelsen, 2011). He is editor (or co-editor) of many books including *Bioenergetics of Photosynthesis* (1975); *Photosynthesis*, two volumes (1982); *Light Emission by Plants and Bacteria* (1986); *Chlorophyll a Fluorescence: A Signature of Photosynthesis* (2004); *Discoveries in Photosynthesis* (2005); and *Non-Photochemical Quenching and Energy Dissipation in Plants, Algae and Cyanobacteria* (2015).

Since 2007, each year a **Govindjee and Rajni Govindjee Award** is given to graduate students, by the Department of Plant Biology (odd years) and by the Department of Biochemistry (even years), at the UIUC, to recognize excellence in biological sciences. For further information on Govindjee, see his website at http://www.life.illinois.edu/govindjee.



Photograph by Sean E. Weise, 2017

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, USA. After 2 years as a research technician, Tom entered a Ph.D. program in the Department of Energy Plant Research Laboratory at Michigan State University under the mentorship of Klaus Raschke and finished in 1979. Postdoctoral research was carried out with Graham Farquhar at the Australian National University, in Canberra, where he coauthored a landmark review on photosynthesis and stomatal conductance. For 5 years, he worked at the Desert Research Institute, Reno, Nevada. After Reno, Tom spent 20 years as professor of botany at the University of Wisconsin in Madison. In 2008, Tom became professor and chair of the Department of Biochemistry and Molecular Biology at Michigan State University. In 2017, Tom stepped down as department chair and moved to the MSU-DOE Plant Research Laboratory completing a 38-year sojourn back to his beginnings. Tom's research interests center on the exchange of gases between plants and the atmosphere and carbon metabolism of photosynthesis. The biochemistry and biophysics underlying carbon dioxide uptake and isoprene emission from plants form the two major research topics in his laboratory. Among his contributions are measurement of the carbon dioxide concentration inside leaves, an exhaustive study of short-term feedback effects in carbon metabolism, and a significant contribution to elucidation of the pathway by which leaf starch breaks down at night. In the isoprene research field, his laboratory has cloned many of the genes that underlie isoprene synthesis, and he has published many important papers on the biochemical regulation of isoprene synthesis. Tom's work has been cited over 26,000 times according to Google Scholar in 2017. He has been named an outstanding faculty member by Michigan State University, and in 2015, he was named a University Distinguished Professor. He is a fellow of the American Society of Plant Biologists and of the American Association for the Advancement of Science. Tom has

co-edited three books, the first on trace gas emissions from plants in 1991 (with Elizabeth Holland and Hal Mooney), volume 9 of this series (with Richard Leegood and Susanne von Caemmerer) on the physiology of carbon metabolism of photosynthesis in 2000, and volume 34 (with Julian J. Eaton-Rye and Baishnab C. Tripathy) entitled *Photosynthesis*: *Plastid Biology*, *Energy Conversion and Carbon Assimilation*. Tom has been co-editor of this series since volume 31.

Julian J. Eaton-Rye is a professor in the Department of Biochemistry at the University of Otago, New Zealand. He received his undergraduate degree in botany from the University of Manchester in the UK in 1981 and his Ph.D. from the University of Illinois in 1987, where he worked with Govindjee on the role of bicarbonate in the regulation of electron transfer through photosystem II. Before joining the Biochemistry Department at Otago University in 1994, he was a postdoctoral researcher focusing on various aspects of photosystem II protein biochemistry with Professor Norio Murata at the National Institute of Basic Biology in Okazaki, Japan, with Professor Wim Vermaas at Arizona State University, and with Dr. Geoffrey Hind at Brookhaven National Laboratory. His current research interests include structure-function relationships of photosystem II proteins both in biogenesis and electron transport as well as the role of additional protein factors in the assembly of photosystem II. Julian has been a consulting editor for the *Advances in Photosynthesis and Respiration* series since 2005, and he edited volume 34 (with Baishnab C. Tripathy and Thomas D. Sharkey) entitled *Photosynthesis*: *Plastid Biology*, *Energy Conversion and Carbon Assimilation*. He is also an associate editor for the *New Zealand Journal of Botany* and an associate editor for the Plant Cell Biology section of *Frontiers in Plant Science*. He edited a Frontiers Research Topic on the *Assembly of the Photosystem II Membrane-Protein Complex of Oxygenic Photosynthesis* (with Roman Sobotka) in 2016, and this is available as an eBook [ISBN 978-2-88945-233-0]. Julian has served as the president of the New Zealand Society of Plant Biologists (2006–2008) and as president of the New Zealand Institute of Chemistry (2012). He has been a member of the International Scientific Committee of the Triennial International Symposium on Phototrophic Prokaryotes (2009–2018) and is currently the secretary of the International Society of Photosynthesis Research.

Contents

Preface: The Importance of Leaves to Life and Humanity

Whether looking down on our planet from space or up at the sky from its surface, blue is the dominant color in the absence of condensed water vapor. The primary color of life on Earth, however, is green due to the ubiquitous presence of leaves as the drivers of primary productivity on land and in shallow aquatic habitats. Given the order of magnitude loss of energy through each transformation as it makes its way through a food web, the overwhelming prevalence of primary producers is inevitable. As Richard Dawkins (2009) pointed out, "It is no accident that we see green almost wherever we look … Without green plants to outnumber us at least ten to one there would be no energy to power us."

Following the emergence of leaves between 380 and 360 million years ago (Kenrick and Crane 1997; Boyce and Knoll 2002; Osborne et al. 2004a, b), there was an explosion in diversity of leaf shape and size as plants evolved to fill virtually every available niche. At the heart of that variation in leaf form is its functionality as an organ optimized for (1) intercepting and harvesting photons from sunlight to fuel the energization of electrons in photosynthetic light harvesting and electron transport, (2) allowing the inward diffusion of carbon dioxide from the atmosphere in order to convert the energized electrons into a relatively stable chemical form (reduced sugars), (3) passive and active movement of photosynthate (primarily sugars) into foliar pipelines (sieve elements of the phloem) that provide for the distribution of these energy-rich, reduced carbon compounds throughout the plant, (4) distribution of water and nutrients through pipelines (tracheary elements of the xylem) within the leaf, and (5) exercising considerable control over the transpirational loss of water molecules to the atmosphere. Three key features that allowed these specialized organs to arise and proliferate include the ability to deposit a waxy cuticle on the outside surface of the epidermis virtually impenetrable to water loss from the water-laden tissue, a vascular system capable of transporting fluids into and out of the leaf, and stomatal pores with guard cells responsive to internal and external cues permitting regulation of water efflux and carbon dioxide influx. The mere presence of stomata, however, was insufficient to permit evolution of broad lamina for intercepting sunlight; only with development of sufficient stomatal numbers that could facilitate a rate of transpirational cooling capable of preventing overheating and death did large, flat leaves become abundant concomitant with declining atmospheric CO_2 concentrations (Beerling et al. 2001; Beerling 2005).

As the key to mitigation of the second law of thermodynamics, thereby permitting the majority of life on earth to persist, the creation of chemically stored energy through the process of photosynthesis has supplied the energy, molecular building blocks, and oxygen necessary for the evolution and proliferation of millions of species. Interestingly, leaves also comprise some of the most flammable components of the ecosystems in which these species coexist (e.g., grasslands, needles of conifer-dominated forests, leaves of *Eucalyptus*-dominated habitats, chaparral vegetation, etc.), with fire constituting a major driver responsible for the maintenance

and even rejuvenation of such habitats (Bond and Keeley 2005; Keane et al. 2008; Bowman et al. 2009, 2014; de Groot et al. 2013). While leaves, as the organs that provide the platform for most of terrestrial photosynthesis, underpin the major land-based biomes (Field et al. 1998), their essential role in humanity's progression is no less remarkable.

Leaves provide the food, either directly through fruits, nuts, roots, grains, leaves, or other plant parts or secondarily through animals, saprophytes, microbes, etc. whose functioning depends on the solar energy harvested by plants, without which we could not survive. The rise of human civilization depended to a large extent on the development of domesticated crop plants (Chrispeels and Sadava 2003). The agricultural products of many of these crops are major sinks for the products of photosynthesis, but humans consume the leaves of quite a few species as well (cultivars of *Lactuca sativa* [lettuces], *Brassica* [cabbages, Brussels sprouts, kale, rapini, collard, turnip, and mustard greens], *Beta vulgaris* [chards], *Spinacia oleracea* [spinach], *Cichorium* [endive, radicchio], a variety of ferns, grape leaves, and a number of others) and use just as great a variety of leafy herbs to enhance the flavor, nutrition, and even aesthetic value of our cuisine (basil, bay laurel, chives, cilantro, dill, fennel, garlic, lavender, lemon grass, marjoram, a number of mints, onion, oregano, parsley, rosemary, tarragon, thyme, etc.). Moreover, the leaves of many grasses and other species serve as forage for our domesticated animals.

As important as leaves are in generating the food upon which we (and our fellow heterotrophs) rely to fuel our bodies, they have played an equally important role in providing the energy for civilization's development. The cellulose-infused, and thus energy-rich, products of photosynthesis (wood, peat, etc.) constituted a fuel source for fire used in cooking food (increasing the accessibility of energy and nutrients for uptake by our digestive system; Wrangham and Carmody 2010; Carmody et al. 2016), making fire-strengthened and ultimately glazed pottery, heating of habitations that could also bring light to the night, smelting of metal ores and the formation of metal-alloy implements, and melting of silica to form useful glass products (Hough 1932). The industrial revolution, fueled by fossil biofuels (and particularly the converted remains of plants in the form of coal that was laid down during the carboniferous era; Lee 2017), spurred technological innovation at an increasing rate (Fernihough and O'Rourke 2014; Nelsen et al. 2016). This led to generation of electricity, the lighting and heating of our businesses and dwellings, and the invention of electricity-powered machines and devices for any number of purposes. The tapping of the buried reserves of compact energy derived from photosynthetic products also yielded unprecedented changes in transportation, from steam-powered ships and trains to automobiles and, airplanes. Although the shift from renewable to fossil fuels transformed much of the developed world, between two and three billion people still rely on wood, charcoal, leftover biomass from agriculture, and plant biomass processed by livestock (dung) as a source of fuel for cooking and heating (Ekouevi and Tuntivate 2012; Zuzhang 2017). The use of such renewable biofuels may be laudable, but it can nonetheless have negative consequences for the environment (Draper 2011; Sawe 2012) and health of those who are exposed to excessive levels of indoor pollution (Fullerton et al. 2008; Nijhuis 2017).

Leaves also fuel the generation of many products that have proved useful to humans and contributed to the advancement of civilization. Wood has been and is fashioned into various useful implements (e.g., utensils, tools and handles for tools, pencils, and even jewelry); used to build houses, boats, furniture, railroads, fences, etc.; and pulped to make paper, cardboard, and paperboard for packaging, toilet paper and facial tissues, etc. Natural plant fibers (e.g., cotton, linen) are woven into clothing, blankets, etc. A number of leaves have been and are used directly, including those of a number of species that serve as thatching material for the

protection of shelters from inclement weather or intense sunlight. The leaves of Spanish moss (*Tillandsia usneoides*), and especially their resilient, vascular strands, were used to stuff pillows, mattresses, and the upholstery of furniture (a sofa in the childhood home of the author was filled with this material). Leaves have also been woven into baskets, mats, boats, ropes (especially cordage made from the parallel veins of long monocotyledonous leaves), and other useful devices, fashioned into articles of clothing, and used in the preparation and serving of food. Where available, palm leaves have played a prominent role in many of these functions, and more specifically for the generation of manuscripts in India and southeast Asia.

The cultural importance of leaves has been immense. The leaves of the laurel tree (*Laurus nobilis*) held special significance to the ancient Greeks (the mythical god Apollo bore a crown of laurel leaves), branches of which were woven into wreaths and worn around the head as a symbol of achievement or ranking in society (e.g., for those athletes who triumphed in Olympic contests). Wreaths from other species (oak, olive, myrtle, grapevine, some herbaceous species) served as well in Greek, Roman, and some Native American groups. Laurel wreaths were often invoked to fete the founders of the USA (Chernow 2004, 2010) and, upon his death in early 1919, laurel wreaths were dropped from airplanes circling Theodore Roosevelt's residence and laurel branches lined his gravesite (Morris 2010). The use of honorific wreaths has persisted into some modern-day athletic competitions (e.g., Olympic Games, Boston Marathon), and the term laurel and its derivatives has continued to connote great accomplishment in the English language. For instance, as a noun, laurels refer to the achievements earned or accomplished for which one is recognized (it can also be used as a verb). Perhaps most notably, those who are honored for exceptional achievements are bestowed the title of laureate (e.g., Nobel Laureate, poet laureate). While it may be quite acceptable to rest on one's laurels at the end of a long and distinguished career, the phrase can take on a less than positive tenor should one cease to make contributions to an area at an earlier age!

The palm leaf has served as an important symbol to Christians. Furthermore, the use of a fig leaf to cover those things that some might find to be objectionable or embarrassing originated with the opening story of the Bible. The lotus (*Nelumbo nucifera*) leaf (not to be confused with water lily leaves) plays a prominent role in Indian culture and Hinduism (Kintaert 2010, 2011), while mango and banana leaves are made into garlands that adorn entryways to Hindu houses and used in Hindu religious rituals. Likely originating in the British Isles, the belief that a clover leaf with four leaflets (the leaves of which normally consist of only three) imbues the individual who finds or possesses such a leaf with good luck is widespread.

Leaves have played a major role in art and architecture. *Acanthus* leaves were depicted in friezes and other architectural accents on buildings erected by ancient Greeks and Romans. Likewise, leaf scroll is often used as a border or decorative accent (see frontispiece image). Leaf motifs also appear in and on artworks (e.g., paintings, statuary, pottery) or recurring on clothing (see the shirt worn by W. Adams in the photograph that accompanies his biography on p. XXXV), curtains, bedding, towels, wallpaper, etc. Even jewelry is fashioned in the shape of leaves or leaves in the abstract.

Some leaves have been important sources and drivers of commerce while also contributing to human suffering, social strife, political upheaval, and revolution. Tobacco (genus *Nicotiana*), used by native Americans for thousands of years, became a major trade commodity following European colonization of America. During the 1600s and 1700s, large quantities of tobacco leaves were exported to England, playing no small part in the economic development of the American colonies (Chernow 2010). This boon to the colonies, however, came at the horrendous

cost of immeasurable suffering and death to the many enslaved Africans working the fields of this labor-intensive crop. Much suffering has also, of course, befallen those who use(d) tobacco for smoking, chewing, or snuffing. Such use is at the root of numerous diseases and deaths that affect not only the users but also society as a whole. It is estimated that the negative economic impact of tobacco exceeds US$500 billion per annum (Ekpu and Brown 2015).

The leaves of *Camellia sinensis*, an evergreen shrub from Asia, have been used by the Chinese to make tea for thousands of years. Tea also has a rich, although more recent, history in Korea, Japan, and other Asian countries. It became an important trading commodity following its introduction to Europe in the early 1600s, after which the forerunner of the East India Company was established to facilitate global trade in tea and many other products. Although this company's monopoly on tea trade with the American colonies, and the taxes imposed on the American colonies for this desired leaf, instigated a pivotal party in the harbor of Boston that contributed to the start of the Revolutionary War and founding of the USA (Chernow 2004, 2010), the East India Company's activities in other parts of the world were no less profound. The desire to control not only the trade in tea but also its production led to arrangements between the East India Company and Great Britain that brought the planting of tea to major regions of colonial India (and later Africa), contributing to the domination of these areas by the British Empire and the latter's control of global trade.

Leaves from several species have been, and are used, for the psychoactive impact that they impart to the individuals who chew or brew them into teas (or in some cases concentrate the active ingredients for administration through other means). These include coca, khat (qat, chat, or several other terms), betel, etc. Moreover, various pharmaceuticals and medicines are, or at least were originally, derived from plant parts including the leaves of a number of species (e.g., menthol, digitalis [digoxin], caffeine, atropine, cocaine, eucalyptus oil, artemisinin). The use of the leaves (buds and flowers) of the marijuana plant *Cannabis sativa* (and its pharmacological derivatives tetrahydrocannabinol and cannabidiol) for alleviation of nausea (e.g., associated with chemotherapy), pain, depression, anxiety, and symptoms of many medical conditions including Parkinson's, Alzheimer's, HIV/AIDS, glaucoma, etc. has led to its legalization for medical purposes in more than half of the USA and some countries (although growth of the plant and sale of its products may be restricted). Legalization of marijuana for recreational use has even occurred in a number of US states and a few countries, opening new entrepreneurial opportunities and additional sources of revenue for various levels of government.

Leaves have also played a role in the affairs of government and corporate entities. The maple leaf is widely employed as a symbol of Canada, appearing on its currency and the national flag, and is used as a logo by the airline Air Canada, professional ice hockey (Toronto Maple Leafs, Toronto Marlies, and Winnipeg Jets) and lacrosse (Toronto Rock) teams, and national teams (e.g., ice hockey, soccer, basketball, softball, ice skating, swimming, skiing). The shamrock leaf is likewise a symbol associated with Ireland and many things Irish, as well as with several companies (Shamrock Foods, Shamrock Oil and Gas - Diamond Shamrock, etc.). In addition, leaves of various species have been featured on the currency of a number of other countries, including France, Germany, Japan, Poland, Australia, and the USA (van Wie 1999), and have been utilized in military service medals (Elder et al. 2003; Zabecki 2014). Recognizing the unique patterns that leaf veins display, Benjamin Franklin duplicated such designs on the currency of some colonial states and the first US currency in order to make it difficult for individuals to print counterfeit money (Isaacson 2003). In order to tout their "green" and sustainable approach to business, a number of corporations have the word leaf as part

of their name and/or utilize a leaf as their logo. One such example is the LEAF electric car, Nissan's "zero emission" vehicle.

For all of their importance to life, the development of human civilization, and pervasiveness in culture, the scientific study of leaf function was slow to arise but accelerated swiftly once an understanding of the nature of gases and their exchange between atmosphere and organism became established. Theophrastus undertook a cataloging of the variety of leaf shapes in ancient Greece (Hort 1916), while Caroli Linnæi took it to a definitive level with the publication of *Species Plantarum* in 1753, founding modern taxonomy and instituting the binomial nomenclature of genus and species. However, such descriptions lacked any recognition of leaf function. The following (two paragraph) short history of research into leaf function has been compiled from several sources (Walker 1992; Govindjee and Krogmann 2004; Lee 2017). Jan Baptist Van Helmont performed an experiment (published in 1648, 4 years after his death) in which a 5-pound willow shoot was planted in 200 pounds of soil. After growing for 5 years, the willow weighed 169 pounds, but the soil had only lost 0.13 pounds (Hershey 1991). Although the weight gain by the tree was not yet attributed to carbon dioxide acquired from the air (only water added to the soil was implicated at the time), the stage was set for elucidating leaf function during the following century. In 1727, Stephen Hales published a volume describing his observations of leaves interacting with the atmosphere, including their loss of water vapor to the surrounding air (i.e., transpiration; he surmised that plants were able to move water through their tissues and that leaves were involved) and their ability to reduce the volume of surrounding air in a closed container (an impact for which he was unable to provide an adequate explanation). Although Hales speculated that leaves were important for provision of something from the air that was essential to the plant and that light might also be important in that provisioning, he did not have the wherewithal to put everything together in a meaningful way.

In 1754, Charles Bonnet suggested that leaves absorb gas (after observing bubbles exuded by illuminated leaves underwater), and Joseph Priestley, using a device previously employed by Hales, subsequently demonstrated that plants could produce a gaseous substance (later identified as oxygen) that permitted a mouse to live or a candle to burn in a sealed enclosure. Antoine Lavoisier, after learning of these results (as well as those of Carl Wilhelm Scheele), realized that air was comprised of at least two different gases (oxygen and nitrogen). Following on all of these findings, the Dutch scientist Jan Ingen-Housz conducted many experiments showing that those parts of a plant that are green produce oxygen (as it would later be called) when they are illuminated with light and produce much smaller amounts of a different gas (later identified as carbon dioxide) in darkness. By the end of the eighteenth century, Ingen-Housz had clarified not only the role of light in photosynthesis but also suggested that carbon accumulated in plants comes from absorbed CO_2 (the concomitant absorption of CO_2 and production of O_2 by leaves during photosynthesis were demonstrated by Jean Senebier during the 1780s). Shortly thereafter, Nicolas Theodore de Saussure conducted experiments leading him to surmise that water was a direct participant in the process of photosynthesis; he also experimented with cactus cladodes, revealing the essential gas exchange features of what would come to be known as Crassulacean acid metabolism (see Chapter 10) a century and a half later.

Following earlier characterizations of plant anatomy by Marcello Malpighi (1675) and Nehemiah Grew (1682), a structure-function view of the leaf was put on a firm footing by Gottlieb Haberlandt (1884; and subsequent revisions drawing also upon Schimper 1898). Ten years later, Dixon and Joly (1894) put forth the hypothesis that water in leaf cell walls transitioning from liquid into vapor followed by its transpirational

loss via stomata at the leaf-atmosphere boundary resulted in the drawing of water up through the xylem portion of a plant's vascular tissue (due to the cohesion of water molecules for one another). Whereas this cohesion-tension mechanism of water movement through the tracheary elements of the xylem involves a pressure potential gradient that becomes increasingly more negative from soil through the leaves and into the atmosphere, movement of sugars (the product of photosynthesis) from the leaves to distant sinks in the plant involves a positive pressure potential gradient. As proposed by Ernst Münch (1930), sugars in high concentration near the site of synthesis accumulate in the phloem of the leaf and water, moving from higher concentration in neighboring cells (e.g., the xylem) to lower concentration in the phloem (filled with sugars), increase the pressure potential in the phloem. Sugars are simultaneously being removed from the phloem of distant sinks (e.g., fruits, roots), as well as along the intervening route, and water follows those sugars moving from the phloem into the surrounding tissues, thereby lowering the pressure potential of the phloem at those sites. The sugar-laden sap in the sieve tubes (sieve elements aligned end to end) thus moves under positive pressure from sites of sugar loading (where the phloem sap is under higher pressure) to sites of sugar unloading (where the phloem sap is under lower pressure).

In summary, the leaf is an organ optimized for capturing sunlight and safely using that energy through the process of photosynthesis to drive the productivity of the plant and, through plants' position as primary producers, that of Earth's biosphere. It is an exquisite organ composed of multiple tissues, each with unique functions, working synergistically to (1) deliver water, nutrients, signals, and sometimes energy-rich carbon compounds throughout the leaf (xylem), (2) deliver energy-rich carbon molecules and signals to the leaf during its development and then from the leaf to the plant once the leaf has matured (phloem), (3) regulate exchange of gasses between the leaf and the atmosphere (epidermis and stomata), (4) modulate the radiation that penetrates into the leaf tissues (trichomes, the cuticle, and its underlying epidermis), (5) harvest the energy of visible sunlight to transform water and carbon dioxide into energy-rich sugars or sugar alcohols for export to the rest of the plant (palisade and spongy mesophyll), and (6) store sugars and/or starch during the day to feed the plant during the night and/or acids during the night to support light-driven photosynthesis during the day (palisade and spongy mesophyll). Various regulatory controls that have been acted upon through the evolutionary history of each plant species result in an incredible diversity of leaf form across the plant kingdom. Genetic programming is also flexible in allowing acclimatory phenotypic adjustments that optimize leaf functioning in response to a particular set of environmental conditions and biotic influences experienced by the plant. Moreover, leaves and the primary processes carried out by the leaf respond to changes in their environment, and the status of the plant, through multiple regulatory networks over time scales ranging from seconds to seasons. This book brings together the findings from laboratories at the forefront of research into various aspects of leaf function and particularly in relationship to photosynthesis.

The editors are grateful for the dedication and patience of the authors in making this volume possible. They also wish to express their gratitude to the series editors Tom Sharkey and Julian Eaton-Rye and the indomitable Govindjee, who immediately endorsed the pursuit of this volume following the submission of a prospectus.

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The Editors

William W. Adams III (*far right*; note the pattern of leaves on his shirt) **with his wife and colleague Barbara Demmig-Adams** (*second from left*) **and their children Robert** (*far left*) **and Melanie** (*second from right*) **Adams.** (Photograph by Markus Demmig)

A Fascination with Leaves

William W. Adams III is a professor in the Department of Ecology and Evolutionary Biology at the University of Colorado in Boulder, Colorado, USA. The initiation of his interest in leaves began in late summer 1975. He had collected a moss growing in Trimble Wildlife Area (now underwater following the completion of Smithville Dam in 1977 and subsequent flooding to create Smithville Lake, north of Kansas City, Missouri), with an eye toward using it for his project in an advanced biology high school course. Before the start of the school day, William used a mirror to reflect direct light from the rising sun up through a microscope stage and was mesmerized by the brilliant green of the moss' simple leaves, setting the stage for a lifelong focus on plants and investigation of leaves.

During his junior year at the University of Kansas, William became the first student to join the laboratory of a recent hire, Prof. Christopher H. Haufler, who suggested a project on the fern *Gleichenia bifida*. After collecting *G. bifida* fronds on a trip to Costa Rica, William conducted a study of gametophyte development that culminated in an honors thesis, half of which was published (Haufler and Adams 1982, William's least-cited publication). A rigorous course on plant physiological ecology (taught by Prof. Rolf Borchert; see http://www.kuonlinedirectory.org/endacott/data/OralHistoryTranscripts/Borchert.wpd.pdf and, e.g., Borchert et al. 2015) proved pivotal in generating a desire to

work in the area of plant ecophysiology. The course also featured presentations by finalists for a plant ecophysiologist position in the Department of Botany. William subsequently became the first student to join the laboratory of the individual chosen for that position, Prof. Craig E. Martin. For his master's thesis, William suggested a comparative ecophysiological study of a pair of *Tillandsia* species (see Martin and Adams 1987 for one of them) he had encountered during trips to Mexico, but the two proved to be too similar in their adaptations to the epiphytic habitat. As an alternative, Craig suggested a study of the juvenile (with trichome-covered atmospheric leaves; see Figs. 1.4 and 5.9i) and adult (with relatively glabrous, overlapping leaves that create a tank for impoundment of water and detritus) forms of the epiphyte *Tillandsia deppeana*, resulting in a successful characterization of the structure and function of its heterophyllous leaves (Adams and Martin 1986a,b,c).

Prof. C. Barry Osmond generously served as William's Ph.D. mentor, providing resources for multiple studies on the potential role of high CO_2 levels (produced by diurnal malic acid decarboxylation in leaves and cladodes of Crassulacean acid metabolism plants) in mitigating photoinhibition (e.g., Adams and Osmond 1988). During the first half of his Ph.D. tenure (undertaken at the Biological Sciences Center of the Desert Research Institute, Reno, Nevada), William was also fortunate to have both Prof. Stanley D. Smith (Adams et al. 1987a) and one of the editors of this book series, Prof. Thomas D. Sharkey (Adams et al. 1987b), as mentors. During the second half of his Ph.D., William joined the other editor of this book (Prof. Ichiro Terashima, then a postdoctoral research associate) in Barry's laboratory at the Australian National University's Research School of Biological Sciences. William solicited Ichiro's assistance with field work, with which his future wife and colleague (Prof. Barbara Demmig-Adams) also became involved (Adams et al. 1988). Almost a decade later, and in conjunction with a Robertson Symposium on Chlorophyll Fluorescence (Adams et al. 1995; Demmig-Adams et al. 1995), Barry was again instrumental in providing financial and logistical support for a 3-month research visit by William, Barbara, their two children, and two of their Ph.D. students to characterize photosynthesis, photoprotection, and photoinhibition of leaves and cactus cladodes in multiple species in three distinctive Australian habitats with varying light and climatic conditions (Logan et al. 1996, 1997; Barker et al. 1998; Adams et al. 1999).

Following the completion of his Ph.D., William pursued various aspects of leaf function including responses to sulfur dioxide (Adams et al. 1989), autumnal senescence (Adams et al. 1990a), and zeaxanthin-associated photoprotection (e.g., Demmig-Adams et al. 1989, 1990; Adams et al. 1990b) with the support of NATO and Alexander von Humboldt fellowships in the laboratory of Prof. Dr. Klaus Winter. This 2-year period in Würzburg solidified the personal and professional collaboration between William and Barbara, yielding two children (see photograph above) and a couple of decades of research on photoprotection (and its sustained variant photoinhibition) under physiologically relevant conditions (for reviews, see Demmig-Adams et al. 1999; Demmig-Adams and Adams 2006; Demmig-Adams et al. 2006, 2012, 2014; Adams et al. 2006, 2013, 2014a,b). The recognition that photoinhibition involved sustained engagement of zeaxanthin in photoprotective energy dissipation accompanied by increased levels of foliar carbohydrates led William to contemplate the role of foliar carbon export in contributing to the response of the photosynthetic apparatus to excess light. In addition, his growing awareness of the different mechanisms through which sugars are loaded into the phloem (through the classes he was teaching) prompted him to initiate a collaboration with one of the preeminent

phloem loading experts, Prof. Robert Turgeon (Amiard et al. 2005, 2007; Adams et al. 2007; see Ayre and Turgeon 2018 Chapter 3).

This collaboration catalyzed an evaluation of the relationship between foliar minor vein features (of both phloem and xylem) and photosynthetic capacity (and transpiration) involving multiple summer annual species, winter annual species, biennial species, and both apoplastic and symplastic phloem loaders. These characterizations revealed that anatomical and ultrastructural features of the phloem that serve as proxies for the capacity to load and export sugars vary among species, exhibit acclimatory adjustment to different growth light and temperature regimes, and are consistently and significantly correlated with photosynthetic capacity (Adams et al. 2018 Chapter 2 and multiple references therein). Features of the tracheary elements that serve as proxies for the water flux capacity of the xylem were consistently correlated with transpiration rate among all species across all growth conditions and typically (but not exclusively) with photosynthetic capacity as well. The latter association was not present for winter annuals that exhibited an increased capacity for sugar loading/export and photosynthesis in response to growth under low temperature, a condition under which evaporative demand and the flux capacity for water were both diminished (Adams et al. 2018 Chapter 2). Moreover, the level of foliar vascular and photosynthetic phenotypic plasticity exhibited by *Arabidopsis thaliana* in response to different growth conditions during plant development varied depending on the climatic conditions prevailing in the habitats from which different ecotypes were obtained (i.e., the extent of acclimatory adjustment was dependent on the evolutionary history of each; Adams et al. 2016, 2018 Chapter 2).

As a professor at a public state university, William's energy has also been directed toward the education and training of students, including thousands that have participated in his introductory general biology class over more than a decade. He has been honored for his efforts in this realm by students (Mortar Board Certificate of Recognition for Exceptional Teaching in 2000), faculty peers (Boulder Faculty Assembly Excellence in Teaching Award in 2004), and administration (Chancellor's Award for Excellence in STEM Education 2013–2014). In the laboratory, he has mentored and co-mentored (with Barbara) over 40 undergraduate students and volunteer workers, 22 graduate students, and 10 postdoctoral research associates and collaborated with 4 colleagues as their host during periods of sabbatical leave.

William has co-edited three books, the first resulting from the inaugural Robertson Symposium on the Ecology of Photosynthesis in Sun and Shade (held in 1987, published in 1988) with John R. Evans and Susanne von Caemmerer. The other two have been books in this series, including volume 21 on photoprotection and photoinhibition (with Barbara Demmig-Adams and Autar K. Matoo) in 2006 and volume 40 on non-photochemical chlorophyll fluorescence quenching and energy dissipation (with Barbara Demmig-Adams, Győző Garab, and Govindjee) in 2014.

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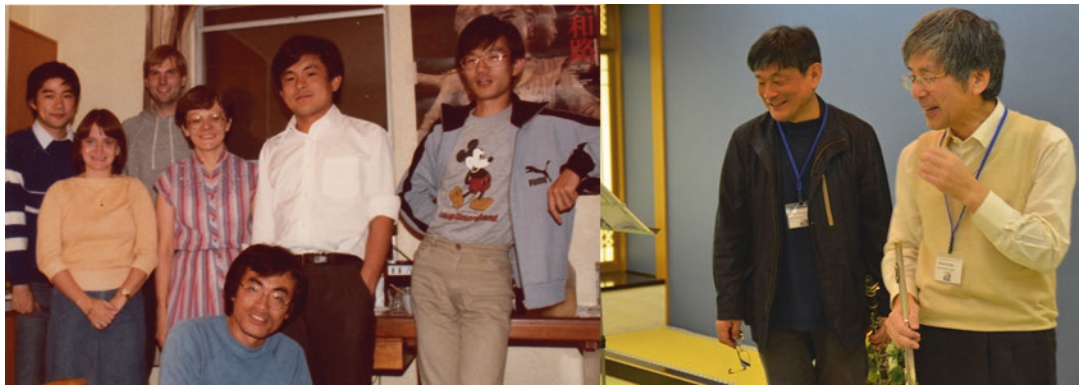

Left: A 1986 photograph taken in Terashima's room at the Australian National University's Bruce Hall. Top row from left to right, Takao Fujikawa (historian), Melodye A. Rooney (stable isotope chemist), William Adams, Catherine M. Brennan (sociologist), Ichiro Terashima, and Takashi Saito (Japan Business Federation). First row, Masashi Hirosue (historian). Right, a recent photo of Terashima (left) after playing a duet piece (clarinet and flute) with Tatsuo Omata (right) on the occasion of a Japan-Germany binational seminar on photosynthesis (March 2016, held at the Atami hot spa, Japan).

Ichiro Terashima entered the University of Tokyo in 1976 and started his studies on the light environment within leaves and its effect on leaf photosynthesis under the supervision of Toshiro Saeki, culminating in a Doctor of Science degree in 1985. He miniaturized his supervisor's study on the light environment and photosynthesis of leaf canopies (Monsi and Saeki 1953, 2005) to the individual leaf scale. He conducted his first postdoctoral study in the laboratory of Noboru Hara, an anatomist specialized in the early phases of leaf development, to study effects of light direction on differentiation of palisade and spongy tissues in bifacial leaves. He then moved to the Australian National University and studied effects of light and nitrogen nutrition on leaf photosynthesis with John R. Evans, patchy leaf photosynthesis in abscisic acid-treated leaves with Graham D. Farquhar, and photoinhibition at chilling temperatures with C. Barry Osmond. He shared an office room with William W. Adams III (see left photograph above), another editor of this volume, and participated in William's field studies. In 1988, he got a position as an assistant professor in the laboratory of Sakae Katoh at the University of Tokyo and studied with Kintake Sonoike. They found that photoinhibition of photosystem I and uncoupling of H^+-ATPase occur in chilling sensitive plants subjected to chilling temperatures in moderate light. He moved to Tsukuba University as an associate professor in 1994 and to Osaka University as a full professor in 1997. With his students and Ko Noguchi, he revealed that CO_2 conducting aquaporins (cooporins) facilitate CO_2 diffusion across the plasma membrane. He also studied systemic regulation of leaf photosynthetic properties, delayed greening of evergreen tree species, roles of alternative oxidase, and extrinsic proteins in photosystem II. In 2006, he moved back to the University of Tokyo and studied the influences of (1) green light in leaf photosynthesis; (2) mesophyll tissue in stomatal responses to environmental conditions; (3) soil dryness, high CO_2, and ABA application on mesophyll conductance; and (4) fluctuating light on photoinhibition of photosystem I. His students who have been studying in the field of photosynthesis and respiration include Kouki Hikosaka, Kiyomi Ono, Momoe Ishibashi, Ko Noguchi, Sachiko Funayama-Noguchi, Shin-ichi Miyazawa, Satoshi Yano, Youshi Tazoe, Wataru Yamori, Takao Araya, Keisuke Yoshida, Takushi Hachiya, Chihiro Watanabe, Yin Wang, Masaru Kono, Yuki Okajima, Kazunori

Miyata, Yusuke Mizokami, and Takashi Fujita. Yuko Hanba, Haruhiko Taneda, Danny Tholen, Riichi Oguchi, and Daisuke Sugiura studied in his laboratory as postdoctoral fellows. W.S. Chow, Poonam Vyas, Ala Druta, Narayan Misra, and Detelin Stefanov spent periods of time in his laboratory as visiting fellows. He stayed in Agu Laisk's laboratory as a visiting fellow in 2003. Light and CO_2 environments within leaves, the role of green light in photosynthesis, photoinhibition of photosystems I and II, and the effects of elevated CO_2 on photosynthesis have been his favorite research subjects. He is an amateur clarinet player (see right photograph above) and is practicing Bach's cello suits and various pieces by Weber, Brahms, Beethoven, Schubert, and, of course, Mozart! He also loves sake and rakugo (Japanese vaudevillian one-man play).

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The Life of a Leaf
by *William W. Adams III*

Throughout the winter leaves remain
Quiescent in seed and bud ‘til rain
Emerging with positive pressure potential
Cells burst forth from every axil

Seamlessly shifting from import to export
Sugars through phloem continuously transport
As chloroplasts reach their competency
The leaf attains full maturity

Water from soil to root is drawn
Passing through strip Casparian
Entering xylem to travel on
Held one to another by strong cohesion
Almost breaking with increasing tension
Reaching the leaf for distribution
Through a network of fine venation
Fluxing out stomates via diffusion
In the process of transpiration
As CO_2 enters assimilation
Fueled by solar illumination

Light of wavelengths from blue to red photons
In chlorophyll absorbed, exciting electrons
Splitting water to oxygen/protons
Electron transport ATP spawns
NADPH for use within
The chemical cycle of Calvin
Let’s not fail to also mention
The intrepid pair of Bassham and Benson

The C_3 cycle ubiquitous is
Among all leaves with photosynthesis
A C_4 cycle yields increased fitness
Where heat and drought contribute to stress
For C_4 plants between cells spatial
Whereas for CAM a divide temporal

CAM also found in pools vernal
As CO_2 falls each day diurnal
And rises again each night nocturnal
Revealing CAM as thoroughly versatile

Chloroplasts move from dawn to twilight
Fully exposed when low is the light
Self-shaded at midday, whenever too bright
Leaves too may shift, with the sunlight
Horizontal in morning, at noon quite upright
The reactive oxygen not to incite
Others may track from morning to night
Returning to east by predawn starlight

Thermal dissipation also steps up
When high light leads to proton backup
As protons gather in thylakoid lumen
Begetting formation of zeaxanthin
For strong engagement in photoprotection
Through increased levels of dissipation

Chemical energy in sugars stored
Used by the leaf or transferred to gourd
Fuel for the plant or herbivore
Microbe, fungus, or omnivore
Even passed on to carnivore
After death to detritivore

From little fern to sycamore
Springing from seed or tiny spore
From mountain top to far seashore
Across the land, we so adore

Some even manage the life aquatic
Others, in treetop, on branch, epiphytic
Many herbaceous, short-lived, mesophytic
Found on ephemeral, annual, biennial
Others much tougher, long-lived, sclerophytic
Among those evergreen, some perennial
From forest near equator, of the tropic
To those circumpolar, the boreal
Few are so touchy, oh so, thigmonastic
Others get sleepy, at night, nyctinastic

Though some remain green regardless of season
Others transform, such as oak, maple, aspen
Draining resources with great abandon
As leaves turn yellow or start to redden

Some blaze brightly with orange or crimson
Still others more subtle, akin to salmon
In petiole develops the zone of abscission
The bond between leaf and plant to weaken
Until the whole leaf from branch is riven
In its wake leaving cellulose, pectin, and lignin
A bud scale endures drought or vernalization
A woody plant's wondrous adaptation
Awaiting its future full activation

During its life, be it weeks, months, or years
Each leaf serves, through adversity, perseveres
Though some may go while others appear
All play a role through every tier
Removing CO_2 from atmosphere
Supporting life in the biosphere

To all life's relief
Whether long or brief
The life of a leaf
Is nature's motif

Author Index

Chapter 1

A Consideration of Leaf Shape Evolution in the Context of the Primary Function of the Leaf as a Photosynthetic Organ

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Summary

Leaf shape evolution in angiosperms is reviewed from an evolutionary developmental (evo/devo) viewpoint. Leaves have evolved as photosynthetic organs in land plants, while leaves in some angiosperms have lost their photosynthetic role, e.g., in some cacti, saprophytes/mycoheterotrophs, and parasitic plants. Although the roles are the same among leaves, their morphologies and developmental systems vary significantly, in part because of the need to maximize their photosynthetic efficiency for survival under particular environmental constraints. An example is the narrow leaves of the rheophyte plants located along river banks where frequent flooding occurs. Narrow leaves are less efficient at absorbing sunlight than

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are leaves with wider blades; however, they can withstand the destructive force of the water flow in full flood. In contrast, in some xerophytic epiphytes, the narrow leaves are effective at catching water from fog. Narrow leaf blade formation is also present in submerged amphibious plants, likely an adaptation to the underwater conditions. The leaf index, i.e., the ratio of leaf length to width, is regulated by several genetic factors, mutations of which may be driving the evolution of leaf shape. However, not all leaf shapes can be explained by environmental adaptation. For example, data on the relationship between leaf shape diversification and environmental habitats in terms of thermo regulation remain controversial.

In contrast, understanding of the genetic/cellular mechanisms underlying leaf blade shape is increasing. Leaf blade organogenesis is governed by adaxial/abaxial identities, in which the plate meristem, responsible for flat leaf blade development, is activated along the interface between the adaxial and abaxial domains. Thus, some morphological diversity in leaves can be partly attributed to changes in the adaxial/abaxial patterning. For example, unifacial leaves, which have only an abaxial identity in the leaf blade, develop into stick-like or terete forms. They can stand vertically and thus live in densely populated areas, unlike plants with bifacial leaves which extend, in most cases, horizontally. Interestingly, some unifacial leaves are flat or ensiform, as seen in the genera *Iris* and *Juncus.* In such cases, since the plate meristem is not available, they rely on thickening growth to create flat lamina from the terete primordia. Lotus-like or peltate leaves are also derived from partial alterations in the adaxial/abaxial patterning; however, the pitcher leaves of carnivorous plants, once considered an extreme deformation of the peltate leaves, result from local alterations in cell division. In compound leaves, the major genetic regulatory components are shared with those of serrated leaf margins. The proportion of serrated-leaf species in any particular habitat precisely correlates with the average air temperature, and thus serration mechanisms are likely linked to physiological adaptations to the external environment. The evolution of determinate and indeterminate leaves in angiosperms is also discussed. While leaves are usually determinate and discarded periodically, some species develop indeterminate leaves, with some looking like twigs or lateral branches and functioning as twigs. Some are simple leaves and have an intermediate nature between a leaf and a shoot. We also see the evolution of leaf-like, photosynthetic organs. Some plants develop cladodes, which are modified lateral shoots that resemble leaves. Molecular analysis revealed that recruitment of dorsiventral control mechanisms in leaves has resulted in lateral shoots changing into leaf-like cladodes in the genus *Asparagus.* All of the above can be interpreted from an evo/devo viewpoint, but the question of why such great diversification occurred in angiosperm leaves remains.

I. Introduction: Basic Mechanisms of Leaf Blade Formation

A. Basic, Common Mechanisms

Before reviewing the diversified evolution of leaf shape from an evo/devo view point, we review the basic genetic mechanisms of leaf organogenesis deduced from studies of the model species *Arabidopsis thaliana* (L.) Heynh. (hereafter, arabidopsis) **(**Fig. 1.1**)**. For details of each gene, please see other reviews (e.g., Tsukaya 2006, 2013).

Leaf primordia occur in the flanking, peripheral zone of the shoot apical meristem (SAM) in a regulated, species-specific manner. Phyllotaxis, or leaf arrangement around the shoot axis, is believed to be strongly dependent on auxin flow in the shoot apex (de Reuille et al. 2006; Jönsson et al. 2006; Smith et al. 2006). In short, PIN-FORMED 1 (PIN1)-regulated polar auxin transport in the epidermis (Layer 1: L1 layer) produces a dynamic auxin flow in the L1 layer of the shoot apex, resulting in the appearance of auxin maxima, or the center for auxin accu-

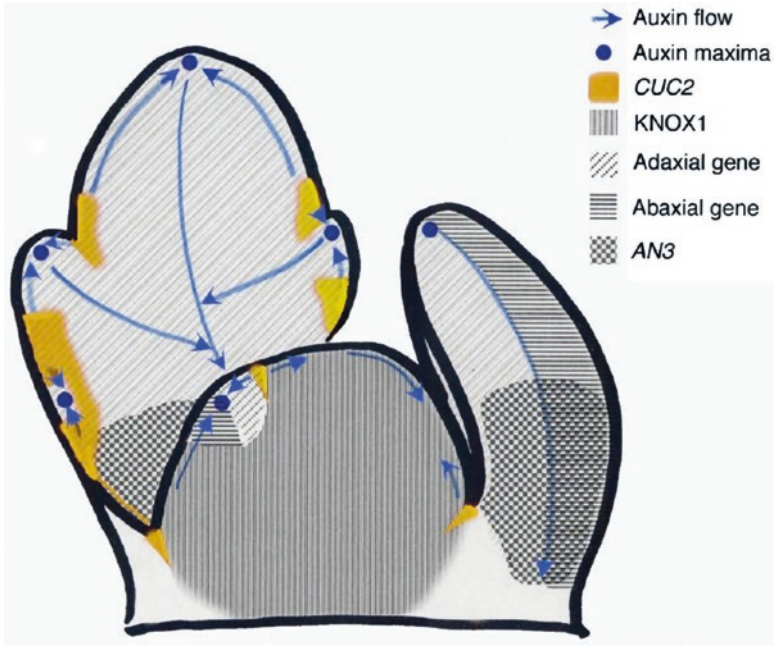

Fig. 1.1. Diagram depicting leaf organogenesis on a longitudinal section image. Please see text for details

mulation (Fig. 1.1). The site of the auxin maxima, identified as a future leaf primordium region, ceases to express the class I KNOTTED-LIKE HOMEOBOX (KNOX) genes (KNOXI). The auxin-maximum area is destined to form a new leaf primordium, and a very young leaf primordium slowly becomes a rod-like protrusion by extensive activation of cell proliferation. At this stage, cell divisions are mostly perpendicular to the longitudinal axis of the primordium (Horiguchi et al. 2011). The leaf primordium starts to release auxin into the attached stem, resulting in a rapid decrease in auxin concentration around the primordium. Due to this sudden change in auxin levels, a new auxin maximum is formed in the other areas of the SAM, resulting in formation of the next leaf primordium.

The rod-shaped leaf primordia acquire bilateral identity in relation to their position against the center of the SAM (Fig. 1.1). From surgical experiments, it has been shown that the SAM might emit some factor(s) ("anlagen factor") necessary for the acquisition of adaxial identity in the primordia (Sussex 1951). The genetic mechanisms for identification of adaxial and abaxial fates are complex (see Tsukaya 2013 for the systems in *A. thaliana*). In short, the adaxial fate is determined by the presence of the HOMEODOMAIN ZIPPER III (HD-ZipIII) family proteins and the abaxial fate by the KANADI (KAN) family and ETTIN (ETT)/AUXIN RESPONSE FACTOR (ARF)3-ARF4 proteins. HD-ZipIII mRNAs are targets of the microRNA miR165/166 that is expressed in the abaxial domain of the leaf primordia; *ETT/ARF3* and *ARF4* mRNAs are targets of trans-acting siRNA *tasiR-ARF* that is expressed in the adaxial domain. Establishment of both adaxial and abaxial fates in a leaf primordium is necessary to activate cell proliferation for flat leaf lamina formation seen in the border between the two dorsiventral fates (Waites and Hudson 1995). If either adaxial or abaxial fate is lost, leaf lamina formation is not seen, and leaves become terete or stick-like in form. The most intense cell proliferation in the leaf primor-

dia in arabidopsis occurs in the leaf meristem, the junction region between the leaf blade and petiole (Ichihashi et al. 2011). A single cell file extends in both directions to the leaf lamina and petiole. In the leaf lamina region, cell division is randomized to expand the flat structure in the form of the plate meristem (Donnelly et al. 1999), and in the leaf petiole, cell division is almost strictly perpendicular to the longitudinal axis, which results in a stalk-like structure (Horiguchi et al. 2011; Ichihashi et al. 2011).

The leaf meristem can be divided into two parts: the plate meristem that extends two-dimensionally and easily marked with active cell proliferation (Donnelly et al. 1999) and the marginal meristem that is restricted to leaf margin and short-lived in arabidopsis leaf primordia due to repressions by *NGATHA* (*NGA*) and *CINCINNATA-class-TCP* (*CIN-TCP*) transcriptional factor genes (Alvarez et al. 2016). Due to its short active period in model species such as arabidopsis, presence and absence of the marginal meristem has been subject to debate for a long time (Nardmann and Werr 2013). Alvarez et al. (2016) proposed an analogy that "the marginal and plate meristem represent two zones of a leaf meristem, analogous, or perhaps homologous, to the central and peripheral zones of the SAM". In this view, the marginal meristem is the organizing center and the plate meristem is its derivative zone for cell proliferation.

The plate meristem region overlaps with the middle domain where transcripts of PRESSED-FLOWER (*PRS*) and its paralog *WUSCHEL-LIKE HOMEODOMAIN 1* (*WOX1*), which encode transcription factors, are expressed (Nakata et al. 2012). In a longitudinal axis, the plate meristem region is marked by the presence of *ANGUSTIFOLIA3* (*AN3*)/*Arabidopsis thaliana GROWTH REGULATING FACTOR-INTERACTING FACTOR1* (*AtGIF1*) transcriptional co-factor mRNA (Horiguchi et al. 2005; Fig. 1.1). The leaf meristem region is often present in the proximal part of leaf primordia, as described above, but in some cases it is present in the distal section or scattered along the leaf primordia (Gupta and Nath 2015). Based on comparative studies of fossil plants, it has been proposed that leaf meristem formation occurred at the distal end of the leaf primordia in ancestral species, like shoot apices from which the leaf primordia arise (Boyce 2007). Indeed, fern leaf primordia have meristematic regions in the distal margin (Zurakowski and Gifford 1988; Nardmann and Werr 2013; see Tsukaya 2014).

The leaf meristem has a fundamentally determinate nature and is active only for a limited period of time. In the course of leaf primordium development, the position and area of the leaf meristems are maintained at a constant position (Kazama et al. 2010). During the active period of leaf meristem formation, meristematic or mitotic cells in the primordia are partitioned by an "arrest front", where cell proliferation and expansion alternate. The genes involved in the positioning of the arrest front remain unclear, but mutations in *BLADE-ON-PETIOLE* (*BOP*) *1/2* and others show prolonged activation of the leaf meristem to cause abnormally elongated leaf lamina (Ha et al. 2003). In normal leaves, the arrest front suddenly drops to the base of the leaf primordia to shut down cell proliferation activity (Kazama et al. 2010; Andriankaja et al. 2012), even if cell-cycle regulators are kept active artificially. It is still unknown which genes encode the forced termination. On the regulatory mechanisms of switching between cell proliferation and cell expansion, local maxima in gibberellic acid (GA) level (Nelissen et al. 2012) and exchange of GROWTH REGULATING FACTOR (GRF) transcription factor component in huge AN3 protein complexes (Nelissen et al. 2015) were proposed to be keys in maize leaf primordia.

A conspicuous feature of leaf cross-sections is the leaf thickness. Sun-type leaves have many layers of palisade cells, which are much more elongated, thus adding to the leaf thickness compared with shade-type leaves;

this is also the case in the model plant arabidopsis (Kim et al. 2005). In arabidopsis leaf thickness is influenced also by growth temperature (Adams et al. 2016). In studies of *Chenopodium album* L., mature leaves under high irradiance were shown to emit signals to the SAM to differentiate sun-type leaves, even if the shoot apex received low light intensity (Yano and Terashima 2004). However, the molecular mechanisms underlying this signaling are not yet known. In addition, how and when the number of palisade cell layers is determined are also unclear, even in arabidopsis sun-type leaves. Furthermore, the evolution of succulent plants is an even greater mystery. On the other hand, the elongation of palisade cells in sun-type leaves is controlled by the blue-light receptors phototropins (Kozuka et al. 2011).

B. *Mechanisms for Compound Leaf Formation and Others*

The processes above are based on studies of simple leaves, such as those of arabidopsis, snap dragon, and maize, but are also applicable to compound leaves, which have several independent morphological units (leaflets). One exception to the common processes is the maintenance of KNOXI gene family expression in the primordia of compound leaves (Bharathan et al. 2002). In simple leaf primordia, KNOXI gene expression is shut down after emergence from the SAM where KNOXI are expressed (reviewed in Hay and Tsiantis 2010; Fig. 1.1). KNOXI appears to give the potential for repeated organogenesis in the expressed region. Although KNOXI expression has often been discussed in relation to "meristematic activity" or "indeterminacy", it is the opinion of the author that this is not the case. This is because (1) if the term "meristematic" means active cell proliferation, cell proliferation is much more extensive in the leaf primordia than in the SAM (which is why the leaf primordia can protrude from the SAM flanking region), (2) indeterminacy of the cell-proliferation stage in the leaf primordia results in elongated leaf lamina, as seen in the *bop* mutants (and some other plant species discussed later), but not in compound leaves, and (3) a combination of the ectopic expression of KNOXIs and *bop* mutation results in indeterminate repeats of compound leaf formation, indicating that repeated organogenesis and indeterminacy are regulated by independent pathways.

The downstream targets of KNOXIs include GA biosynthesis (Sakamoto et al. 2001) and cytokinin pathways (Jasinski et al. 2005). Indeed, the GA pathway is deeply involved in KNOXI-dependent serration development and lobe formation in arabidopsis and tomato leaves (Hay et al. 2002). Heteroblastic change from simple to compound leaves in *Rorippa aquatica* (Eaton) E.J. Palmer & Steyerm. is also mediated by the KNOX-GA gene module (Nakayama et al. 2014). The upstream regulators of KNOXI ASYMMETRIC LEAVES (AS)1 and AS2 are also involved in cytokinin regulation (Takahashi et al. 2013). These hormonal pathways are thought to be responsible for repeated organogenesis.

Molecularly, both deep serrations and compound leaf formation are usually associated with the continued expression of the KNOXI genes in the leaf primordia (Bharathan et al. 2002), and pattern formation is associated with the CUP-SHAPED COTYLEDON (CUC) gene family (Blein et al. 2008; Fig. 1.1). While the wild-type Columbia accession of arabidopsis leaves have a smooth margin, they actually have a serrated structure (Tsukaya and Uchimiya 1997; Kawamura et al. 2010). The pattern of serration tip positioning depends on the flow of auxin that is regulated by PIN1 (Kawamura et al. 2010, Bilsborough et al. 2011). Similar to phyllotaxy mechanisms in the SAM, autoregulation of auxin maxima formation determines the position of the serrations on the leaf margin; this pattern is further stabilized by additional expression of *CUC2* (Kawamura et al. 2010; Bilsborough et al.

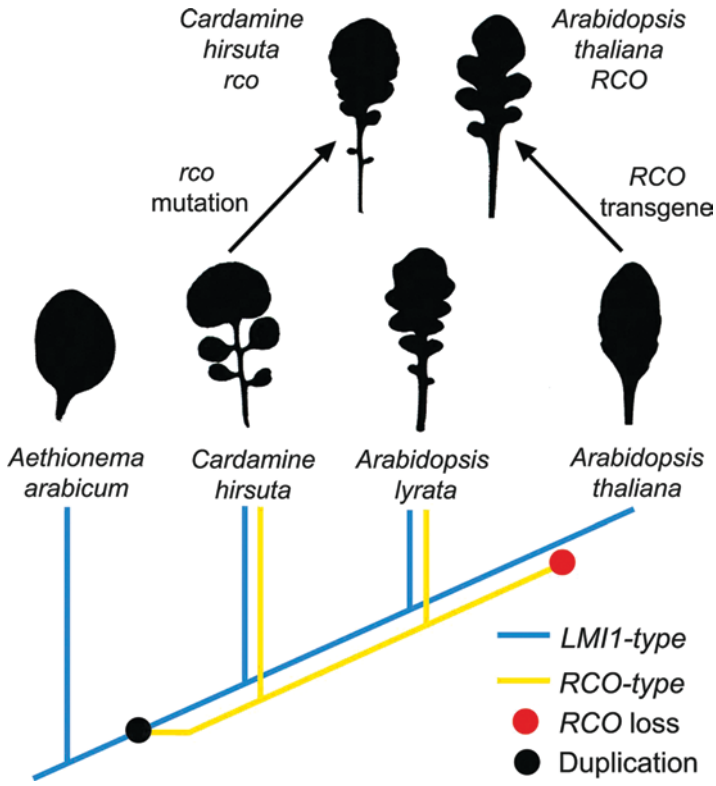

Fig. 1.2. *RCO* regulates the complexity of leaf shape in Brassicaceae. (This figure was reproduced from Vlad et al. 2014, with permission)

2011; Fig. 1.1). During early stages of development, the serrations are very evident, showing pointed tips, and tip growth is accelerated by the function of *CUC2*; thus, the loss-of-function *cuc2* mutant has a smaller serration height than that of the wild type, which shows a smoother leaf margin (Kawamura et al. 2010). During later stages of development, serration becomes unclear due to anisotropic growth of the leaf lamina or the widening of each unit of serration. The patterning of leaflet formation in a compound leaf primordium in tomato and *Cardamine hirsuta* L. is also regulated by formation of auxin maxima along the leaf margin by the PIN-mediated auxin flow, that is stabilized by CUC gene expression (Barkoulas et al. 2007; Koenig et al. 2009; Wang et al. 2005).

Because ectopic expression of KNOXI does not promote the switch from a simple to a compound-leaf type in arabidopsis (deeply serrated with creation of only a few leaflets), it has been speculated that the genetic factors responsible for compound leaves are lacking in arabidopsis. From comparative analyses between the compound leaves of *C. hirsuta* and arabidopsis, Vlad et al. (2014) revealed that REDUCED COMPLEXITY (*RCO*), a homeobox gene, is a key gene (Fig. 1.2). The *RCO* gene was lost from the simple leaf arabidopsis lineage, and the loss-of-function mutant of *RCO* in *C. hirsuta* developed only a few deep leaf serrations. Sustained *SHOOT MERISTEMLESS* (*STM*)-type KNOXI in leaf primordia is also the key difference between *C. hirsuta* and arabidopsis leaf primordia. With regard to leaflet organogenesis, activity of the marginal meristem was shown to be important (Barkoulas et al. 2007).

Interestingly, not all compound leaves are formed by the action of KNOXI. An excep-

tional case in compound-leaf formation has been seen in some legumes, including *Pisum sativum* L., which does not express KNOXIs but rather the LEAFY (LFY)/FLORICAULA (FLO) homolog (Hofer et al. 1997). The unique mode of compound leaf formation in palms, by means of orchestrated cell death (Dengler and Dengler 1984), is also independent of KNOXI (Nowak et al. 2011). Diversity in the positioning of the meristematic regions along the longitudinal axis is also seen in compound leaves, but its molecular background appears to be species-specific and is not shared among taxa (Ikeuchi et al. 2014; reviewed in Tsukaya 2014).

Finally, age-dependent or physiological condition-dependent shape and size differences are a common feature of leaves in angiosperms and gymnosperms (heteroblasty or heterophylly). The mechanisms underlying heteroblasty involve microRNA-mediated control of transcription factors (reviewed in Poethig 2013). In short, cell size and numbers in the leaf lamina are controlled by the *miR156*-mediated pathway (Usami et al. 2009), and the overall leaf shape and trichome distribution pattern are controlled by both the *miR156*- and *miR-ARF*-mediated pathways; this system is conserved among weeds such as arabidopsis and in maize and trees such as *Acacia colei* Maslin & L.A.J. Thomson and *Eucalyptus globulus* Labill. (Wang et al. 2011).

Based on the above, it is important to review the evolutionary diversification of leaf shapes. While the leaves in some angiosperms have lost their photosynthetic roles, as seen in some cacti, saprophytes/mycoheterotrophs, and parasitic plants, which have evolved very unique morphologies, they were omitted from this review, which is focused on the leaves of photosynthesizing plants. Other review articles on closely related topics are available (e.g., Cronk et al. 2002; Nicotra et al. 2011; see Tsukaya 2018 for saprophyties/mycoheterotrophs).

II. Natural Variation in Leaf Width

Leaf index is defined as the ratio of leaf length to width (Tsukaya 2002a). Although a larger total area is more advantageous in terms of photosynthetic productivity, it is not always an advantage to have wider leaves. The optimal leaf width, or the distance from the midvein to the leaf margin, has been driven evolutionarily by the growth habitat and other external conditions experienced by a species. For this reason, leaf index diversity can be discussed in terms of natural selection. First, we will examine some examples of natural selection for leaf width, and then we will investigate the genetic systems possibly underlying natural variation in this feature.

The narrow leaf shape (larger leaf index = stenophylly) of rheophytes is understood to be an adaptive response to the strong shearing forces produced when river water levels rise (van Steenis 1981; Fig. 1.3**)**. The areas along streams are open and obtain plenty of sunlight; however, if the water level rises, the plants growing there must be able to tolerate the strong shearing forces created underwater. Because of this, the rheophytes are characterized by a combination of narrow leaves, a dense root system and a horizontally elongating shoot system (van Steenis 1981; Kato and Imaichi 1992a, b; Imaichi and Kato 1992; Usukura et al. 1994). Rheophytes have evolved repeatedly in many families of mosses, ferns, and angiosperms, indicating that these morphological changes can be achieved through few mutations. As seen in a typical example of the rheophyte fern, *Dipteris lobbiana* (Hllk.) T. Moore, in comparison with the terrestrial species *D. conjugate* Reinw., the leaf area of rheophytes is significantly reduced by stenophylly (Fig. 1.3a, b). This is also present in angiosperm rheophytes, as seen in the comparison between terrestrial *Ainsliaea apiculata* Sch. Bip. and rheophytic *A. linearis* Makino (these

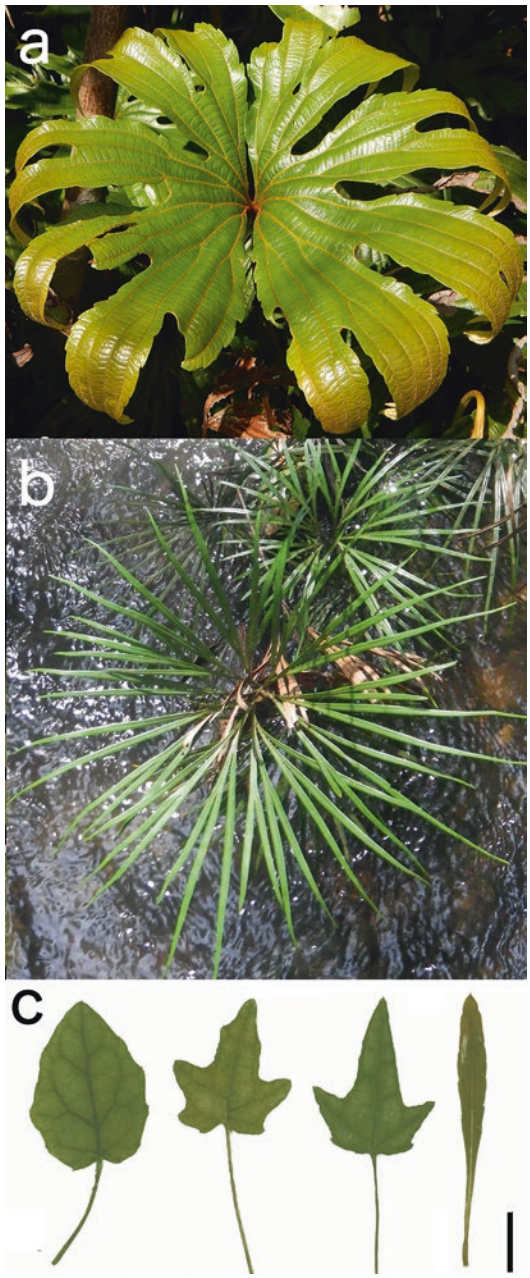

Fig. 1.3. Rheophytes have narrower leaves. (**a**) Terrestrial *Dipteris conjugate* Reinw. compared with (**b**) a closely related rheophyte species, *D. lobbiana* (Hook.) T. Moore. (**c**) Leaf shape diversity in *Ainsliaea apiculata* Sch. Bip. and its close relative *A. linearis* Makino, a rheophyte (far right). Scale bar in (**c**) = 1 cm

two species can interbreed; Tsukaya et al. 2007; Fig. 1.3c), in which there is no compensation in leaf area by an increase in leaf length.

Because a reduction in leaf area is disadvantageous for photosynthetic productivity, the disadvantage of having the rheophytic morphological characteristic is counterbalanced by the advantages gained by the ability to tolerate the shear forces during periods of flood. Because of this trade-off, rheophytes show clear habitat isolation from non-rheophytes in natural habitats. Curiously, rheophyte ferns and angiosperms differ in the anatomical changes associated with the rheophytic habit (Tsukaya 2002a). In ferns, comparative studies between rheophyte species and the most closely related non-rheophyte species have shown that differences in cellular shape and/or size underlie the differences in leaf indexes. In contrast, most angiosperm rheophytes examined to date appear to differ in the number of cells per leaf blade in comparison with closely related non-rheophyte species (Usukura et al. 1994; Tsukaya 2002b). There are, however, some exceptions, such as those seen in an intraspecific variation in *Arundina graminifolia* (D. Don) Hochr. (Orchidaceae), in which not only cell numbers but also cell sizes differ between the rheophyte- and non-rheophyte forms (Yorifuji et al. 2015). From such comparative analyses, it is strongly suggested that two major pathways control the leaf index: 1) differences in cell shape and/or size (as seen in ferns) and 2) differences in cell number (as seen in angiosperms). Possible genetic pathways for these differences will be discussed later.

Narrow leaves also have an advantage in harvesting water from fog under particular climatic conditions. Martorell and Ezcurra (2007) reported that "narrow-leaf syndrome" is seen in some xerophytic species of the genera *Tillandsia* (Fig. 1.4**)** and *Agave* that depend on the interception of fog for obtaining water. Since the interception of fog depends on leaf area, and the efficiency of water capture increases with decreasing thickness of the leaf boundary layer, the best adaptation is long, narrow leaves. Such effect of the narrowness is also greatly aided by dense trichomes on the surface that increase

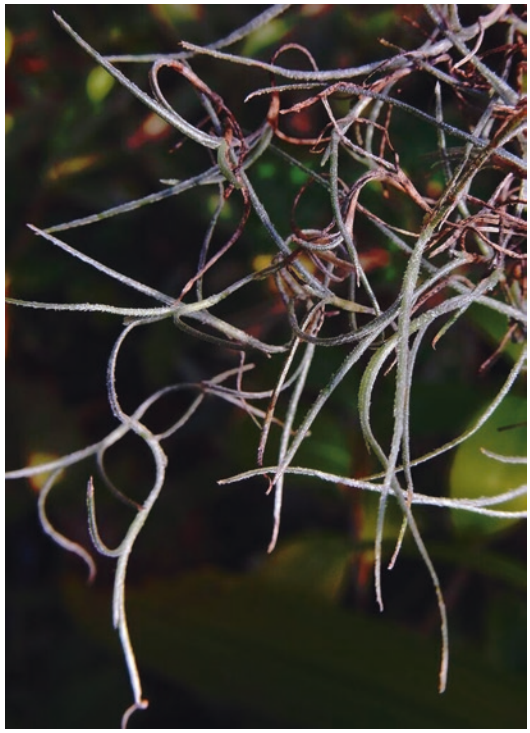

Fig. 1.4. Narrow-leaf syndrome seen in a shoot of *Tillandsia usneoides* (L.) L. Note its filamentous leaves with silvery hairs

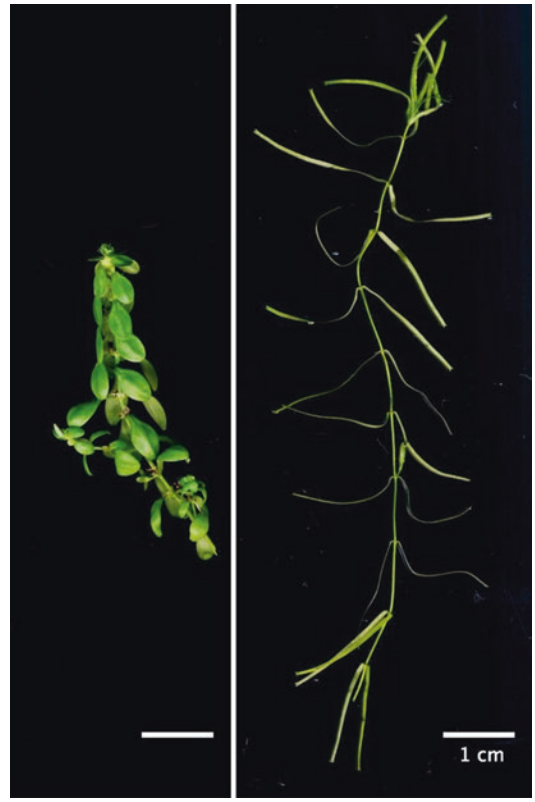

Fig. 1.5. Heterophylly of amphibious plants. Aerial (left) and submerged (right) shoots of *Callitriche palustris* L. Note the morphological differences in leaf shape and size between the two growth conditions. (Photograph courtesy of Dr. Hiroyuki Koga (Univ. Tokyo))

the boundary layer (Fig. 1.4). Other factors are also thought to influence the leaf index in nature. For example, in *Eucalyptus globulus* (Myricaceae), heteroblastic differences in leaf index (short ovoid leaves in juveniles and long sickle-shaped leaves in adults) are associated with differences in oviposition of two herbivorous insects (Brennan et al. 2001). Unlike the isobilateral adult leaves in *E. globulus*, juvenile leaves are non-isobilateral, with different stomatal distribution, palisade distribution, leaf thickness, and wax content. In this particular case, it is possible that the differences between juvenile and adult leaves lie not only in leaf shape but also in chemical composition, leading to altered behavior of the herbivorous insects. However, the morphology of the leaves could also affect herbivory in some conditions. The factors influencing the selection of a particular leaf index in a given species may thus differ from case to case.

Other than speciation-dependent leaf index differences, heterophyllic leaf width differences are seen between aerial and submerged shoots in many amphibious plants (Fig. 1.5). In *Hippuris vulgaris* L., *Callitriche* spp., *Ludwigia arcuata* Walter, and other simple-leafed species, the leaf index is smaller in aerial shoots and larger in submerged shoots. In *Rorippa aquatica* and some species of the genus *Ranunculus,* such as *R. flabellaris* Raf., aerial shoots have simple leaves and submerged shoots have deeply serrated compound leaves. In all cases, the distance to the leaf margin is shorter in the submerged leaves, and because the dispersal speed of CO_2 or HCO_3^- is relatively slow in water compared with air, the submerged-

type leaf shapes are thought to be adaptive. To date, the phytohormonal regulators abscisic acid, GA, and ethylene underlie heterophyllic development in response to water conditions (e.g., Deschamp and Cooke 1984, Kane and Albert 1987; Young et al. 1987; Kuwabara et al. 2003; Nakayama et al. 2014), while recruitment of the switching mechanisms in these amphibious plants remains to be elucidated.

As mentioned above, narrow and compound leaves or deeply serrated/lobed leaves may provide for similar interactions with the surrounding environment, including factors beyond the submerged conditions for CO_2 uptake. The boundary layer surrounding the leaf surface is thickest at the point most distant from the leaf margin. The thickness of the boundary layer also influences leaf temperature under low wind speeds, when other factors such as evaporation/transpiration-dependent cooling effects are restricted by a dry atmosphere: namely, a thicker air boundary layer can contribute to over-heating. A leaf energy balance model showed that large or wide leaves have an advantage in cooler environments (Okajima et al. 2012). In contrast, at high temperatures in the absence of wind, leaves with a thinner boundary layer are thought to be more adaptive than those with a thicker boundary layer. Indeed, comparative analyses have shown that narrow-leaf species are often small-leaved, and wider-leaf species are often larger (Tozer et al. 2015). Thus, the evolution of the leaf index can be discussed in line with the evolution of compound leaves. However, it should be emphasized that smaller or narrower leaves are not always adaptive under high-temperature conditions; under dry, low-temperature conditions, smaller leaves dominate (Givnish 1987). There is, moreover, a 10^3-fold variation in leaf area in any one particular habitat (Fonseca et al. 2000), indicating that leaf area has been selected not only by temperature but also as a result of many other factors.

Finally, leaf asymmetry is based mostly on unequal leaf widths between the left and right halves of the lamina. A typical left-right asymmetry in the leaf lamina is seen in species of the genera *Begonia* and *Elastema.* Here, asymmetry is maintained at the shoot level, wherein the smaller side is located in the inner position and the larger side in the outer position. This positioning seems to be effective in maximizing light absorption by minimizing self-shading. Auxin-dependent phyllotaxy patterning has been shown to result in an unequal distribution of auxin between the left and right sides of the leaf, causing a slight but apparent asymmetry in the leaf blades, even in tomatoes and arabidopsis, which appear to have symmetric leaves (Chitwood et al. 2012). This type of unequal auxin distribution in the leaf primordium of the left and right sides may also underlie the strong asymmetry of the leaf blades in *Begonia* and *Elastema.* Along with leaf asymmetry, as seen in the genus *Elastema,* anisophylly is also often observed (Dengler 1999). Anisophylly is shoot asymmetry manifested as different leaf sizes and shapes between the dorsal and ventral sides. The mechanisms underlying anisophylly are largely unknown, but since anisophylly is associated with asymmetry of each leaf and shoot, it is plausible that an uneven distribution of auxin is involved.

III. Genetic Factors Underlying Leaf Index Variation

As summarized above, many factors are thought to be involved in the selection of a particular leaf width for any given species. The question arises as to which genes are involved in the evolution of the leaf index. As suggested from comparative morphologies in rheophytes, developmental genetic studies in a model species of eudicot, arabidopsis, have shown that leaf length and width are controlled independently; two major pathways control either the leaf length or

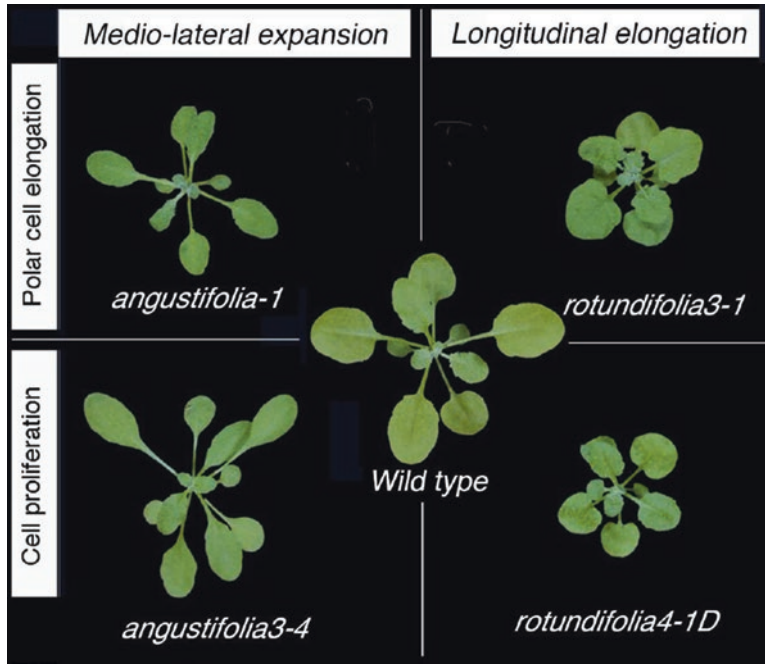

Fig. 1.6. Four typical examples of arabidopsis mutants with altered leaf indexes. Underlying alterations in cell growth are depicted along the left side and resulting impacts on leaf development are depicted along the top. (This figure was slightly modified from Tsukaya 2006)

width via regulation of cell shape or number (Tsukaya 2013; Fig. 1.6). Examples of genes that regulate cell shape and affect the leaf index were identified from studies of mutants with defects in leaf width or length (Tsuge et al. 1996). The *angustifolia* (*an*) mutation causes narrower and thicker leaves, which is very similar to the leaf shapes seen in rheophytes, but the leaf length is maintained by changes in the shape of each cell in the leaf lamina (Tsukaya et al. 1994; Tsuge et al. 1996). The AN protein is a homolog of the animal C-terminal Binding Protein/BFA–ADP-Ribosylation Substrate (CtBP/BARS) protein family, but its intercellular localization differs from those in animals, and it is uniquely located neighboring to the trans-Golgi network (Minamisawa et al. 2011). Studies of its molecular function are underway. The *rotundifolia3* (*rot3*) mutant shows the opposite phenotype to that of the *an* mutant, having shorter leaf blades of normal width (Tsuge et al. 1996). Molecular cloning of the responsible gene revealed that the *ROT3* gene encodes a cytochrome P450 (Kim et al. 1998) and catalyzes C-23 hydroxylation involved in the biosynthesis of brassinosteroid, an important phytohormone (Ohnishi et al. 2006). LONGIFOLIA (LNG) 1/LNG2 also affects leaf length: *LNG* overexpression results in long, slender leaves, and loss-of-function mutants have shorter leaves due to changes in cell size (Lee et al. 2006). In all of the mutants mentioned here, there was no particular decrease in plant activity or seed production at least under artificial growth conditions, and thus it is possible that similar mutations might have contributed to the natural evolution of leaf shape under environmental selection.

In arabidopsis, many genes control cell number, resulting in leaf index changes. There are 23 members of the ROTUNDIFOLIA FOUR-LIKE/DEVIL (RTFL/DVL) gene family in arabidopsis (Guo et al. 2015), most of which encode

peptides that, upon over-expression in leaves, affect the number of leaf cells along the longitudinal axis (Narita et al. 2004; Wen et al. 2004; Yamaguchi et al. 2013). A homolog of this gene from rice also shows a similar effect when expressed in arabidopsis (Guo et al. 2015). Because of the presence of many paralogs with similar functions, the phenotypes of the loss-of-function mutations in this gene family in arabidopsis are unknown (Narita et al. 2004; Wen et al. 2004). An interesting example of leaf index mutants is seen in the *an3*/*atgif1* mutant (Kim and Kende 2004; Horiguchi et al. 2005). The AN3 protein, a transcriptional co-activator, interacts with the AtGRF5 transcription factor to promote cell proliferation in the leaf meristem (Horiguchi et al. 2005), which is seen in the junction between the leaf blade and petiole (Ichihashi et al. 2011). While the AN3 protein itself does not regulate the direction of cell division, its loss-of-function mutants have narrower and smaller leaves, with a more severe defect in cell number in the medio-lateral or leaf-width direction than in the longitudinal direction. The reason the *an3* mutant has a larger leaf index and fewer cells in the medio-lateral axis than in the longitudinal axis is because the activation of AN3 is only seen during the developmental stages at which the cell-division planes are randomized to expand the leaf lamina; however, it is not required during the early stage of leaf development at which most cell-division planes are regulated perpendicularly to the longitudinal axis (Horiguchi et al. 2011). Therefore, the shortage of cells is much more evident in the medio-lateral direction than in the longitudinal direction, leading to a larger leaf index in the *an3* mutant. In this case, fertility and growth are normal, and it is possible such a mutation could be involved in natural evolutionary changes in the leaf index.

It should be emphasized that not all genes regulating leaf size act in an anisotropic manner. Most genes affect leaf size in an isotropic way. For example, over-expression of *AN3* results in larger leaves with the same proportion/leaf index as that of the wild-type leaves (Horiguchi et al. 2005). *DA1,* which encodes a ubiquitin receptor, also controls cell proliferation in the leaf primordia, and overexpressing and loss-of-function mutants show changes in the leaf area without significant effects on the leaf index (Li et al. 2008). The *extra-small sisters* (*xs*) mutants, which show specific defects in the cell expansion processes in leaves, also have smaller leaves compared with the wild-type leaf index (Fujikura et al. 2007). This is also the case for the *oligocellula* (*oli*) mutants, which show specific defects in the number of cells per leaf lamina, but no defects in cell size (Fujikura et al. 2009). Only a subset of genes regulating leaf development are involved in anisotropic growth and are thought to cause the leaf-index variations involved in environmental adaptation.

IV. Diversity in Compound Leaves and Leaves with Serrated Margins

Entireness of the leaf margin (i.e., smooth margins) in trees is more common in areas with a higher annual mean temperature (Bailey and Sinnott 1916; Royer et al. 2005). This has been confirmed in many areas and has been used to estimate ancient climates from fossil evidence, as serrated leaves are more common in areas with lower annual mean temperatures. This tendency has also been confirmed in two different phylogenetic lineages of the genus *Viburnum* (Schmerler et al. 2012), indicating a strong correlation between marginal serration and air temperature. The correlation of leaf tooth variation with climate of the native habitat was also seen in an intraspecific study of leaf variation in *Acer rubrum* L. (Royer et al. 2009).

What has led to this leaf margin-air temperature correlation? Serrations can affect the temperature of the leaf surface if the ser-

rations/lobing are sufficiently deep to reduce the distance across the lamina, i.e., create a narrower leaf lamina. The narrower or smaller lamina width in simple leaves and the short distance to the leaf margin in compound or deeply serrated leaves could be regarded as the same adaptive form in terms of heating (e.g., Leigh et al. 2006). But the logic is contrary to the observed correlation if we consider the effect of boundary layer thickness on leaf temperature, evolutionary trends in morphological complexity, and leaf index, all of which are correlated among species. It has also been argued that gas exchange is enhanced around the toothed margins; however, Feild et al. (2005) did not detect such an effect. Instead, Feild et al. (2005) found that the toothed margins are important to release root pressure; if guttation from the teeth hydathodes is blocked, mesophyll intercellular spaces are flooded resulting in decreased photosynthetic activity. In the case of the genus *Vibrunum,* the southern species have evergreen, narrow, and integrated leaves, while the northern species have seasonally deciduous, round, and serrated leaves (Schmerler et al. 2012). The authors therefore speculated that the round leaves of the northern species might have adapted to the cooler environment by altering leaf morphology to increase the air boundary layer and minimize any damage caused by low temperature; the narrower leaf shape in the southern species could be an adaptation to the warmer environment. The serrations may increase sap flow and/or gas exchange, but data on this are controversial.

As Nicotra et al. (2011) stated, the relationship between serration/lobing and adaptation to air temperature is not simple. Leaf temperature can be controlled by not only the thickness of the boundary layer that is lost under moderate wind but also by transpiration. The adaptive significance of leaf serration/lobing can be discussed in relation to adaptation to a shortage of water and/or wind-caused draught. Case-by-case discussions may be required to understand the reasons for differences in leaf margins in terms of adaptations to different external environments. For example, Nicotra et al. (2008) examined leaf shape variations in South African *Pelargonium* species in relation to environmental adaptation and discussed that the deeply serrated leaves may be an adaptation to the combination of the hot, dry summer season and cool, wet winter season in their native habitat in South Africa.

Recently, Givnish and Kriebel (2017) compared six major hypotheses on the role/function of serrations based on published data, and discussed that "support-supply hypothesis" is the most plausible idea among them. This support-supply consideration is based on calculation of the optimal lamina area for a major vein to mechanically support the mesophyll tissue while also supplying it with water and nutrients; it is said to explain the commonly observed tapering of the leaf around its midrib toward the tip. This idea also predicts that thinner leaves should have serrated margins. Of course, the support-supply hypothesis does not encompass every feature that is relevant to leaf size and shape, and the authors proposed a model in which additional factors considered in other hypotheses can contribute to the morphology of leaf margins (Givnish and Kriebel 2017).

V. Proximal-Distal Pattern Variation

One of the major factors contributing to the diversity of leaf-shape variation, other than the leaf index and leaf complexity, is the positioning of the leaf lamina along the proximal-distal axis of the leaf primordium. Some species do not have a petiole, while others have prominent petioles. The presence of a long petiole is costly, but it is an adaptation to minimize self-shading by leaves, particularly in rosette plants (e.g., Yamada et al. 2000). Re-positioning of the leaf lamina in response to the direction of the light is also easier for leaves with petioles (e.g., Mano et al. 2006). It is unclear which genes are

involved in this, but in arabidopsis, loss-of-function mutations in the *BOP* genes cause a reduction in the naked petiole region and an extension of the leaf lamina to the proximal section of the leaves (Ha et al. 2003). Analysis of gene expression patterns suggested that *bop* mutations cause a shift in the expression domain of *AN3,* a key regulator of the leaf plate meristem, to the more basal regions (Ichihashi et al. 2011). Since the *bop* mutations also cause prolonged organogenesis of the leaf lamina, this mutation cannot be attributed to natural variation in the absence/presence of the leaf petiole. However, other related systems might also be involved in the variation.

A minor variation observed in the development of the leaf lamina along the proximal-distal axis relates to the positioning of the widest part, as seen in the difference between ovate and obovate leaves. Obovate leaves can minimize self-shading due to leaf overlap as seen in leaves with long petioles. This variation may reflect the distribution pattern of the most active cell proliferation zone in the leaf primordia. An extreme example is seen in a gymnosperm, *Ginkgo biloba* L., in which cell proliferation is most extensive in the apical margin of the primordia, resulting in fan-shaped leaves (Fig. 1.7), while its marginal positioning of leaf meristem may suggest that it is not distal replacement of the plate meristem but rather a long-lived marginal meristem. How the positioning of the leaf meristem varies along the proximal-distal axis is not yet fully understood, but spatial changes in miR396 expression reportedly alter the positioning of the leaf-meristem zone in arabidopsis (Gupta and Nath 2015). miR396 regulates the mRNA levels of certain AtGRFs that bind to AN3/AtGIF1 to activate the leaf meristem; AN3/AtGIF1 is expressed in the basal part of the leaf primordia marking the position of the leaf meristem; over-expression of miR396 or loss-of-function of certain *AtGRFs* or *AN3/AtGIF* results in poor leaf meristem activity and smaller leaves (reviewed in Kim and Tsukaya 2015; Omidbakhshfard et al. 2015). Therefore, it is possible that variations in spatio-temporal expression patterns of GRF-GIF-miR396 underlie the positional variations in the leaf meristem in some species showing variations in the widest part along the proximal-distal axis.

Fig. 1.7. Altered patterning in the leaf lamina positioning of fan-shaped leaves of *Ginkgo biloba* L. along the proximal-distal axis

A more drastic variation in the leaf-lamina positioning along the proximal-distal axis is seen in peltate leaves. For example, the lotus (*Nymphaea nucifera* Gaertn.) and nasturtium (*Tropaeolum majus* L.) have typical peltate leaves (Fig. 1.8a, b), defined by the attachment of the leaf petiole in the abaxial face of the leaf lamina. This morphology seems to be adaptive for positioning the leaf surface parallel to the horizon to maximize light capture, under the assumption that no other plants shade them. Indeed, lotus grows in muddy ponds and does not cohabitate with other plants; rhizomes extend parallel to the horizon, positioning each leaf at an appropriate distance to minimize self-shading. In arabidopsis, such peltate or cup-like leaves are found in mutants with defects in dorsiventral control. For example, the *as2* mutant with a defect in dorsiventrality often forms peltate or cup-shaped leaves, particularly in the presence of enhancer mutations (Xu et al.

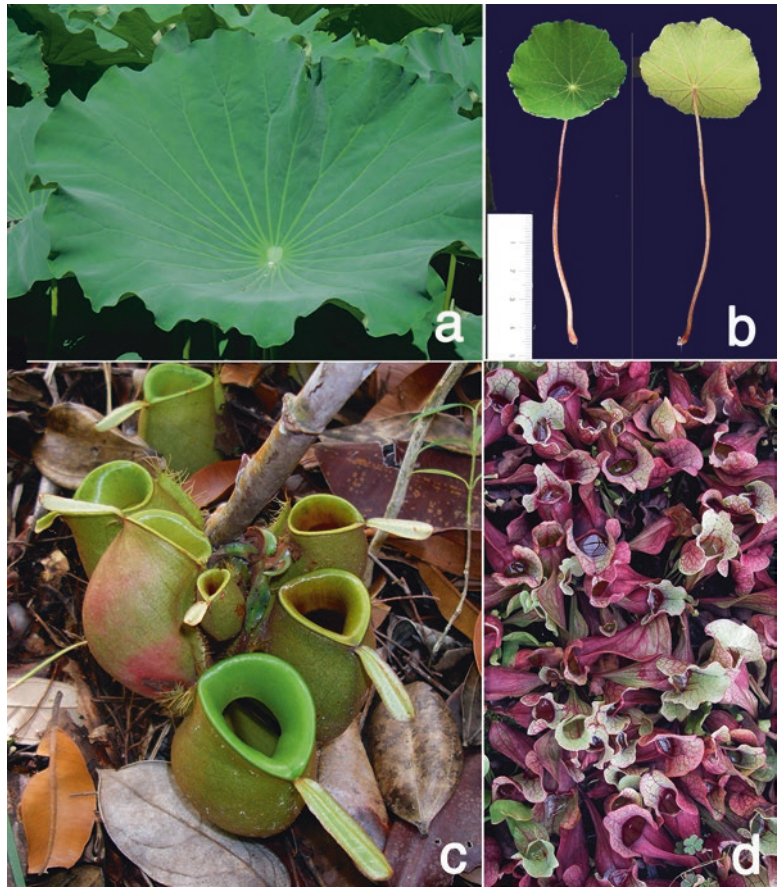

Fig. 1.8. (**a**) a peltate lotus leaf and (**b**) adaxial (left) and abaxial (right) views of a peltate nasturtium leaf. Pitcher leaves of (**c**) *Nepenthes ampularia* Jack (photo taken at Bako National Park, Sarawak, Malaysia) and (**d**) *Sarracenia purpurea* L.

2003; Horiguchi et al. 2011), suggesting that peltate leaf formation is caused by altered patterning of dorsiventrality in nature. According to Gleissberg et al. (2015), this is the case for the peltate leaves of nasturtiums. The basal portion of the nasturtium leaf primordium exhibits only abaxial identity without the adaxial domain, and the boundary between the adaxial and abaxial fates is formed in the distal portion of the primordium. Because plate meristem formation for flat lamina development depends on the establishment of the abaxial-adaxial boundary (Waites and Hudson 1995), Gleissberg et al. (2015) showed that this altered positioning of the abaxial-adaxial boundary is reflected in the delayed initiation of the leaf lamina, resulting in peltate leaves.

A similar variation mechanism underlying the positioning of the leaflets along the proximal-distal axis in compound leaves was proposed by Kim et al. (2003). Specifically, the differences among pinnate, non-peltately palmate, and peltately palmate leaves are attributed to the varied spatial expression pattern of the*AS1*/*PHANTASTICA* (*PHAN*) gene, which regulates the adaxial domain (see also Efroni et al. 2010). Variation in the positioning of the abaxial-adaxial boundary is one of the major factors that has enabled the evolution of many leaf shapes.

VI. Pitcher Leaves

One of the most curious leaf shapes seen in angiosperms are the pitcher leaves of carnivorous plants (Fig. 1.8c, d). These pitcher leaves have a dual function: photosynthetic production of sugars and acting as a trap to absorb nutrients from insects. From comparative studies, it was speculated that pitcher leaves have evolved by a similar mechanism to that of peltate leaves mentioned above. In fact, in arabidopsis, some mutants with a defect in dorsiventral patterning often develop not only flat peltate-like leaves, but also cup-shaped leaves. It was therefore speculated that changes from flat, lotus-like peltate leaves to cup-shaped leaves were extended to result in the evolution of pitcher leaves.

Fukushima et al. (2015) examined this theory using *in situ* hybridization analysis of adaxial and abaxial gene expression in the pitcher leaf primordia of *Sarracenia purpurea* L. but found no proximal-distal abnormality in the spatial establishment of the dorsiventral domain in this species. Namely, compared with the nasturtium leaf primordia in which the abaxial domain surrounds the basal section (Gleissberg et al. 2015), *S. purpurea* leaf primordia have an adaxial domain from the proximal to the distal end, although the leaves are narrower than normal. Therefore, pitcher leaf formation in *S. purpurea* differs from the formation of the peltate leaves in nasturtiums. Instead, Fukushima et al. (2015) identified a cell division pattern that strongly differed locally in the primordia and that transformed the rod-shaped initial primordia into a tube-like, pitcher form. Thus, pattern changes in dorsiventrality do not necessarily underlie the evolutionary diversification of leaf shapes.

VII. Unifacial Leaves – Terete and Ensiform Types

Another evolutionary trait in leaf morphology is also present in relation to dorsiventral control. If the photosynthetic productivity of leaves is to be maximized per individual leaf, most leaves should expand horizontally to receive as much sunlight as possible, when the photon density is not too high for photosynthesis. Indeed, in arabidopsis, a typical rosette plant, positioning of the leaf lamina is under the control of negative gravitropism and nastic movements, which are both suppressed by light perception, resulting in horizontal expansion of leaves perpendicular to the shoot axis (Mano et al. 2006). On the other hand, if photosynthetic productivity depends on the sum of all leaves in the individual, then the leaves can stand straight up to form a denser plant, compared with those with horizontal expansion, as seen in needle leaves or rice. In this case, leaf shape is also different from that of horizontally positioned leaves.

In rice, as often seen in members of Gramineae, the leaf blade is ribbon-like in form, and dorsiventral differentiation of the adaxial (palisade) and abaxial (spongy) tissues in the lamina is poor. Poor dorsiventrality is reasonable if we consider that rice leaves usually stand perpendicularly to the horizon, via the presence of a strong and hard midrib tissue in the center of the leaf lamina. The development of the thick midrib depends on the activity of the *DROOPING LEAF* (*DL*) gene, a member of YABBY (YAB) transcription factor gene family (Yamaguchi et al. 2004). If *DL* activity is lost, the leaves cannot stand up and droop. Photosynthetic efficiency is much lower under these conditions. Densely arranged, straightly elongated leaves are also seen in needle-type leaves of conifers.

The dense positioning of standing leaves is typically seen in monocots, particularly in species with unifacial leaves (Fig. 1.9). Unifacial leaves only have an abaxial identity in the leaf blade, as seen in many *Allium* and *Iris* species of monocots. It is not yet known why unifacial leaves are dominant in monocots but rare in eudicots. As mentioned above, the establishment of both adaxial and abaxial identities is necessary for the lateral expansion of the leaf lamina;

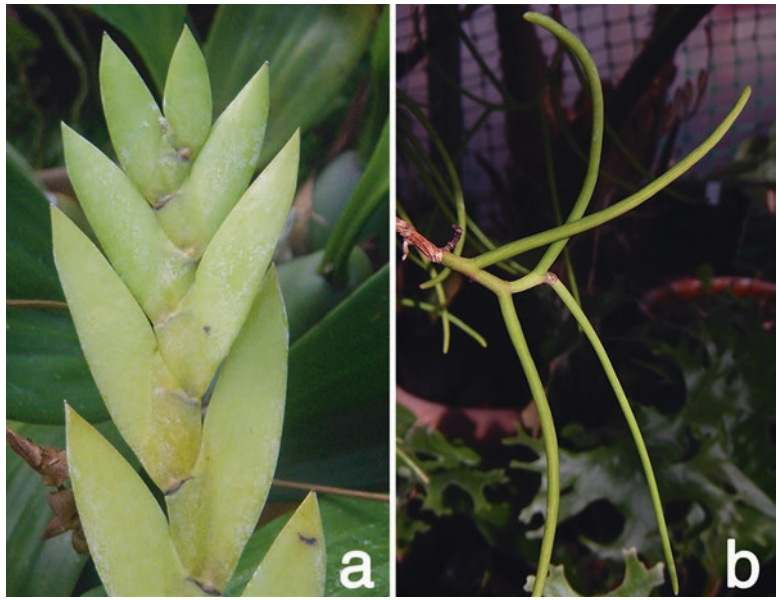

Fig. 1.9. (**a**) *Dendrobium anceps* with flat, ensiform unifacial leaves arranged in a fan-shaped manner. (**b**) *Luisia teres* (Thunb.) Blume shoot with terete, stick-like unifacial leaves

thus, the loss of adaxial identity results in the loss of the flat leaf lamina. In fact, many unifacial-leaf species develop terete or stick-formed leaves as seen in *Allium fistulosum* L. and *Luisia teres* Blume. However, a flat leaf lamina is also seen in many species with unifacial leaves, such as in the ensiform leaves of *Iris germanica* L. and Dendrobium anceps Sw. The unique feature of the flat lamina in these unifacial leaves is recognized in their developmental direction. In bifacial leaves with normal dorsiventrality, the leaf lamina expands along the adaxial-abaxial boundary of the leaves to result in radial expansion from the shoot axis. This directional expansion is adequate to arrange the leaves around the shoot axis and for horizontal extension. On the contrary, the flat lamina of unifacial leaves are arranged as a fan-like shape, expanding parallel to the shoot axis (Fig. 1.9a). Thus, the orientation of expansion in bifacial and unifacial leaves is perpendicular to each other.

Comparative molecular genetic studies of two closely related *Juncus* species (Juncaceae), *J. prismatocarpus* R.Br. with ensiform leaves and *J. wallichianus* Laharpe, revealed that flat lamina formation in the ensiform species depends on the activity of a *DL* homolog (Yamaguchi et al. 2010) that functions to thicken the midrib in rice leaves, as mentioned above. Namely, the unusual arrangement/directional growth of the lamina in the unifacial, ensiform leaves is due to a thickening growth of the central domain in leaves by *DL*. This is a co-option of the developmental system for thickening of the midrib to create a flat lamina (Nakayama et al. 2013). Interestingly, the role of *DL* in thickening the growth of leaves seems to have been acquired in monocot clades (Nakayama et al. 2010), but its ortholog in eudicots (CRABS CLAW (CRC) in arabidopsis) does not have a comparable role. The repeated evolution of ensiform unifacial leaves in monocots may depend on this monocot-specific function of *DL.* Besides *DL,* at least in the above *Juncus* cases, the *PRS* gene is also involved in the flattened growth of the ensiform lamina (Yamaguchi et al. 2010).

VIII. Indeterminate Leaves – Intermediate Form of a Shoot and a Leaf

These final sections discuss those forms of leaves that lie between typical leaves and other organs. Leaves are characterized by their determinate fate that is the exception in plant organs. Shoots and roots grow indeterminately using their apical meristems. In contrast, the leaf meristem is controlled to maintain its activity for a certain period of time during the development of the leaf primordia, after which the activity is terminated. However, some tropical species exhibit indeterminacy in their leaves. There are two types of indeterminate leaves (Fig. 1.10): basal and apical (reviewed in Tsukaya 2000). The former type has an indeterminate leaf meristem in the basal section of the leaves, as seen in all species of the genus *Monophyllaea* and in some species of the genus *Streptocarpus* (both Gesneriaceae) or the well-known gymnosperm *Welwitschia mirabilis* Hook.f. The indeterminancy of leaves of genus Monophyllaea is acquired by a competition between two cotyledons after the germination (Tsukaya 1997). The latter type has SAM-like apical meristems in the leaves, as seen in members of the genera *Chisocheton* and *Guarea* (both Meliaceae; Steingraebear and Fisher 1986; Fisher and Rutishauser 1990; Fukuda et al. 2003). The basal type appears to have acquired SAM-like activity in the meristematic zones of the leaf primordia to keep its indeterminacy; the apical type appears to have acquired a SAM-like structure on the tip of the leaf primordia. Interestingly, while expression of an *STM* ortholog is not detected in the basal, meristematic zones of the indeterminate leaves (or 'phyllomorph') of *Streptocarpus* (Harrison et al. 2005), *Monophyllaea glabra* Ridl. of the same Gesneriaceae expresses an *STM* ortholog in their indeterminate leaves at the base (Ishikawa et al. 2017).

What is the adaptive advantages of these indeterminate leaves? The basal type may be an extreme evolutionary form of the rosette-type status. The continuous production of rosette leaves around a short axis often leads to self-shading of the old leaves by new

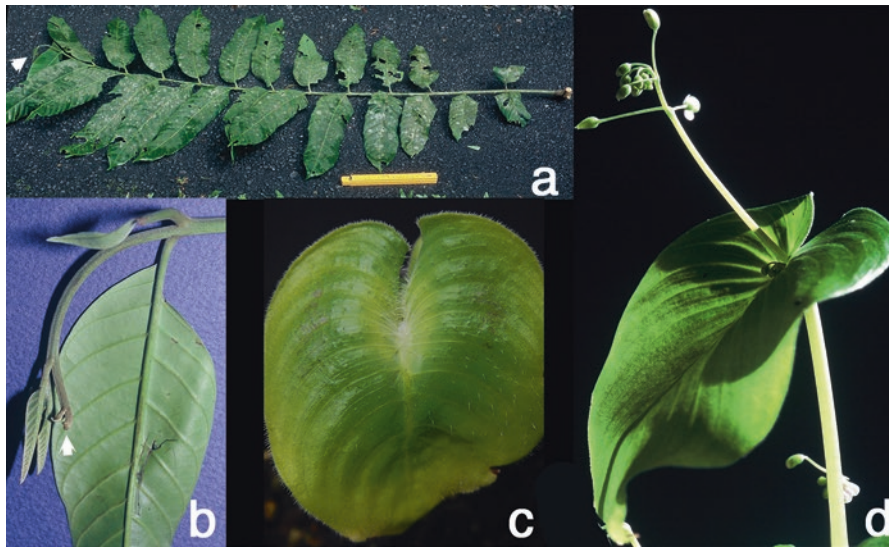

Fig. 1.10. (**a**, **b**) The apical type of indeterminate leaves of *Chisocheton perakensis* (Hemsl.) Mabb. The white arrowheads indicate the position of the leaf apical meristem. (**c**, **d**) *Monophyllaea gralba* Ridl. with basal type of indeterminate leaves. (**c**) An individual at the vegetative stage. (**d**) A plantlet at the reproductive stage with inflorescences on the indeterminate leaf

leaves. One possible solution to overcome this would be to maintain the activity of morphogenesis in the old leaves without the addition of new leaves, i.e., a reduction in the number of rosette leaves instead of immortalization. Indeed, all basal-type indeterminate leaves continue to supply new tissue from their base, allowing the old section that has moved distally to be lost. This strategy can decrease phyllotaxy regulatory costs; instead, the leaf meristems become non-exchangeable and a potential weak point. Since all indeterminate-leaf species in Gesneriaceae grow in or near caves where sunlight comes from only one direction, they require only one leaf to be facing the sunshine. This also seems to accelerate the extreme reduction of rosette leaf numbers.

The advantage of apical-type indeterminate leaves is unclear, but they are functionally equivalent to twigs/shoots. The only difference between the apical-type indeterminate leaves and shoots is the absence/presence of lateral buds. For example, in *Chisocheton macrophyllus* King, a large-leaved species, young trees often develop leaves over 2 m in length that continue to develop for years. These long leaves function in a similar way to lateral branches. Later, when the trunk becomes thick and tall enough to sustain true branches, the indeterminacy of each leaf is seen only for 1 or 2 years (personal observations in the Malay Peninsula and Bogor Botanical Gardens, Indonesia). Therefore, the indeterminate nature of these apical-type leaves may serve as a replacement for lateral twigs with disposable leaves during the younger stages, and with lower costs. Molecular phylogeny analysis revealed that the acquisition of indeterminacy in these two genera of Meliaceae occurred only once (Fukuda et al. 2003), suggesting that this morphological evolution was the result of a rare mutation.

The genetic mechanisms underlying indeterminacy are still unknown, although expression analyses of developmental genes, such as KNOXI in basal-type indeterminate leaves (e.g., Harrison et al. 2005), have been performed. Further extensive studies are required to understand these unusual developmental issues, which will help elucidate how determinacy, a key feature of leaves, is controlled in almost all plant species.

IX. Cladodes and Other Leaf-Like Organs

Many unusual organs exist that resemble leaves in their morphology and function. Typical examples are seen in cladodes or phylloclades in *Phyllacradus hypophyllus* Hook.f. of gymnosperms, members of the monocotyledonous *Asparagus* and *Ruscus* (Fig. 1.11a, b); and leaf-like flat, green roots in some Podostemaceae, such as *Hydrobryum puncticulatum* Koidz. (Fig. 1.11c), and some orchids (Fig. 1.11d). They have a flat, dorsiventral structure with full photosynthetic capability. There is the question of how these organs obtained their leaf-like forms. Investigation of this question will provide us with information about what systems are essential for leaf organogenesis and leaf functions. In studies of the "green roots" phenotype in arabidopsis, it was shown that auxin transported from the shoot to the root is involved in suppressed expression of the photosynthetic machinery in roots (Kobayashi et al. 2012). A simple detachment of a shoot from the roots can accelerate the greening of the roots in arabidopsis; constitutive overexpression of the transcription factors *GOLDEN2-LIKE* (*GLK*)*1* and *GLK2* also causes greening of the roots. Therefore, acquisition of photosynthetic activity in the roots appears to be due to changes in the auxin-related- or *GLK*-related pathways. In contrast, exactly how the cylindrical roots become flat, ribbon-like forms in some Podostemaceae and orchids is still unknown.

In cladodes and phylloclades, greening and photosynthesis do not require any special mechanisms, since they are originally lateral shoots or stems with green tissues.

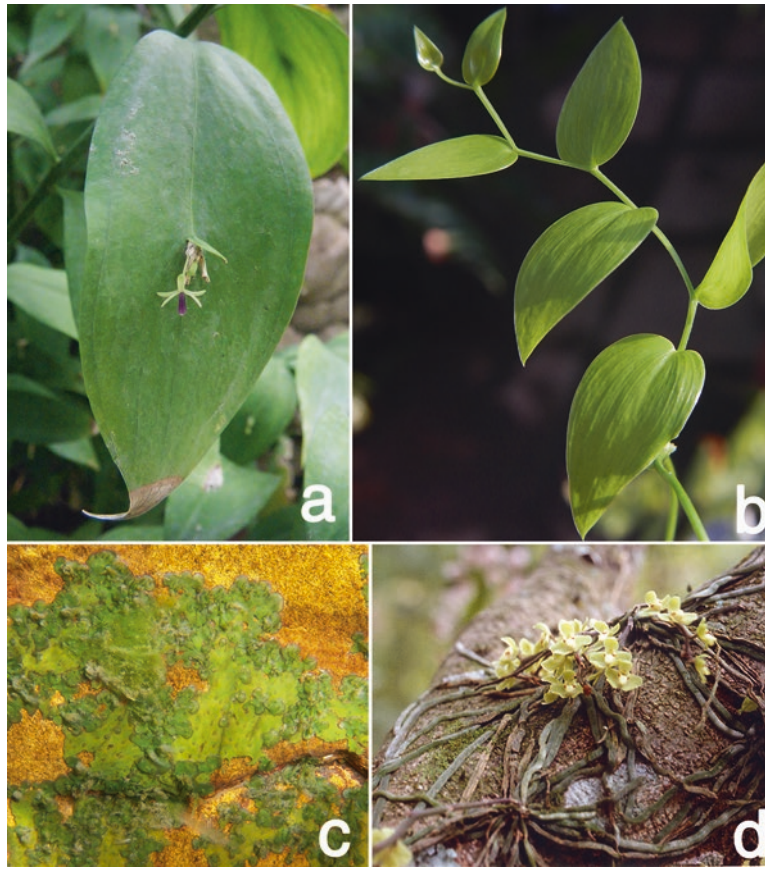

Fig. 1.11. (**a**) A leaf-like cladode of *Ruscus hypophyllum* L. with flowers, (**b**) a leaf-like cladode of *Asparagus asparagoides* (L.) W.F.Wright, (**c**) leaf-like green, flat roots of *Hydrobryum puncticalatum* Koidz., and (**d**) ribbon-like green roots of the orchid *Sarcochilus segawae* Masam

The problem is how to obtain the dorsiventral structures. A comparative analysis of flat-cladode *Asparagus asparagoides* (L.) W.Wight and terete-cladode *A. officinalis* L. revealed that the cladodes of both species express the shoot-apical-meristem gene KNOXI and the leaf-primordia gene *AS2*, indicating that the cladodes share a heterogeneous identity in their genetic expression of both shoot and leaf systems (Nakayama et al. 2013). Reverse transcription polymerase chain reaction analysis of KNOXI and *YAB2* genes also suggested this in *Ruscus aculeatus* L. (Hirayama et al. 2007). Interestingly, *DL,* which is also a leaf primordium gene, is not expressed in cladodes but is expressed in the carpels and leaves of *A. asparagoides* (Nakayama et al. 2010), indicating that not all leaf systems are recruited in cladodes. Further analyses showed that *A. asparagoides* ectopically expresses certain leaf dorsiventral genes in the cladodes, such as the adaxial HD-ZipIII genes and abaxial *ARF3* and *miR166* genes, whereas the terete *A. officinalis* cladodes express only the abaxial *miR166* gene. This strongly suggests that the leaf-like dorsiventral structures in the *A. asparagoides* cladodes are created by the dorsiventral systems used for the leaf primordia. It appears that in the ancestral species of these *Asparagus* species, flat leaf-like cladodes evolved by recruiting leaf systems to the lateral shoots. This may have been caused by an adaptation to drier envi-

ronments. Molecular phylogeny analyses have suggested that *A. officinalis* is more derived than *A. asparagoides,* and thus *A. officinalis* may have lost its ectopic expression of the adaxial gene systems, resulting in the terete form of their cladodes. The evolution of leaf-like organs and their morphological diversification seem to be attributed to the ectopic recruitment of a subset of leaf organogenesis genes in the other organs, followed by their partial modification.

X. Conclusions

In this review, I have provided an overview of leaf shape diversification/evolution in angiosperms from an evo/devo viewpoint. At present, most of our evo/devo understanding of leaf shape diversification is based on knowledge deduced from developmental genetic studies of model systems such as arabidopsis, rice, and the snapdragon. This approach, although powerful, has its limitations. For example, we know that *Sarracenia* pitcher leaves are formed by localized changes in the orientation of cell division and not by altered dorsiventrality patterning, but we do not know which genes are involved in this unique organogenesis. Arabidopsis leaves, which do not have such a curious morphogenesis, are not able to provide any clues. Therefore, an alternative strategy is needed to analyze leaf morphogenesis directly in non-model species. Recently, new RNAseq methods have gained sufficient power to address this question. For example, Ichihashi et al. (2014) carried out comparative transcriptomic analyses of three species in the genus *Solanum* with different levels of complexity in their compound leaves (tomato, *S. habrochaites* S.Knapp & D.M.Spooner and *S. pennellii* Corell), and identified several important key genes responsible for the differences in leaf shape. In the future, this kind of approach will dramatically enhance our understanding of which genetic changes have contributed to leaf shape diversification in plants. This, in combination with a deeper understanding of the functions/roles of each gene, will also resolve the long-standing debate on the physiological and adaptive implications of a particular leaf shape.

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Chapter 2

Leaf Vasculature and the Upper Limit of Photosynthesis

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Summary

The foliar vascular network is responsible for (1) structural support of the lamina as a platform for absorbing photons of light to drive photosynthesis, (2) transfer of information carriers like hormones and other signaling molecules between the leaf and other parts of the plant, (3) distribution of water and nutrients to leaf tissues via the xylem, and (4) movement of photosynthetic products, as well as chemical components remobilized during senescence, from mesophyll tissue into the phloem, and from source leaves to the plant's many sinks. Foliar venation is thus central to the leaf's primary role as a photosynthetic organ. Positive relationships between hydraulic conductance of the xylem, foliar vein density, and photosyn-

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thesis have been studied, and close links between foliar phloem capacity and intrinsic photosynthetic capacity were identified more recently. In this chapter, the relationship between various features of the foliar vasculature and photosynthetic capacity in mesophytic species with high rates of photosynthesis is explored. These metrics include foliar vein density, numbers and/or cross-sectional areas of xylem, phloem, and companion (including intermediary) cells, tracheary and sieve elements, and expansion of cell membrane area due to cell wall ingrowths in phloem transfer cells. Total xylem conduit volume per leaf area (the product of vein density and xylem cell metrics) of minor foliar veins exhibited a strong positive relationship with photosynthetic capacity per leaf area among multiple summer annuals. In the winter annual *Arabidopsis thaliana*, acclimation to contrasting growth temperatures involves differential acclimation of photosynthesis versus transpiration and is matched by similar differential acclimation of phloem versus xylem features. Photosynthetic capacity was positively correlated with various phloem metrics among all species and conditions examined, including summer annuals, winter annuals, and biennial species under various temperature and light conditions during growth. Given the essential role of vasculature in leaf functioning, it is not surprising that foliar vascular metrics are adjusted in response to environmental conditions (temperature, light levels, etc.). The vascular grid of the leaf and its xylem and phloem components thus underlies efficient leaf and plant functioning by facilitating the exchange of water, nutrients, and energy and information carriers between photosynthetic and non-photosynthetic parts of the plant. Recognition of this centrality of the foliar vasculature is critical to the effective selection, breeding, and engineering of crop plants to meet the nutritional, energy, fiber, material, and pharmaceutical needs of an expanding human population.

I. Introduction

The rich variety of leaf shapes and sizes found across the plant kingdom, the venation network within the lamina, and their developmental flexibility within a single species, have been a source of fascination and inquiry for well over a century (Gray 1848; von Ettingshausen 1861; Bower 1884; Fischer 1884, 1885; Strasburger 1891, 1894; Zalenski 1902, 1904; Schuster 1908; Bailey and Sinnott 1916; Lebedincev 1927; Keller 1933; Foster 1936; Wylie 1951; Pray 1954, 1955; Esau 1934, 1967; Melville 1969; Efroni et al. 2010). Despite their remarkable phenotypic variability, most leaves share a basic set of features. Encased in a cuticle-covered epidermis coupled with stomata to finely control the exchange of gases between leaf and surrounding atmosphere (for all non-aquatic species), the mesophyll tissue comprising the bulk of the leaf is linked to the rest of the plant by veins that carry vital substances. The majority of vein patterns in broad-leafed species can be placed into three broad categories (Roth-Nebelsick et al. 2001): (1) a mesh-like reticulated network prominent in dicots or eudicots, (2) parallel veins with small cross-linking veinlets in most monocots, or (3) veins forming a dichotomous (bifurcating, tree-like, or dendritic) pattern in most ferns and broad-leafed gymnosperms. The central role of this vasculature in facilitating and supporting the leaf's role in absorbing sunlight and converting this energy to chemical energy for export to the rest of the plant will be explored in the following.

What is the relationship of the foliar vasculature to photosynthesis? Beyond providing structural support to the photosynthetic tissues, transport of water into the leaf through the xylem and transport of photosynthetic products out of the leaf via the

phloem are both essential processes without which photosynthesis would cease. Delivery of water at a sufficient rate to maintain the hydration status of the leaf and prevent stomatal closing is necessary to keep the stomata in the leaf's epidermis open for diffusional influx of carbon dioxide from the atmosphere to the chloroplasts as photosynthesis proceeds (Chaps. 5, 6 and 7). Moreover, the flux of water along its water potential gradient from xylem to phloem of the foliar vasculature creates the positive pressure potential in the sieve elements that drives the sugar laden phloem sap out of the leaves (Boersma et al. 1991; Patrick et al. 2001; Höltä et al. 2006; Sevanto et al. 2011; Höltaä and Nikinmaa 2013; Nikinmaa et al. 2013; Jensen et al. 2016).

Through thorough literature reviews, Gifford (Gifford and Evans 1981; Gifford et al. 1984) and Wardlaw (1990) evaluated the possibility that long-distance phloem conduits connecting source and sink tissues might pose a limit to transport of the products of photosynthesis (photosynthate). Based on various manipulative experiments, they concluded that long-distance translocation of photosynthate was rarely, if ever, limiting. Gifford et al. (1984) stated that "Long-distance transport seems unlikely to impose appreciable limitation on the rate of transport from sources to sinks. .." and Wardlaw (1990) "suggest[ed] that the carrying capacity of the phloem is not a major factor in determining yield" and that some species even have "an excess carrying capacity in the phloem". This conclusion may well be true for the majority of the phloem transport system throughout the plant. The network of phloem within the plant has to be extremely flexible with regard to both direction of flow and changes in total flux. For instance, sugar flows into emerging leaves as long as these function as sinks, but flow reverses once leaves become photosynthetically competent sources of photosynthate (see Chap. 3). Likewise, during vegetative growth, sugars typically flow into stems, roots, and tubers, whereas during bolting, flowering, and seed/fruit development, sugars can flow out of tubers, roots, and stems into reproductive tissues. Furthermore, the relative strength of sources and sinks, which drives total flux, continuously changes as older leaves become shaded, produce less photosynthate, and are sacrificed for their nutrients while new leaves develop. Over this time course, photosynthetic capacity is up- or downregulated, roots and tubers shift from sinks to sources, and plants transition from strictly vegetative growth to reproductive growth. However, the conclusion that long-distance phloem flux capacity is nonlimited did not address potential limitations of the phloem capacity within the lamina of the leaf itself to load and export photosynthate and how that capacity might relate to the leaf's photosynthetic capacity.

It is well established that the rate at which the products of photosynthesis are utilized by the plant (sink activity) modulates the source leaves' photosynthetic capacity (Körner 2013; Fatichi et al. 2014). Krapp and Stitt (Krapp et al. 1993; Krapp and Stitt 1995) demonstrated that feedback inhibition of photosynthesis could be induced at the individual leaf level by cooling the petiole with a refrigerated sleeve leading to increased viscosity of the phloem sap, slowed foliar sugar export, carbohydrate accumulation in the leaf, and downregulation of photosynthesis by repression of photosynthetic genes. Our group subsequently demonstrated that the foliar phloem network can limit leaves' photosynthetic capacity under some conditions. In species in which vein density responds to the light intensity during leaf development, mature leaves that had developed in low light (with a relatively low vein density) suddenly transferred to high light accumulated starch and failed to fully increase photosynthetic capacity to the level observed in high-light-grown leaves (with a relatively high vein density; Amiard et al. 2005; Adams et al. 2007). However, it cannot be excluded that an inability to increase leaf thickness might have also

contributed to the limited upregulation of photosynthetic capacity (Amiard et al. 2005). While mature leaves show varying and limited anatomical adjustment to a sudden, dramatic change in light environment (Wimmers and Turgeon 1991; Amiard et al. 2005), developing leaves possess substantial plasticity to respond to conditions prevailing during leaf development (Sultan 2000; Valladares et al. 2007; Niklas 2009; Nicotra et al. 2010; Palacio-López et al. 2015; Zhu et al. 2015). It is, therefore, not surprising that the foliar vasculature exhibits considerable phenotypic plasticity. The relationship between foliar minor vein density, several minor vein phloem metrics, and photosynthetic capacity, as dependent on species and growth conditions, will be explored in Sect. 4.

Flux capacity of the conduit components of the vascular system can be estimated by similar measures of cell numbers and sizes for the tracheary elements of the xylem and sieve elements of the phloem. In contrast, evaluation of the phloem cells involved in transfer of photosynthate into the sieve elements needs to take into consideration the different mechanisms of phloem loading (Rennie and Turgeon 2009; Turgeon 2010; Slewinski et al. 2013; Chap. 3). In passive phloem loading, photosynthates diffuse from cell to cell through plasmodesmata from sites of higher concentration in photosynthesizing mesophyll cells to lower concentration in the area around exporting phloem tissue. For species that do not employ an active loading mechanism, diffusion through plasmodesmata continues into the phloem, and bulk flow through the sieve elements (sieve tubes) is driven by a pressure gradient from source leaves to sinks as originally envisioned by Münch (1930).

Active loading of photosynthate into the foliar phloem, on the other hand, creates a strong step gradient in solute concentration between the cells immediately adjacent to the companion cell-sieve element complexes of the phloem and the latter complexes (Rennie and Turgeon 2009; Turgeon 2010; Slewinski et al. 2013; Chap. 3). The impact of this active loading step is two-fold: (1) enhancement of the gradient from photosynthesizing mesophyll cells to companion cells and sieve elements increases the rate at which photosynthates diffuse from their sites of synthesis to the phloem and (2) elevation of solute levels in the companion cell-sieve element complex. This concentration of solutes results in greater pressurization of the source phloem to drive bulk flux of phloem sap laden with sugars or sugar alcohols from source leaves to sinks. This bulk flow is supported by water flux along a gradient from adjacent cells into companion cells and sieve elements; this gradient is generated by active solute transfer from surrounding cells (in which solute concentration is decreased and water potential increased) into the phloem complexes (in which solute concentration is increased and water potential decreased).

Two general mechanisms can serve to actively load photosynthate into the phloem (Rennie and Turgeon 2009; Turgeon 2010; Slewinski et al. 2013; Chap. 3). Symplastic loading involves diffusion of sucrose via plasmodesmata from bundle-sheath or mesophyll cells to companion cells and addition of one, two, and sometimes even three galactose units to sucrose, forming raffinose, stachyose, and verbascose, respectively. This active step of synthesizing the raffinose-family sugars enhances the diffusion gradient for sucrose from the surrounding tissues into the veins' companion cells. The larger sugars formed by conversion of disaccharides to trisaccharides, tetrasaccharides, or pentasaccharides cannot move back through the relatively small plasmodesmata between the companion cells and the adjacent bundle-sheath or mesophyll cells; as part of the mechanism of symplastic loading, this process is also called polymer trapping (Haritatos and Turgeon 1995). These larger sugars can, however, pass through the larger plasmodesmata at the companion cell-sieve element interface. These features of the companion cells of symplastic loaders led to

their distinguishing designation as intermediary cells.

For the second active loading mechanism, called apoplastic loading, the first step is movement of sugars or sugar alcohols through efflux channels from cells surrounding the phloem's companion cells and sieve elements into the apoplastic cell wall space of phloem cells (Chen et al. 2012; Eom et al. 2015). Uptake of these apoplastically localized photosynthates into the phloem via sugar alcohol- or sucrose-proton symporters is energized by active pumping of protons into the apoplastic cell wall space by phloem-localized ATPases (Kühn 2003; Offler et al. 2003). Secondary active transport of photosynthates by such symporters takes place in companion cells and, in some species such as *Arabidopsis thaliana*, sieve elements as well (Srivastava et al. 2008; Duan et al. 2014). For more detail, see Sect. 3.3 below.

II. Foliar Venation as a Structural Scaffold

Although this review focuses mainly on features of the xylem and phloem essential to their function as conduits for the transport of aqueous fluids, foliar venation is also critical to the mechanical integrity of the leaf (Roth-Nebelsick et al. 2001; Sack and Scoffoni 2013). The venation acts as a structural scaffold between which the mesophyll cells are packed, thus supporting optimal display of photosynthetic tissue for the absorption of sunlight while also providing the stability necessary for such a large and thin organ to withstand the vagaries of gravity, wind, precipitation, and even trampling (Vogel 1989; Niklas 1999). While all vascular cells contribute to the vein's rigidity, the specialized sieve and tracheary elements through which bulk flow occurs likely provide the greatest level of structural buttressing. The small sieve elements have thickened cell walls (Esau and Cheadle 1958), a feature that can extend to surrounding phloem parenchyma and companion cells in those apoplastic loaders that develop wall ingrowths (Gunning and Pate 1969; Pate and Gunning 1969, 1972; Amiard et al. 2007; Adams et al. 2014a; Fig. 2.1e, f). The lignified cell walls of the tracheary elements, however, undoubtedly provide the greatest rigidity for the veins of all species (Niklas 1992). For C_4 plants with Kranz anatomy, suberization of bundle-sheath cell walls would also be expected to contribute to the structural integrity of the foliar veins and leaves (Botha 1992). In addition, leaves of some woody and herbaceous species possess bundle sheath extensions (columns of cells that extend from the vascular bundle to the epidermis on either side of the leaf; Wylie 1952). These transparent cells have been shown to transmit a broad spectrum of light into the interior of the leaf, thus enhancing photosynthesis in the surrounding mesophyll cells (Karabourniotis et al. 2000), as well as to facilitate a higher flux of water from the xylem to the epidermis, thereby accelerating the rate at which the stomata may open (Buckley et al. 2011) as well as influencing the extent to which they open (Zsögön et al. 2015). Although not yet supported by any evidence, it is possible that these bundle sheath extensions could also contribute to foliar structural support.

Upon loss of turgor, leaves of herbaceous plants collapse and wilt, whereas those of woody plants typically retain all or most of their shape. Until the mesophyll and epidermal cell turgor loss point is reached, it is possible that the sclerophytic leaves of woody plants rely primarily on the structural components of cell walls of both vascular and non-vascular cells for support of the mesophyll tissue, whereas the mesophytic leaves of many herbaceous species may rely primarily on the greater turgor pressure within the phloem tissue that arises from the concentration of much higher levels of photosynthate due to active phloem loading. Many herbaceous species (lacking woody tissue) characterized thus far actively load

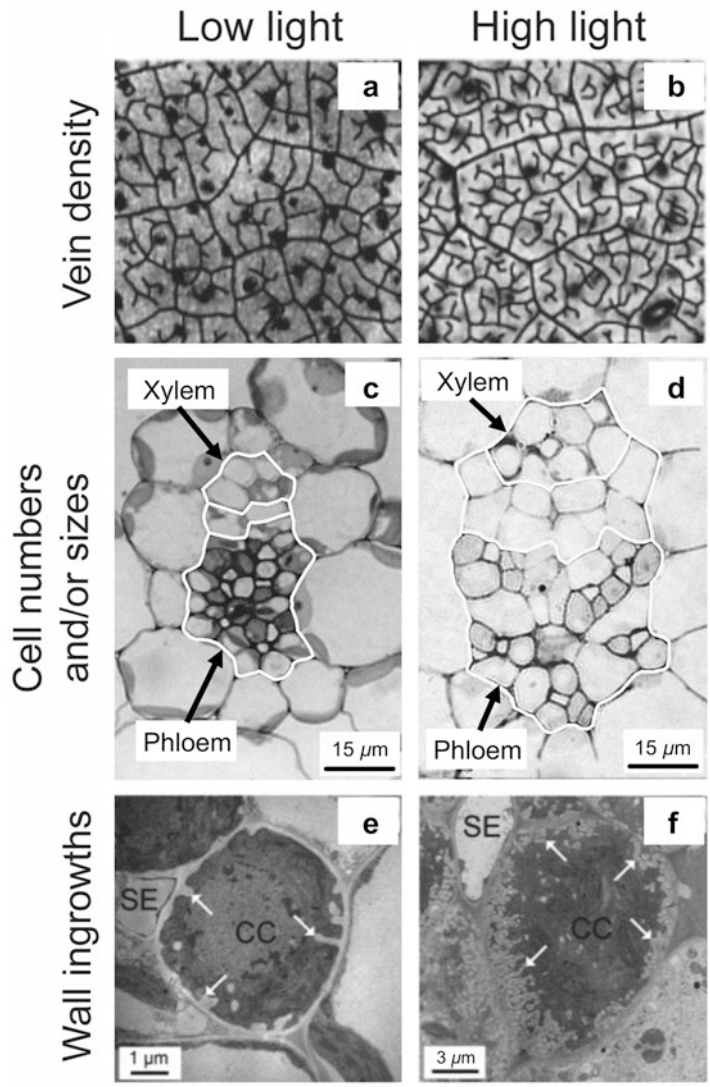

Fig. 2.1. Vascular features of leaves grown in low light (100 μmol photons m^{-2} s^{-1}; **a**, **c**, **e**) versus high light (1000 μmol photons m^{-2} s^{-1}; **b**, **d**, **f**). (**a, b**) Illustration of low vein density in pumpkin (*Cucurbita pepo*) leaves that developed in low light (10 h photoperiod) versus high vein density in pumpkin leaves that developed in high light (14 h photoperiod), both under a 25 °C/20 °C day/night temperature regime (from Amiard et al. 2005, Copyright [2005] National Academy of Sciences). (**c**, **d**) Illustration of larger and more numerous vascular cells in the minor vein of an *Arabidopsis thaliana* (Col-0) leaf that developed under high light compared to the minor vein of a leaf that developed under low light (9 h photoperiod, day/night temperature regime of 20 °C/12 °C; from Stewart et al. 2017a). (**e**, **f**) Illustration of the level of cell membrane magnification via cell wall ingrowth that is greater in the companion cell of a minor vein in leaves of *Senecio vulgaris* that developed in high (14 h photoperiod) compared to low (10 h photoperiod) light under a 25 °C/20 °C temperature regime. (From Amiard et al. 2007)

photosynthate into the phloem apoplastically or symplastically, whereas many woody species rely on passive flux of photosynthate along a concentration gradient without an active loading step (Turgeon et al. 2001; Davidson et al. 2011; Fu et al. 2011).

Vein density (vein length per unit leaf area) also plays a role in the structural integrity of the leaf, with a greater vein density

providing a greater level of support (Roth-Nebelsick et al. 2001). Furthermore, thicker leaves can be supported by vascular girders that are either more ramified or thicker but not more numerous, depending on species. For instance, in both pumpkin (Fig. 2.1a, b) and *Verbascum phoeniceum*, leaves grown in high compared to low light were thicker and had higher densities of veins (due in part to greater numbers of free-ending veinlets) of similar sizes (Amiard et al. 2005). In two winter annuals (*A. thaliana* and spinach), on the other hand, leaves grown under high light and low temperature (compared to moderate light or moderate temperature) had higher rates of photosynthesis and were thicker, but had similar vein densities. While these leaves thus did not have more numerous veins, each individual minor vein had a larger cross-sectional area due to a greater number of vascular cells (Figs. 2.1c,d and 2.2; Cohu et al. 2013a, b, 2014). Either approach – smaller, more numerous veins versus fewer, more substantial veins – presumably provides a scaffold to support a greater volume of photosynthetically active cells in leaves acclimated to conditions allowing higher rates of photosynthesis.

III. Flux Capacity of Foliar Veins

A. Vein Density

In addition to a more extensive and supportive structural scaffold, a higher vein density will have the potential to provide a greater flux of water, nutrients, hormones, etc. to the leaf tissue and a greater capacity to export photosynthates, hormones, amino acids, etc. from the leaf tissue to the rest of the plant (Sack and Scoffoni 2013). The latter may be particularly important in facilitating an export capacity that permits upregulation of photosynthesis under conditions favorable for high rates of growth and a high capacity for the utilization and/or storage of photosynthetic products in distant sinks (Adams et al. 2005, 2007, 2013b, 2014b). The more highly ramified minor veins are throughout the photosynthetic mesophyll tissue of the leaf (i.e., the higher the vein density), the

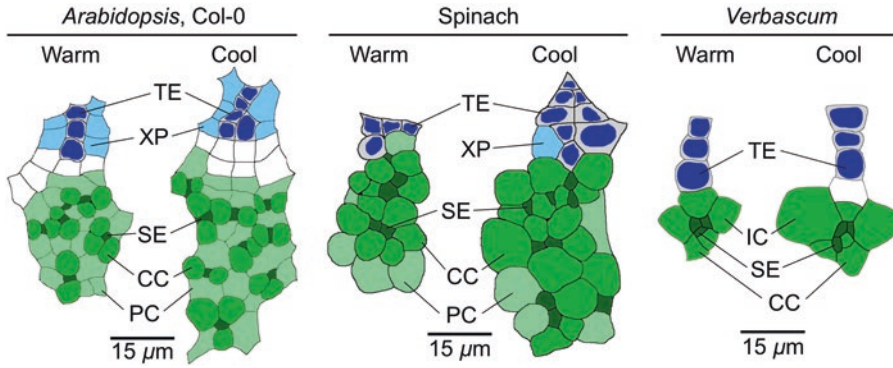

Fig. 2.2. Sketches (based on light microscopic images) of minor veins from fully expanded leaves of the winter annual *Arabidopsis thaliana* Col-0 (an apoplastic loader), the winter annual spinach (an apoplastic loader), and the biennial *Verbascum phoeniceum* (a symplastic loader) grown under a 9-hour photoperiod of 400 μmol photons m^{-2} s^{-1} at a day/night air temperature of 25 °C/20 °C (warm conditions) or 8 °C/12 °C (cool conditions). Small dark green cells are sieve elements (SE), medium green cells are companion (CC) or intermediary (IC) cells (in the minor veins of *V. phoeniceumm*, the two largest companion cells are ICs, whereas the rest of the companion cells may or may not be ICs), light green cells are phloem parenchyma cells (PC), dark blue cells are tracheary elements (TE), light blue cells are xylem parenchyma cells (XP), and white cells are undifferentiated parenchyma cells. (Modified from Adams et al. (2013a), Cohu et al. (2014), and Muller et al. (2014b))

shorter the transit distance for sugars from sites of photosynthesis to entry into the phloem for those cells most distant from the minor veins. Minimization of this distance from the sites of sugar synthesis, coupled with a greater overall flux capacity for feeding sugars from the more numerous minor vein tributaries into the major veins, likely contributes to the ability of leaves to exhibit maximal rates of photosynthesis. On the other hand, if there are an insufficient number of pipelines for moving photosynthates out of the leaf, the potential for upregulation of photosynthesis can be limited (Amiard et al. 2005; Adams et al. 2007).

B. Vascular Pipelines: Tracheary and Sieve Elements

Cells of the foliar minor veins exhibit a range of cross-sectional shapes, from round to oval to rhomboidal to polygonal, depending on their development and constraints by surrounding cells. The cross-sectional size is likewise variable, varying among different species, within a species depending on growth conditions, and, to some extent, even within a vein (Figs. 2.1c, d and 2.2).

Assuming no difference in the average cross-sectional area, the capacity for bulk flow of sap through minor vein tracheary or sieve elements is presumably proportional to the number of such cells. In such a scenario, a minor vein with six sieve elements could transport twice as much phloem sap as a minor vein with three sieve elements. On the other hand, the relationship between flux capacity and cell cross-sectional area is expected to be different. A tracheary element with a cross-sectional area that is 100% greater than another would have a flux capacity for the transport of xylem sap that is more than 100% higher than that of the smaller tracheary element. This is due to a smaller proportion of the water flowing through the cell being under the influence of frictional resistance to flow around the periphery of the cells in the larger tracheary elements compared to those of the smaller tracheary elements. The physics of these relationships is straightforward, based on the Hagen-Poiseuille Law, wherein the resistance to flow is inversely proportional to (assuming a perfectly circular cell) the tube radius to the fourth power (Nobel 2009). However, this physical law fails to perfectly describe the relationships in tracheary and sieve elements because both vascular pipelines are inherently leaky (Canny 1993; Cabrita et al. 2013).

Beyond its impact on the flux capacity, the cross-sectional area of a tracheary element can also influence its susceptibility to the phenomenon of cavitation, whereby introduced pockets of water vapor create an embolism in the water-conducting cells of the xylem that can prevent water transport through the conduit of the impacted cell. Such embolisms may arise when the tension (negative pressure potential) within the xylem becomes too great (due, e.g., to an extreme imbalance between the supply of water from the roots and its loss from the leaves to the atmosphere) or in response to freeze-thaw cycles. One might expect a vein with larger tracheary elements to be more vulnerable to cavitation than one with smaller tracheary elements (Hargrave et al. 1994; Langan et al. 1997; Davis et al. 1999; Hacke and Sperry 2001; Hacke et al. 2009; Sterck et al. 2012), whereas those with similarly sized tracheary elements but varying in number of such elements per minor vein might be similarly susceptible to cavitation.

C. Phloem Loading

In addition to vein density, three additional key vascular cell metrics can contribute to sugar-flux capacity through their contribution to phloem loading capacity. These three features of individual phloem cells include cell size, cell number per minor vein, and, for some species that employ apoplastic phloem loading, increase of total cell membrane area through development of cell wall ingrowths.

Larger cells (e.g., Figs. 2.1c,d and 2.2) provide for both a greater total membrane area around the perimeter of the cells as well as a greater cross-sectional area for bulk flow of phloem sap (through sieve elements), both of which can contribute to the flux capacity of the phloem. For those species that do not employ an active phloem loading step, as well as those that load the phloem symplastically, sugars flow into the phloem and into the sieve elements exclusively via plasmodesmatal openings in the cell walls. While we found no difference in the number of plasmodesmata per unit cell wall length in the foliar minor vein companion cells (intermediary cells) of pumpkin that developed under low versus high light (Amiard et al. 2005), a greater total cell wall length around the perimeter (and thus presumably a greater total wall area) of the phloem cells presents a greater area for placement of plasmodesmata, thus presumably supporting a greater flux of sugars into and through the phloem. Moreover, larger intermediary cells (Fig. 2.2) provide a greater volume within which to package a greater number of the enzymes responsible for the addition of galactose to sucrose for synthesis of higher levels of the large raffinose-family sugars as the active step in symplastic phloem loading (Rennie and Turgeon 2009; Slewinski et al. 2013; Chap. 3). As mentioned in the Introduction, these (tri- to pentasaccharide) sugars are too large to flow back through the small plasmodesmata through which the smaller disaccharide sucrose enters the intermediary cells; the larger sugars can, however, proceed through the larger plasmodesmata leading from intermediary cells into the sieve elements. This active step contributes to creating a gradient in sucrose concentration from the photosynthesizing leaf mesophyll cells to the phloem. From among the features of foliar minor vein phloem cells characterized, we found larger individual cell sizes to be the primary phloem feature associated with higher rates of photosynthesis among those species that actively load sugars into the phloem symplastically (Adams et al. 2013a; Muller et al. 2014a, b; Polutchko et al. 2018; see Sect. 5 below).

Larger phloem cells can likewise contribute to a greater flux of sugars into and through the phloem of species that actively load sugars apoplastically. As is the case in symplastic loaders, larger sieve elements provide both a greater cross-sectional area for bulk flux of phloem sap as well as a larger interface shared with surrounding companion cells for placement of a greater number of plasmodesmata through which sugars and/or sugar alcohols can flux into the sieve elements of apoplastic phloem loaders. What is specific to apoplastic loaders, however, are the membrane-spanning transport proteins that facilitate the transfer of sucrose (or sugar alcohols) from symplast to apoplast and back to symplast (Rennie and Turgeon 2009; Slewinski et al. 2013; Chap. 3). In the model species *A. thaliana*, this includes efflux channels (SWEET proteins; Chen et al. 2012; Eom et al. 2015) through which sucrose moves from the cytosol of mesophyll cells and phloem parenchyma cells into the cell walls, ATPases in the cell membranes of phloem parenchyma and companion cells that actively pump protons from the cytosol into the cell wall space (Kühn 2003; Offler et al. 2003; Sondergaard et al. 2004; Gaxiola et al. 2007; Falhof et al. 2016), and symporters that actively move protons and sucrose from the apoplastic cell wall space into the cytosol of companion cells and sieve elements (Srivastava et al. 2008; Rennie and Turgeon 2009; Slewinski et al. 2013; Duan et al. 2014). Larger phloem cells provide for a greater total cell membrane surface area into which a greater number of all of these membrane-spanning proteins can be placed to facilitate a greater level of sucrose flux, thereby contributing to the steep gradient from photosynthesizing mesophyll cells to the phloem.

Although larger-sized cells of minor veins can contribute to a greater sugar flux capacity of the phloem, we found that it is the

number of phloem cells per foliar minor vein that is most consistently associated with photosynthetic capacity among apoplastic phloem loaders (Figs. 2.1c, d and 2.2; Adams et al. 2013a, 2016; Cohu et al. 2013a, b, 2014; Muller et al. 2014a, b; Stewart et al. 2016). Even at constant cell size, four compared to two sieve elements have twice the capacity for bulk flow of phloem sap and twice the membrane area for placement of proton-sucrose symporters, and four compared to two companion cells likewise have twice the membrane area for placement of ATPases and proton-sucrose symporters.

The three vascular features discussed thus far (vein density, individual cell size, and cell number per minor vein) apply equally well to the phloem and its associated flux capacity to move sugars and the xylem and its associated conductance of the tracheary elements for water transport. A greater vein density and larger and/or more numerous tracheary elements will all contribute to a higher foliar hydraulic conductance (Sack and Scoffoni 2013; Muller et al. 2014a,b; Adams et al. 2016; Stewart et al. 2016). However, the fourth feature of foliar minor veins (cell wall ingrowths) that contributes to the sugar flux capacity of some species is limited to phloem cells of apoplastic loaders. Cell wall ingrowths or invaginations that lead to magnification of the cell membrane area for the potential placement of greater numbers of membrane-spanning sucrose efflux channels, ATPases, and proton-sucrose symporters can be found in the phloem parenchyma cells of some species (e.g., *A. thaliana*; Amiard et al. 2007), the companion cells of some species (e.g., pea, sunflower; Gamalei 1989; Gamalei et al. 2000; Amiard et al. 2005, 2007), and both phloem parenchyma cells and companion cells of yet other species (e.g., *Senecio vulgaris*; Fig. 2.1e, f; Amiard et al. 2007).

IV. Foliar Hydraulic Conductance, Minor Vein Xylem Features, and Photosynthesis

Correlative and experimental studies over the course of almost two decades have repeatedly shown that there are strong, positive relationships between foliar hydraulic conductance, foliar vein density, and photosynthetic CO_2 uptake among many vascular plants (Hubbard et al. 2001; Santiago et al. 2004; Brodribb et al. 2005, 2007, 2010; Nardini et al. 2005; Franks 2006; Sack and Holbrook 2006; Maherali et al. 2008; Boyce et al. 2009; Beerling and Franks 2010; Brodribb and Feild 2010; McKown et al. 2010; Blonder et al. 2011; Walls 2011; Zhu et al. 2013; Chap. 4). Water is lost as carbon dioxide is taken up from the atmosphere through stomata when leaves are engaged in the process of photosynthesis (Chap. 6), and water loss is often proportional to the rate of photosynthetic CO_2 fixation (Wong et al. 1979; Jarvis and Davies 1998; Jones 1998; Tuzet et al. 2003; Giuliani et al. 2013). The vascular system is constructed to provide the level of hydraulic conductance to facilitate such transpirational water loss. In the summer annual species depicted in Fig. 2.3 (a,b), photosynthetic capacity and the product of foliar vein density and the number of tracheary elements per minor vein (Fig. 2.3a) and the cross-sectional area of xylem per minor vein (Fig. 2.3b) are strongly correlated. A similar positive association among photosynthetic capacity, transpiration rate, and both phloem and xylem anatomical features is seen in high- versus low-light grown leaves of the winter annual *A. thaliana* (Stewart et al. 2017a).

On the other hand, winter annuals exhibit differential acclimation of photosynthetic capacity versus transpiration to growth temperature, with upregulated photosynthetic capacity and lower transpiration activity in cool- versus hot-grown leaves (Adams et al. 2016; Stewart et al. 2016, 2017a). This differential acclimation of photosynthetic

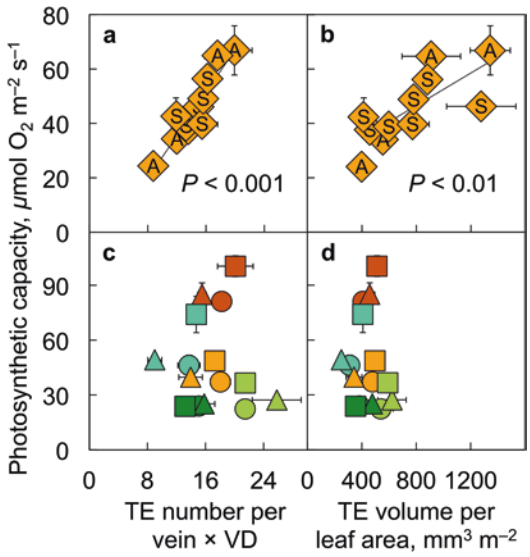

Fig. 2.3. Relationship between photosynthetic capacity (determined at 25 °C) and the mathematical product of vein density (VD) and either (**a**,**c**) the number of tracheary elements (TE) per minor vein or (**b**,**d**) the cross-sectional area of tracheary elements per minor vein for four species of apoplastically loading summer annuals (orange diamonds labeled with an "A" for apoplastic) and four species of symplastically loading (orange diamonds labeled with an "S" for symplastic, i.e., polymer trapping) summer annuals (**a**, **b**) grown under 1000 μmol photons m^{-2} s^{-1} at an air temperature of 25 °C, as well as (**c**, **d**) three ecotypes (circles = Italian, triangles = Polish, squares = Swedish) of the winter annual *A. thaliana* grown under 1000 μmol photons m^{-2} s^{-1} at an air temperature of 25 °C (orange symbols) or 8 °C (orange-red symbols) or 400 μmol photons m^{-2} s^{-1} at an air temperature of 35 °C (olive-green symbols), 25 °C (green symbols), or 8 °C (blue-green symbols). Mean values ± standard deviation for photosynthesis and ± standard error for tracheary elements. (Data from Cohu et al. (2013a,b), Muller et al. (2014a), Adams et al. (2016), Stewart et al. (2016), and unpublished data)

capacity versus transpiration is mirrored by corresponding differential acclimation patterns of phloem versus xylem anatomical features (Adams et al. 2016; Stewart et al. 2016, 2017a). Thus, for several ecotypes of the winter annual *A. thaliana* grown under different temperature regimes, photosynthetic capacity is not significantly correlated with xylem anatomical features (Fig. 2.3c, d).

In addition to the water lost through stomata, a certain fraction of the water that flows through the xylem tissue into the leaf is diverted to the phloem to support export of photosynthate from the leaf to the rest of the plant (Boersma et al. 1991; Patrick et al. 2001; Hölttä et al. 2006; Sevanto et al. 2011; Hölttä and Nikinmaa 2013; Nikinmaa et al. 2013; Jensen et al. 2016). Under cool, moist conditions with a low vapor pressure deficit between leaves and atmosphere but very high photosynthesis rates in winter annuals, the water potential gradient from the xylem to the phloem could increase due to high levels of phloem loading while water loss from the leaves to the atmosphere is low, thus altering the relationship between foliar water transport capacity and photosynthesis. This is apparently the case in *A. thaliana* leaves that have developed under contrasting temperature regimes (Cohu et al. 2013b; Adams et al. 2016; Stewart et al. 2016). These findings were recently corroborated when no association between foliar vein density and photosynthesis was found among multiple accessions of *A. thaliana* (Rishmawi et al. 2017).

As a winter annual, *A. thaliana* upregulates photosynthetic capacity in response to development under low temperature (Strand et al. 1997, 1999; Adams et al. 2013a; Cohu et al. 2013b, 2014), while transpiration rate is elevated in leaves of *A. thaliana* that developed under high temperature (Adams et al. 2016; Stewart et al. 2016). These differential acclimation patterns between summer and winter annuals underlie the differences in foliar vascular acclimation seen between these two groups of plants. While acclimation patterns of the foliar vasculature, of photosynthesis, and of transpiration thus vary not only among environmental factors but also among species, a robust positive correlation is consistently maintained between photosynthetic capacity and phloem features, albeit depending on phloem-loading mechanism, as is detailed in the following Sect. 5.

V. Minor Vein Phloem Features and Photosynthetic Capacity

While the study of multiple summer annuals grown under common conditions yielded a single relationship between photosynthesis and the product of vein density and tracheary element features of minor veins (Fig. 2.3a, b), the relationship between minor vein phloem features and photosynthetic capacity differed depending on the mechanism of phloem loading employed (Muller et al. 2014a; Polutchko et al. 2018). For species that load sugars symplastically, photosynthetic capacity was linearly correlated with the mathematical product of leaf vein density and the cross-sectional area of minor vein intermediary cells (Fig. 2.4a) or sieve elements (Fig. 2.4c). In symplastic loaders, one might indeed expect a higher photosynthetic capacity to be associated with larger phloem cells and a higher vein density as features that could accommodate a greater flux of sugars through a greater number of plasmodesmata around the perimeter of the larger cells (the walls of which have equal numbers of plasmodesmata per unit length; Amiard et al. 2005), a greater number of raffinose-synthesizing enzymes per intermediary cell, and a greater flux volume per cross-sectional area of sieve elements.

On the other hand, the mathematical product of vein density and the number of companion and phloem parenchyma cells per minor vein (Fig. 2.4b) or number of sieve elements per minor vein (Fig. 2.4d) was correlated linearly with photosynthetic capacity among species that load sugars apoplastically. In apoplastic loaders, a higher photosynthetic capacity can presumably be supported by the greater vein density and number of cells per minor vein providing a greater membrane area for placement of sugar efflux channels (in phloem parenchyma cell membranes), ATPases to pump protons into the apoplast (in phloem parenchyma and companion cell membranes), and proton-sucrose symporters (in companion cell membranes), all of which can contribute to moving sugars into the phloem. The greater vein density and number of sieve elements per minor vein can likewise provide a greater total cross-sectional flux area per unit area of leaf in support of higher rates of photosynthesis. The relationships revealed in Fig. 2.4 suggest a certain level of genetic variation depending on the mechanism of active phloem loading employed by species that load sugars apoplastically versus species that load sugar symplastically. Furthermore, phenotypic plasticity in various vascular metrics dependent on growth conditions is also possible (e.g., Figs. 2.1 and 2.2).

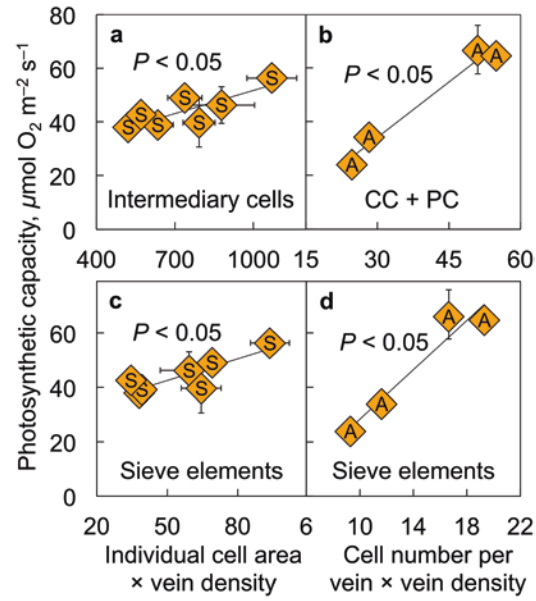

Fig. 2.4. Relationship between photosynthetic capacity (determined at 25 °C) and the mathematical product of vein density and (**a**) cross-sectional area of minor vein intermediary cells, (**b**) the number of companion (CC) and phloem parenchyma (PC) cells per minor vein, (**c**) cross-sectional area of minor vein sieve elements, and (**d**) the number of sieve elements per minor vein among four species of summer annual symplastic loaders (orange diamonds labeled with an "S" for symplastic, i.e., polymer trapping, in panels **a**,**c**) and four species of summer annual apoplastic loaders (orange diamonds labeled with an "A" for apoplastic in panels **b**,**d**) grown under 1000 μmol photons m^{-2} s^{-1} at an air temperature of 25 °C. Mean values ± standard deviation for photosynthesis and ± standard error for phloem cell metrics. (Data from Muller et al. (2014a))

For instance, higher photosynthetic capacities in the leaves of two herbaceous biennials (a symplastic and an apoplastic loader) that developed in response to growth at low compared to higher temperature (Fig. 2.5a, b) was associated with acclimation of minor vein features that largely mirrored the features associated with photosynthetic capacity among different species (Fig. 2.4). For the symplastic loader *Verbascum phoeniceum* (see minor vein cross-section in Fig. 2.2), a higher foliar photosynthetic capacity (Fig. 2.5a) was associated with a higher vein density (Fig. 2.5c) and larger minor vein phloem cells (Fig. 2.5e, g), features that are consistent with those associated with higher photosynthetic capacities among symplastic loaders grown under common conditions (Fig. 2.4a, c). For the apoplastic loader *Malva neglecta*, a higher foliar photosynthetic capacity (Fig. 2.5b) was associated with a higher vein density (Fig. 2.5d) and more numerous phloem cells per minor vein (Fig. 2.5j, l) – features consistent with those associated with higher photosynthetic capacities among apoplastic loaders grown under common conditions (Fig. 2.4b, d) – but also larger sieve elements (Fig. 2.5h). Larger and more numerous sieve elements per unit leaf area might be especially important to permit sufficient flux despite increased viscosity of the sugar-laden phloem sap during nocturnal sugar export (Adams et al. 2001a; see also discussion in Cohu et al. 2013a) under low temperatures of Colorado winters.

Acclimatory adjustments of photosynthesis coordinated with those of foliar phloem in response to growth temperature and photon flux density have been found to be particularly pronounced in winter annuals (Adams et al. 2013a, 2014a, 2016; Cohu et al. 2013a, b, 2014; Stewart et al. 2016, 2017b). Strong relationships between the number of phloem cells (for cells involved in phloem loading and/or sugar export) per minor vein and photosynthetic capacity have been observed in both spinach (Adams et al. 2013a; Cohu et al. 2014) and *A. thaliana* (Fig. 2.6a, b), and between photosynthetic capacity and the level of membrane-area-enhancing phloem cell wall ingrowths in *A. thaliana* (Fig. 2.6c) and other species (Amiard et al. 2005, 2007; Adams et al. 2014a). While all three *A. thaliana* ecotypes depicted in Fig. 2.6c exhibited correlations between photosynthetic capacity and the level of phloem parenchyma cell membrane area, photosynthetic capacity was consistently higher in the Swedish ecotype that also featured more numerous phloem cells of all types (either per vein or per leaf area; Cohu et al. 2013a, b; Adams et al. 2016; Stewart et al. 2016). As outlined in Sect. 3.3 above, greater numbers of phloem cells per minor vein and levels of phloem cell wall ingrowths can both contribute to increasing the total cell membrane area available for placement of transport proteins facilitating phloem loading and for sugar flux through these cells, and a greater number of sieve elements provides for a greater sugar-export capacity from the leaves, features that might be expected to accommodate higher rates of photosynthesis in apoplastic loaders. As was noted for the parallel acclimation of larger sieve elements and higher rates of photosynthesis in leaves of the apoplastic loader *M. neglecta* under winter conditions (Fig. 2.5b, h), larger minor vein phloem cells is also a feature associated with the acclimation of *A. thaliana* leaves to high compared to low light (Fig. 2.1c, d). The significance of the mathematical product of vein density (length of all individual minor veins per leaf area) and total vascular cell cross-sectional area per individual vein lies in that this product describes the total pipeline volume available for flux per leaf area irrespective of the extent to which changes in total vein cross-sectional area stem from changes in cell number or in cell size.

Across all species and environmental conditions examined thus far, foliar phloem metrics that serve as proxies for loading and flux capacity have been shown to vary proportionally with photosynthetic capacity.

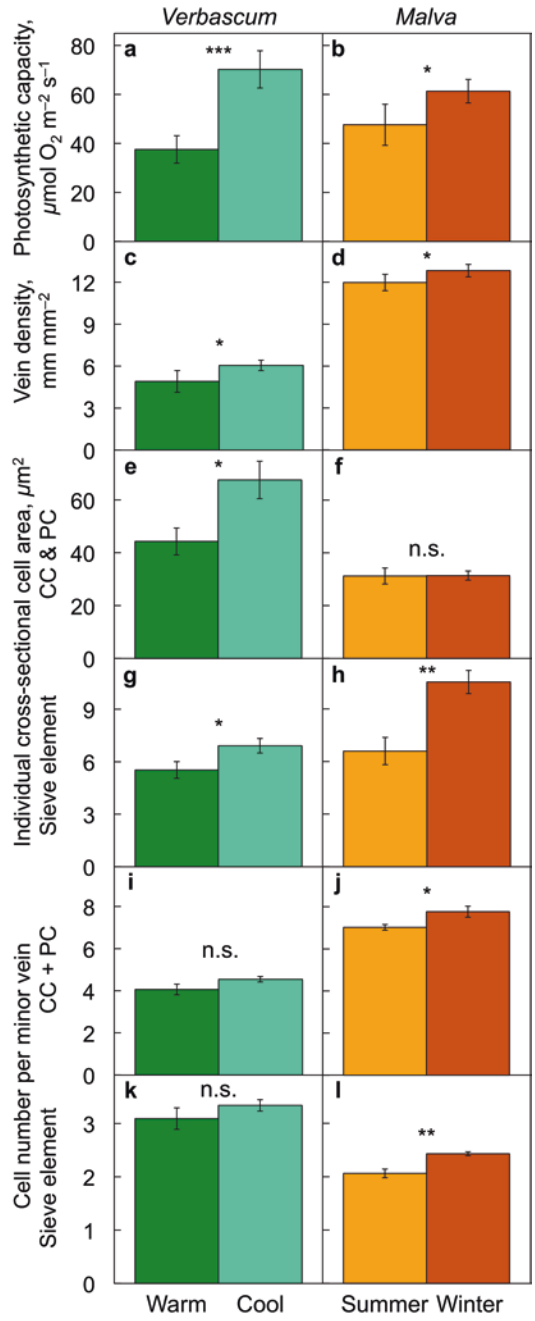

Fig. 2.5. Impact of growth conditions on foliar metrics of two biennials, Verbascum phoeniceum (a symplastic loader; **a**, **c**, **e**, **g**, **i**, **k***) and Malva neglecta (an apoplastic loader;* **b**,**d**,**f**,**h**,**j**,**l***), including (***a**, **b***) the light- and CO_2-saturated rate of photosynthetic oxygen evolution determined at 25 °C, (***c**,**d***) vein density, (***e**,**f***) the cross-sectional area of companion (CC; intermediary in the case of V. phoeniceum) and phloem parenchyma (PC) cells, (***g**, **h***) the cross-sectional area of sieve elements, (***i**,**j***) the number of companion (intermediary in the case of V. phoeniceum) and phloem parenchyma cells per minor vein, and (***k**,**l***) the number of sieve elements per minor vein. Verbascum phoeniceum was grown under 400 µmol photons m^{-2} s^{-1} and a leaf temperature of 29 °C (Warm; green columns) or 13 °C (Cool, blue-green columns). Malva neglecta leaves were obtained from plants growing in full sunlight in Boulder, Colorado following 112 days of summer temperatures (mean ± standard deviation maximum air temperature of 30.2 ± 4.2 °C; orange columns) or 98 days of winter temperatures (mean ± standard deviation maximum air temperature of 8.7 ± 5.5 °C; orange-red columns). Mean values ± standard deviation for* **a-d** *and ± standard error for* **e-l**. *** = $P < 0.001$; ** = $P < 0.01$; * = $P < 0.05$; and n.s. = not significantly different. (Data from Muller et al. 2014b)*

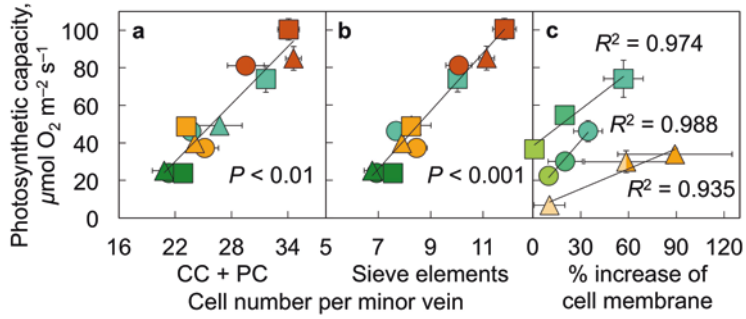

Fig. 2.6. Relationship between photosynthetic capacity and (**a**) the number of companion and phloem parenchyma cells per minor vein, (**b**) the number of sieve elements per minor vein, and (**c**) the estimated increase in cell membrane area of phloem parenchyma cells (transfer cells) due to wall ingrowths in three ecotypes (circles = Italian, triangles = Polish, squares = Swedish) of *Arabidopsis thaliana* grown under 1000 μmol photons m^{-2} s^{-1} at an air temperature of 25 °C (orange symbols) or 8 °C (orange-red symbols) or of 400 μmol photons m^{-2} s^{-1} at an air temperature of 35 °C (olive-green symbols), 25 °C (dark-green symbols), 15 °C (green symbols), or 8 °C (blue-green symbols) or of 100 μmol photons m^{-2} s^{-1} at an air temperature of 25 °C (light orange triangle) or leaves that had developed under the latter and then transferred to 1000 μmol photons m^{-2} s^{-1} at an air temperature of 25 °C for one week (a half light orange and half orange triangle). Mean values ± standard deviation for photosynthesis and ± standard error for phloem cell metrics. (Data from Cohu et al. (2013b) and Adams et al. (2014a))

While hydraulic conductivity and foliar xylem metrics correlate with photosynthetic capacity in many species under many conditions, this was not the case for foliar xylem metrics and photosynthetic capacity in *A. thaliana* plants grown under contrasting temperature regimes (Fig. 2.3c, d). The role of the evolutionary history of *A. thaliana* ecotypes in influencing the acclimatory responses of foliar vascular metrics and photosynthetic capacity to environmental conditions during growth will be explored in the following Sect. 6.

VI. Phenotypic Plasticity Underlying Photosynthetic Acclimation of Ecotypes from Varying Climatic Conditions

A. Response to Growth Light Intensity

Evaluation of foliar acclimation to growth light intensity in the context of environmental conditions prevailing in the sites from which different ecotypes originate offers further insight into the complex relationship between xylem flux capacity and photosynthetic capacity in leaves of *A. thaliana* (Fig. 2.3). With respect to the dependency on the conditions in the habitats of ecotype origin, photosynthetic capacity of low light-acclimated leaves increased slightly but significantly among the three ecotypes with decreasing habitat temperature (and thus increasing habitat latitude). Photosynthetic capacity, furthermore, underwent significant upregulation in response to high growth light intensity in all three ecotypes, with high light-acclimated leaves exhibiting a strong and significant positive linear relationship with decreasing temperature in the habitat of ecotype origin (Fig. 2.7a). Higher photosynthetic capacities were associated with thicker leaves with greater leaf mass per area (Fig. 2.7b), due in part to differences in the number of palisade mesophyll layers (two layers in leaves acclimated to low light compared to three layers in the Italian and Polish ecotypes and four layers in the Swedish ecotype acclimated to high light; Fig. 2.7c). Thicker leaves associated with higher rates of photosynthesis are commonly observed as an acclimatory response to high versus low

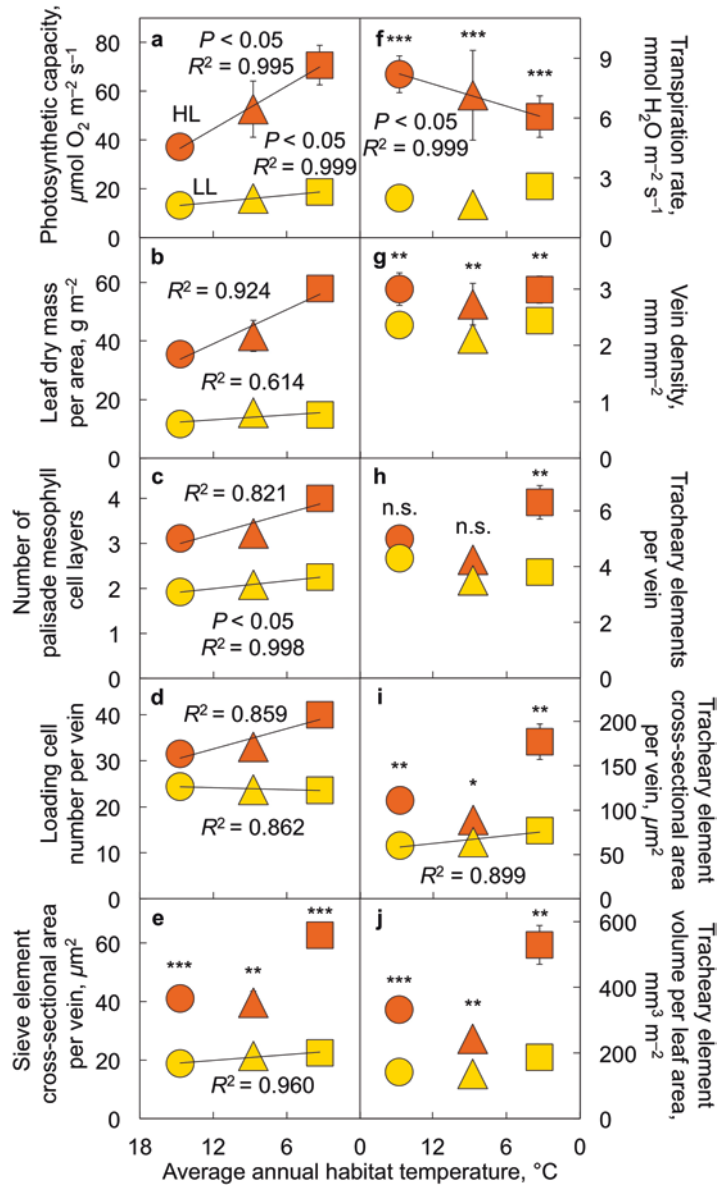

Fig. 2.7. Relationship between average annual temperature in the habitat from which the three ecotypes originated and (**a**) photosynthetic capacity (determined at 25 °C), (**b**) leaf mass per area, (**c**) number of palisade mesophyll cell layers, (**d**) loading cell (companion cells, phloem parenchyma cells, and sieve elements) number per minor vein, (**e**) sieve element cross-sectional area per minor vein, (**f**) transpiration rate determined at a leaf temperature of 27.0 ± 1.0 °C (mean ± standard deviation; $n = 24$) and vapor pressure deficit of 2.03 ± 0.20 kPa (mean ± standard deviation; $n = 24$), (**g**) vein length per area, (**h**) tracheary elements per minor vein, (**i**) tracheary element cross-sectional area per minor vein, and (**j**) the product of vein density and the cross-sectional area of tracheary elements per minor vein (yielding the volume of tracheary elements per unit leaf area) for fully expanded leaves of three ecotypes (circles = Italian, triangles = Polish, squares = Swedish) of *Arabidopsis thaliana* grown under 100 (light orange) or 1000 (orange) μmol photons m^{-2} s^{-1} at a daytime leaf temperature of 20 °C. Mean values ± standard deviation for **a**, **b**, **f**, and **g** and ± standard error for **c**-**e**, and **h**-**j**. Where linear regressions were weak, values for low- versus high-light grown leaves were compared (Student's *t*-test) revealing significant differences indicated with asterisks (* = $P < 0.05$, ** = $P < 0.01$, *** = $P < 0.001$, or *ns* not significantly different). (Data from Adams et al. (2016), Stewart et al. (2017a, b), and Adams et al. (in progress))

light and, in the case of winter annuals and biennials, also in response to low versus moderate temperature (Givnish 1988; Boese and Huner 1990; Terashima et al. 2001; Amiard et al. 2005; Poorter et al. 2009; Gorsuch et al. 2010, Dumlao et al. 2012; Cohu et al. 2014). This acclimation provides a greater number of chloroplasts per unit leaf area, contributing to higher rates of photosynthesis on an area basis.

The number of phloem cells (all of which facilitate the loading of sucrose; see Sect. 3.3) per minor vein was significantly greater in all three ecotypes acclimated to high versus low light. However, the degree of acclimation to growth light intensity varied among the ecotypes, with the least pronounced acclimation seen in the Italian ecotype and the most pronounced acclimation in the Swedish ecotype (Fig. 2.7d). These differential adjustments of phloem cell number presumably correspond to differences in the capacity to actively load sucrose (in support of different photosynthetic capacities) between the two growth light conditions and among the three ecotypes (Fig. 2.7a). Furthermore, acclimation to high versus low light resulted in a greater cross-sectional sieve element area per minor vein (Fig. 2.7e), with such areas presumably corresponding to the minor veins' flux capacity to move phloem sap to major veins. The greater minor cross-sectional sieve element area of Swedish leaves is a likely prerequisite to facilitate their higher photosynthetic capacities compared to the smaller corresponding areas of the Italian leaves.

Another feature of the minor-vein phloem that might further increase the capacity to load and export sucrose is the degree to which phloem parenchyma cells increase their membrane area available for placement of sucrose efflux channels and ATPases via cell wall ingrowths (see Sect. 3.3). Such a contribution of phloem cell ultrastructure may explain the deviation from linearity in the relationship between photosynthetic capacity and loading-cell number per vein (Fig. 2.7d) and especially sieve element cross-sectional area per vein (Fig. 2.7e) for the Polish ecotype among the three ecotypes acclimated to high light. At this time, data on phloem cell ultrastructure are not available for this entire matrix of growth conditions and ecotypes, but a parallel study found that the low temperature-stimulated upregulation of photosynthesis was accompanied by enhanced cell wall ingrowths of minor vein transfer cells. It was indeed the Polish ecotype that exhibited a number of loading cells per minor vein slightly ***below*** the predicted linear relationship among the three ecotypes combined with a level of transfer cell wall ingrowth slightly ***above*** the corresponding linear relationship (Adams et al. 2016; see also Sect. 6.2).

As was the case for photosynthetic capacity (Fig. 2.7a), transpiration rate from leaves of all three ecotypes was significantly greater for plants acclimated to high versus low light (Fig. 2.7f). However, the relationships between either transpiration rate or photosynthetic capacity and temperature in the habitat of ecotype origin exhibited diverging trends (Fig. 2.7a, f). While transpiration rate among the three ecotypes acclimated to high light exhibited a significant and positive linear relationship with increasing habitat temperature (Fig. 2.7f), photosynthetic capacity exhibited a significant negative linear relationship with increasing habitat temperature (Fig. 2.7a). In other words, in response to acclimation to high versus low light, photosynthetic capacity was upregulated to the greatest extent in the Swedish ecotype from the coldest habitat, whereas transpiration rate was greatest in the Italian ecotype from the warmest habitat.

Foliar minor-vein density was also significantly higher in the leaves of all three ecotypes acclimated to high versus low light (Fig. 2.7g). A higher vein density can support higher fluxes of both foliar sucrose export and foliar water intake in support of higher photosynthetic capacities and greater transpirational water loss, respectively.

Furthermore, the number of tracheary elements in those veins was significantly higher in leaves of the Swedish ecotype acclimated to high versus low light (Fig. 2.7h), and the cross-sectional area of the minor veins comprised of tracheary elements was significantly higher in leaves of all three ecotypes acclimated to the higher light intensity (Fig. 2.7i). The product of tracheary cross-sectional area and vein length per leaf area (same as vein density) represents the total volume of tracheary elements per unit leaf area, which was also significantly higher in leaves of all three ecotypes acclimated to high versus low light (Fig. 2.7j).

Water delivered through foliar tracheary elements follows water potential gradients to two major destinations. One is to exit through stomata, driven by the gradient in water vapor pressure between the leaf's water-saturated interior and the atmosphere. The second is into the phloem, driven by active accumulation of sugars and the low water potential resulting from this concentration of solutes in companion cells and sieve elements (Boersma et al. 1991; Patrick et al. 2001; Hölttä et al. 2006; Sevanto et al. 2011; Hölttä and Nikinmaa 2013; Nikinmaa et al. 2013; Jensen et al. 2016). For a comparison of high-light-acclimated leaves of the three ecotypes, photosynthetic capacity was greatest in the Swedish ecotype and lowest in the Italian ecotype – and the same difference could be assumed to exist between the level of sucrose actively pumped into the phloem. If total sucrose flux were proportional to photosynthetic capacity, sucrose flux should be highest in the Swedish ecotype and lowest in the Italian ecotype. Furthermore, given the photosynthetic capacities (Fig. 2.7a) and transpiration rates (Fig. 2.7f) exhibited by the three ecotypes acclimated to high light, a proportionally greater fraction of the water flowing through the xylem may be lost to the atmosphere in the Italian ecotype compared to the other two ecotypes, whereas a proportionally greater fraction of the water flowing through the xylem may follow sucrose into the phloem in the Swedish ecotype. In a summarizing statement, one may speculate that for high-light-acclimated leaves of the three ecotypes, (i) the relatively smallest total volume of tracheary elements per leaf area in the Polish ecotype is sufficient to support this ecotype's intermediate levels of both photosynthesis and transpiration, (ii) the intermediate total volume of tracheary elements per leaf area in the Italian ecotype supports this ecotype's high transpiration rate (highest among the three ecotypes), and (iii) the large total volume of tracheary elements per leaf area in the Swedish ecotype (largest among the three ecotypes) is necessary to support this ecotypes high photosynthetic capacity (highest among the three ecotypes), and by inference, its high sucrose export rate (Fig. 2.7j).

B. Response to Growth Temperature

Whereas the photosynthetic upregulation exhibited by all three ecotypes in response to growth under high versus low light intensity (Fig. 2.7a) is seen in many species, upregulation of photosynthesis in response to low compared to moderate or high temperature typically occurs only in species that remain active during colder months of the year, such as herbaceous winter annuals and biennials (Holaday et al. 1992; Adams et al. 1995, 2001a, b, 2002, 2004, 2013a, 2016; Martindale and Leegood 1997; Strand et al. 1997, 1999; Verhoeven et al. 1999; Amiard et al. 2005; Dumlao et al. 2012; Cohu et al. 2013b, 2014; Muller et al. 2014b; Stewart et al. 2016). *Arabidopsis thaliana* is an archetype for winter annuals in this regard; its leaves exhibit higher photosynthetic capacities (assessed at a common temperature) when grown under low temperature compared to moderate (Cohu et al. 2013b, 2014; Adams et al. 2016) or high (Fig.2.8a; Adams et al. 2016; Stewart et al. 2016) temperature. Moreover, photosynthetic capacity among the three ecotypes exhibited a positive linear relationship with decreasing tem-

perature (and increasing latitude) of the ecotypes' habitat of origin, regardless of the temperature experienced by plants during experimental growth (Fig. 2.8a).

As was the case for acclimation to light intensity (Fig. 2.7a–c), acclimation of photosynthetic capacity to growth temperature among the three ecotypes correlated with leaf thickness (Fig. 2.8a, b), with thicker leaves presumably accommodating greater numbers of chloroplasts per unit leaf area. For leaves of plants acclimated to low temperature, the upregulated maximal rates of photosynthetic oxygen evolution were likely facilitated by upregulated capacities for active loading of sucrose into the minor vein phloem and sucrose export from the leaf. There was a strong, positive linear relationship between decreasing temperature among the ecotypes' habitats of origin and both the number of minor vein phloem cells (Fig. 2.8c) and the level of phloem parenchyma cell wall ingrowths (Fig. 2.8d), as two features that represent means by which total cell membrane surface area for placement of transport proteins associated with phloem loading can vary among the three ecotypes. There was a similar strong positive relationship between cross-sectional area of sieve elements per minor vein (a proxy for the leaf's sucrose export flux capacity) and decreasing temperature of ecotypes' habitats of origin among the three ecotypes acclimated to low temperature (Fig. 2.8e). The differing levels of these three phloem features (Fig. 2.8c–e) are therefore likely to support a relatively high, intermediate, and low capacity for phloem loading and sucrose export in the Swedish, Polish, and Italian ecotypes, respectively, in support of the corresponding range of photosynthetic capacities exhibited by the three ecotypes (Fig. 2.8a).

Whereas photosynthesis, leaf thickness, and features of the phloem that are presumably associated with the capacity for sucrose transport on a leaf area basis were all higher in leaves from plants acclimated to low compared to high temperature and all varied with the temperature of the ecotypes' habitats of origin (Fig. 2.8a–e), transpiration rate and features of the vasculature presumably associated with the capacity for water transport were all higher in leaves acclimated to high compared to low temperature and varied with the precipitation level in the ecotypes' habitats of origin (Fig. 2.8f–j) for leaves experimentally acclimated to different growth temperatures. In this case, the ecotype from the driest habitat exhibited the most pronounced upregulation of transpiration, vein, and xylem features in response to a high growth temperature. One might surmise that the ability to provide for transpirational cooling of the leaves, rather than water conservation, is the stronger imperative for this herbaceous winter annual, and that such acclimation is accompanied by development of a root system that provides for sufficient access to water, especially in habitats that receive relatively low precipitation.

C. Extent of Plasticity Linked to Habitat Environment

Experimental growth of *A. thaliana* at low versus high light intensity under a common moderate temperature regime (Fig. 2.7) and at cool versus hot temperature under a common moderate light intensity (Fig. 2.8) elicited a range of different responses among the three ecotypes. On the other hand, growth of the three ecotypes under a common condition of moderate temperature and moderate light intensity revealed little to no difference among the ecotypes (Cohu et al. 2013a, b; Adams et al. 2016). One must conclude that determination of the full range of genetic potential of a population, as well as of differences between populations (or even species), depends on the growth of plants under multiple contrasting environmental conditions.

To summarize, for many features, the Swedish ecotype exhibited the greatest degree of acclimatory adjustments and the Italian ecotype exhibited the smallest degree

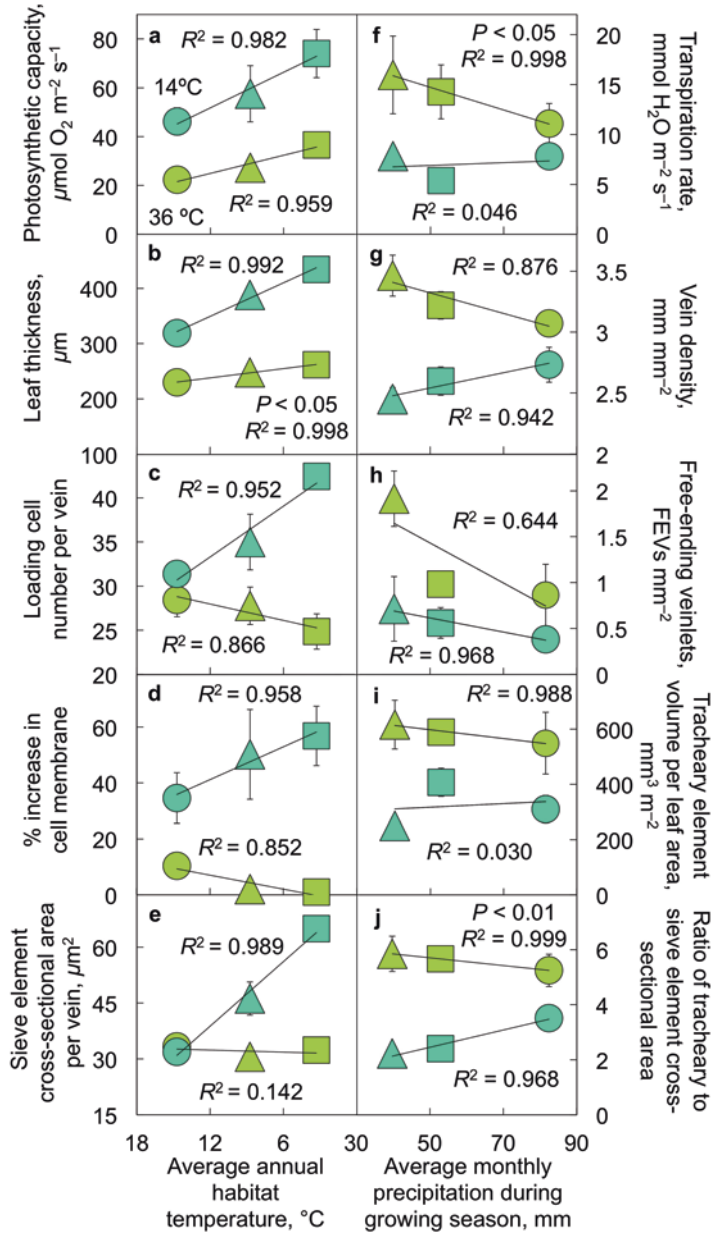

Fig. 2.8. Relationship between the average annual temperature in the habitat from which each ecotype was obtained and (**a**) photosynthetic capacity (determined at 25 °C), (**b**) leaf thickness, (**c**) loading cell (companion cells, phloem parenchyma cells, and sieve elements) number per minor vein, (**d**) percent increase in phloem parenchyma cell membrane, and (**e**) sieve element cross-sectional area per minor vein and between the average monthly precipitation during the growing season and (**f**) transpiration rate determined at a leaf temperature of 28.7 ± 1.6 °C (mean ± standard deviation; n = 29) and vapor pressure deficit of 2.74 ± 0.07 kPa (mean ± standard deviation; n = 29), (**g**) vein length per area, (**h**) free-ending veinlets per leaf area, (**i**) tracheary element cross-sectional volume per leaf area, and (**j**) the ratio of tracheary to sieve element cross-sectional area per minor vein for three ecotypes (circles = Italian, triangles = Polish, squares = Swedish) of *Arabidopsis thaliana* grown under 400 μmol photons m^{-2} s^{-1} at a leaf temperature of 14 °C (blue-green symbols) or 36 °C (olive-green symbols) during the photoperiod. Mean values ± standard deviation for **a**, **f**, **g**, and **h** and ± standard error for **b**-**e**, **i**, and **j**. (Data from Adams et al. (2016) and unpublished data)

of acclimatory adjustments in response to extremes in growth light intensity and temperature. Acclimation of leaf form and function to both cold temperature and high light has been related to a particular family of transcription factors (Thomashow 2010; Hüner et al. 2012, 2016; Kurepin et al. 2013), for which genetic differences were identified between the Swedish and Italian ecotypes (Oakley et al. 2014; Gehan et al. 2015) as well as other sets of *A. thaliana* ecotypes from habitats differing in annual temperature profiles (Alonso-Blanco et al. 2005; Kang et al. 2013; Monroe et al. 2016). A mutation in the C-repeat binding factor (CBF), the overexpression of which resulted in thicker leaves with higher rates of photosynthesis (Savitch et al. 2005; Pino et al. 2008; Dahal et al. 2012), may be responsible for these different responses to growth conditions. Whereas all three *A. thaliana* CBFs are functional in the Swedish ecotype and contribute to its greater freezing tolerance (Oakley et al. 2014), the Italian ecotype was recently shown to encode a non-functional CBF2 (Gehan et al. 2015). The Swedish ecotype, originating from the coldest habitat with the greatest variation in temperature over the course of the year (Adams et al. 2016), exhibited the greatest phenotypic plasticity in leaf thickness/mass (Figs. 2.7b, c and 2.8b), photosynthetic capacity (Figs. 2.7a and 2.8a), and foliar phloem features associated with phloem loading (Figs. 2.7d, e, and 2.8c–e). In a winter annual that thrives during the colder months of the year, such a coordinated response could compensate for the cold-temperature-induced inhibition of the biochemistry of photosynthesis and of transport proteins contributing to active loading of sucrose into the phloem (see Figs. 3.2 and 18.8), as well as the increased viscosity of sugar-laden phloem sap (see also Cohu et al. 2013a, b; Adams et al. 2016; Stewart et al. 2016) that would be progressively more profound with increasing habitat latitude and decreasing habitat temperature. The Polish ecotype exhibited intermediate responses for all of these foliar traits and the Italian ecotype exhibited the smallest responses, consistent with the fact that the habitat of the Polish ecotype from the intermediate latitude is also intermediate in temperature characteristics, and the habitat of the Italian ecotype is warmest and experiences the least variation in temperature over the course of a year (Adams et al. 2016).

On the other hand, the precipitation experienced during the ecotypes evolutionary history in their habitats of origin appears to have strongly influenced the extent of acclimatory adjustment in features contributing to foliar delivery of water. Whereas the Italian ecotype (from the habitat with the greatest precipitation) exhibited the least phenotypic plasticity in transpiration rate, vein density, and ratio of tracheary to sieve elements, the Polish ecotype (from the habitat with the least precipitation) exhibited the greatest phenotypic plasticity in these features related to water transport and loss (Figs. 2.8f, g, j). Thus, the Italian ecotype – from the warmest habitat with the least variation in temperature throughout the year (Adams et al. 2016) and the greatest precipitation – exhibited the least pronounced phenotypic plasticity for most foliar features in response to both growth light intensity and growth temperature. Evolutionarily, the minimal plasticity in the Italian ecotype might have developed in response to an environment in which temperatures are moderate and stable, and water availability is consistently relatively high (rainfall is greatest in Italy among the three ecotype habitats during the months from germination to seed set; Adams et al. 2016). The Polish ecotype receives the lowest precipitation in its original habitat during the months from germination to seed set as well as the lowest annual precipitation among the three ecotypes (Adams et al. 2016) and exhibited the greatest phenotypic plasticity in features associated with water transport and in transpiration rate in response to growth temperature (Figs. 2.8f–j). The latter responsiveness may

ensure continued foliar water delivery and transpirational cooling required by an herbaceous annual species in the face of high temperatures during its relatively short lifespan (see additional discussion in Adams et al. 2016).

VII. Conclusions

The relationship between foliar hydraulic conductance of leaves and photosynthesis is supported by many studies (see Chap. 4). Several foliar vascular metrics (vein density and features of the minor vein tracheary elements) exhibit characteristics consistent with the importance of a sufficient flux capacity for the distribution of water within the leaf in support of photosynthesis and transpirational water loss in a number of species under a range of environmental conditions. However, in a situation unique to winter annuals and biennials, transpiration rate, vein density, and tracheary element features can follow a differential acclimation trend from that of photosynthesis and phloem features in response to low temperature. Under low temperature, transpirational water loss can be greatly diminished, but winter annuals and biennials respond to growth under such conditions with upregulation of their photosynthetic capacity. Conversely, under hot temperature transpirational water loss can be greatly enhanced, but photosynthesis is diminished.

Despite diverging trends in the acclimation of photosynthetic capacity versus transpiration rate, a strong relationship between foliar phloem metrics (likely to underlie the capacity of the phloem to load and export sugars) and photosynthetic capacity has emerged for all herbaceous summer annuals, winter annuals, and biennials that have been characterized thus far under a broad range of growth light intensities and growth temperatures. However, the extent of this characterization has been far from exhaustive and remains in its infancy. Many additional species, representing taxonomic groups with other growth habits, photosynthetic pathways, foliar venation networks, and phloem loading mechanisms, remain to be characterized to verify the universal nature of the role of foliar phloem in contributing to, and possibly setting, the upper limit to photosynthesis.

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Chapter 3

Export of Photosynthates from the Leaf

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Summary

Mature leaves export approximately 80% of the carbon they fix by photosynthesis. Although most attention has been devoted to studying export of recently synthesized sugar, this is only one source of nutrients that enter the export stream. The mesophyll also supplies amino acids and other chemical species; ions and compounds are rerouted from the xylem, and companion cells have the capacity to reconfigure metabolites as they pass along the sieve tubes. The extent to which each of these channels contributes to the complexity of sieve tube content is not easy to estimate, an analytical problem made especially acute by the difficulty in obtaining authentic phloem sap.

Prior to export, the products of photosynthesis, primarily sucrose and, in some plants, sugar alcohol, must be transferred from mesophyll cells to the phloem. To date, three phloem-loading mechanisms are known. Two are metabolically active in the sense that energy is used

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to increase the concentration in the phloem relative to the mesophyll. One of these involves sucrose transfer into the apoplast (cell walls) and subsequent transporter-mediated uptake by phloem cells. The second is an oligomerization or "polymer trap" mechanism in which sucrose enters the phloem through plasmodesmata where it is converted to larger sugars that cannot pass back into the mesophyll cells and are thus vectored out of the leaf. The third loading mechanism is also symplastic (through plasmodesmata) but is passive in the sense that it occurs down the concentration gradient. Polymer trap plants also load apoplastically to a lesser degree and it is possible that heterogeneous patterns of loading are more common in plants than presently realized. The adaptive advantages conferred by the three loading mechanisms are not fully understood. One advantage to active loading is that it allows leaves to maintain overall reduced carbohydrate status, thus freeing up fixed carbon for transport to sinks and increasing growth potential. Symplastic loading mechanisms allow compounds in addition to carbohydrates direct access to the phloem without having to traverse the apoplast.

Export from source leaves needs to be regulated consistent with the dynamic needs of storage and sink organs and with environmental conditions that influence hydrostatic pressure gradients throughout the plant. In principle, export can be regulated at every structure along the path, including chloroplasts, tonoplasts, plasma membranes, plasmodesmata, and sieve plate pores. These regulatory steps could involve transporters as well as biochemical interactions in the phloem and/or in the flanking cells along the phloem path. Examples of control at each of these steps have been described. Notwithstanding, how these diverse processes work together to influence resource partitioning on a whole-plant scale is poorly understood. We present such a model based on sucrose concentration and associated turgor pressure.

I. Introduction

Leaves, like people, pass through successive stages of development, beginning life in a state of total dependence and gradually transitioning into providers. As a young, emerging leaf starts to green, photosynthesis contributes resources in a limited way, like an after-school job held by a daughter or son. But while these initial steps add only trivially to the total resource budget, the path to independence is rapid. When dicot leaves are approximately half grown, they begin to capture more carbon from the atmosphere than they import from the rest of plant and quickly become self-sustaining. (Young adults are less predictable.) This transition from a net importer to an exporter of photoassimilate occurs basipetally (Turgeon and Webb 1973), beginning at the leaf tip and continuing to its base, and is developmentally programmed – in other words it is not driven by the carbon budget itself (Turgeon 1984). Once a leaf becomes an exporter there is no going back. Even complete darkening for many days will not induce a leaf to import more than a small amount of carbohydrate from other leaves (Turgeon 2006). A darkened leaf will soon run out of available carbon and senesce.

Mature leaves export the products of photosynthesis and other nutrients to sinks in various regions of the plant, including newly emerging leaves. Although most mechanistic studies on export have focused on sucrose (Suc), phloem sap is highly complex; the sieve tubes export a wealth of materials from leaves including ions, metabolites, hormones, transcription factors and other proteins, small RNAs and defense compounds (Oparka and Turgeon 1999; Lucas et al. 2013; Van Bel et al. 2013; Notaguchi and Okamoto 2015; Otero et al. 2016; Shabala et al. 2016).

How do we know what is transported in the phloem? This is a very difficult question to answer (Turgeon and Wolf 2009; Dinant and Kehr 2013). Sieve tubes are living entities under extreme pressure. Tapping into the export stream releases the pressure, upset-

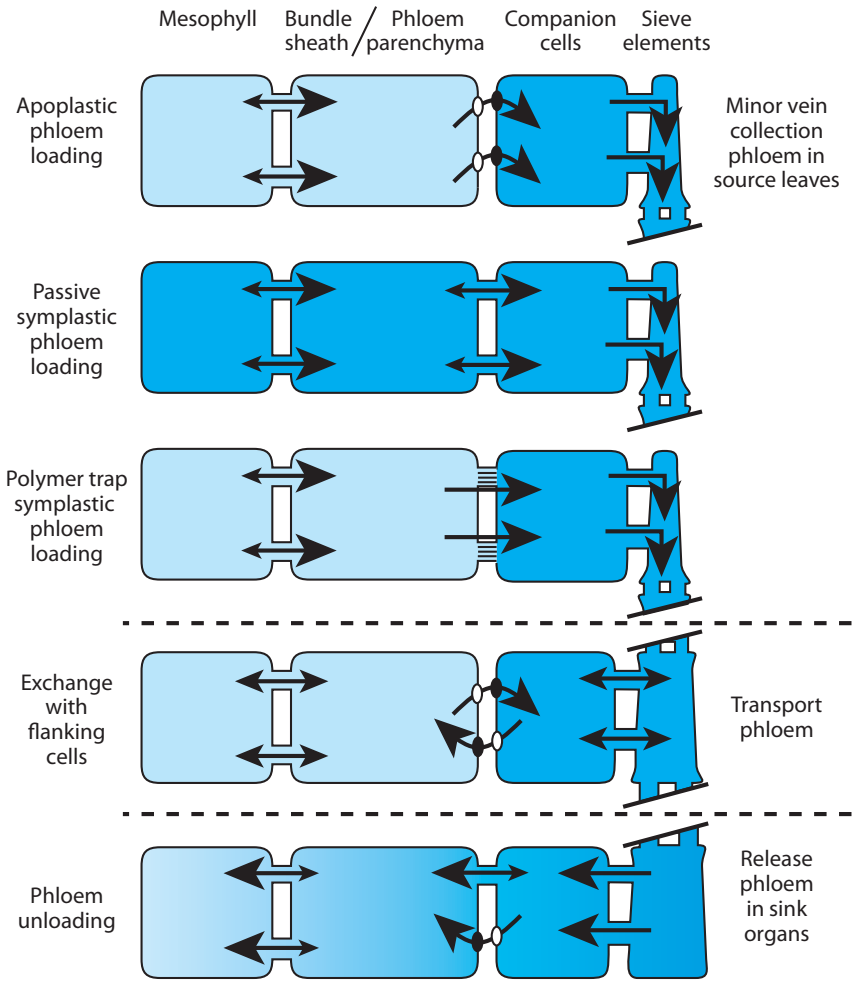

Fig. 3.1. **Principal pathways for entry of solute and solution into sieve elements for long distance transport.** Phloem transport and export from the leaf initiates in the minor vein collection phloem of source leaves. Three loading strategies are represented: apoplastic phloem loading, passive symplastic loading, and polymer trap symplastic phloem loading; mixed loading strategies also exist. Blue color density represents relative solute concentration (primarily Suc, but also raffinose family oligosaccharides in the polymer trap mechanism), which is always high in the phloem. Straight arrows represent flux through plasmodesmata, which may be greater in one direction than the other. Curved arrows indicate apoplastic transport by efflux through SWEET transporters (open ovals) and Suc-proton co-transporters (SUTs, closed ovals). The loss of solute to the sieve elements is unidirectional at the inception of the translocation stream in minor veins. In the transport phloem of larger veins exchange through plasmodesmata keep the sieve elements and companion cells near equilibrium but these are generally symplastically isolated from flanking tissue. In the release phloem of terminal sinks, phloem unloading to adjacent cells is through symplastic domains connected by plasmodesmata or with efflux to the apoplast and influx into adjacent domains catalyzed by membrane transporters. See text for details

ting water balance, rupturing the internal matrix of the sieve tubes, releasing non-mobile materials, and probably dragging companion cell (CC) content into the sieve elements (SE) as well. Furthermore, unless the puncture is made into the sieve tube directly, content from other cells can contaminate collected "phloem sap" immensely. All this in addition to the immediate and long-term synthesis and release of additional compounds that seal and protect the phloem against solute loss and pathogen invasion.

Authentic, mobile constituents of phloem sap come from a number of sources (Fig. 3.1) although the origin of a given ion or compound is not always clear. Some compounds are made in mesophyll cells or other non-phloem cells and loaded into the phloem for export (Braun et al. 2014; Savage et al. 2016). Another site of synthesis is the SE/CC complex itself. CCs are connected to sieve tubes by plasmodesmata with especially large size-exclusion limits, allowing the exchange of materials all along the transport pathway (Kempers and van Bel 1997; Imlau et al. 1999; Stadler et al. 2005). In addition, other materials, especially ions, are captured on their way into the leaf in the xylem and re-routed back out of the leaf again in the phloem (Tegeder 2014; Yamaji and Ma 2014; Savage et al. 2016). All this exchange provides the plant with an opportunity to dynamically modify sieve tube content to satisfy the nutritional and defensive needs of the sinks.

Exchange of metabolites along the phloem transport pathway has deep implications, especially with regard to the range of nutrients and metabolites exported out of the source leaf in the translocation stream. Molecules up to the size of green fluorescent protein are able to pass back and forth from metabolite-rich CC to sieve tubes through the relatively wide plasmodesmata that connect these two cell types, thus allowing mixing of content to occur in both directions (Kempers and van Bel 1997; Imlau et al. 1999; Stadler et al. 2005). Indeed, analyses of phloem sap collected from severed aphid stylets demonstrate that the river of solution in the sieve tubes is rich in ions and organic compounds, including amino acids, organic acids, macromolecules, etc. (Winter et al. 1992; Lough and Lucas 2006; Turgeon and Wolf 2009). How can a plant regulate which low-molecular weight metabolites are to be transported, or not transported?

Loss of metabolites from CC to SE must be most extreme in source leaf minor veins where the contents of the SEs are continually being exported (Fig. 3.1). How are these metabolites replenished? Why do the CCs in minor veins not lose all their small molecules to the transport stream and bleed to death? An approximation of steady state is possibly reached somewhere along the transport pathway as CC metabolites lost to the sieve tubes are replaced by other solutes entering from the sieve tubes, but at the beginning of the transport stream net flux must remove ions and small molecules from the CC. This problem is especially acute in apoplastic loaders (see below) since they are largely divorced symplastically from the mesophyll and must rely to a large extent on membrane transport systems to re-supply CCs with nutrients and metabolites. From this viewpoint, symplastic loaders have an advantage because replacement compounds can migrate into the phloem through plasmodesmata from the much larger number of mesophyll cells. It seems likely that CCs in the collection phloem of leaves of apoplastic loaders expend a lot of energy re-synthesizing or importing metabolites that they lose to the SEs. Perhaps this is a major reason why the CCs in minor veins of apoplastic phloem loaders are generally larger and more metabolically active than the CCs further along the transport route in the leaf, stem and roots of the same plants. This would suggest that the minor vein CCs of plants that load through plasmodesmata are not so large and metabolically active and indeed, in poplar, a passive symplastic loader, minor vein companion cells are difficult to distinguish from phloem parenchyma cells (Russin and Evert 1985). The companion (intermediary) cells in the minor veins of polymer trap plants are also large and metabolically active, but it seems likely this is a consequence of the RFO synthetic activity.

No matter how different sap constituents get into the sieve tubes, sugar provides most of the osmotic potential that motivates transport. In the next section, we discuss three mechanisms by which Suc is loaded into the sieve tubes, a subject that has been reviewed by others in recent years (Turgeon and Wolf

2009; Ayre 2011; Eom et al. 2012, 2015; Atkins 2013; De Schepper et al. 2013; Knoblauch and Peters 2013; Liesche and Schulz 2013a; van Bel et al. 2013; Braun et al. 2014; Chen 2014; McCurdy and Hueros 2014; Ryan and Asao 2014; Stroock et al. 2014; Savage et al. 2016).

II. Phloem Loading Mechanisms

Carbon photoassimilated in leaf mesophyll cells has three principal fates. A portion contributes to the metabolic needs of the cell by contributing to cellular components or being respired for energy. A second portion is transiently stored, primarily as starch in chloroplasts but also as soluble organic molecules in vacuoles, to provide fixed carbon for the heterotrophic phase of the diurnal cycle. The third and remaining principal fate is transport out of the leaf via the phloem. Mature mesophyll cells have relatively low internal needs for self-maintenance, and transient reserves accumulated during the day are remobilized during the night to maintain soluble carbon for maintenance and transport (Graf et al. 2010; Sulpice et al. 2014). The primary fate of photoassimilated carbon is thus long-distance transport from photoautotrophic source leaves to heterotrophic organs, and as much as 80% of the carbon assimilated in mature leaves, and comparable amounts of other nutrients cycling through leaves, such as potassium and amino acids, are exported in the phloem (Kalttorres et al. 1987).

The basic principle of export from leaves via the phloem appears to be osmotically generated pressure flow as described by Ernest Münch (1930) over 85 years ago. In its simplest distillation, Münch's pressure flow hypothesis is based on source organs being connected symplastically to sink organs (Fig. 3.1). The accumulation of solute at the source end promotes osmosis of water to generate pressure, while solute use in sink organs reduces pressure; the pressure gradient drives mass flow of the phloem sap from source to sink. Over the decades, Münch's model has been debated, discarded, and reaccepted (Sjolund 1997; Knoblauch and van Bel 1998; Jensen et al. 2012; de Schepper et al. 2013; Ryan and Asao 2014); refinements have been made and details specific to different plant lineages have emerged, but the basic premise remains intact. One significant refinement has been in defining where and how solute and the resulting hydrostatic pressure is generated in source leaves. Although phloem transport evolved with the earliest lineages of land plants, the diversity of mechanisms employed in extant species implies a survival advantage in modifications of the ancestral state, whatever that may be. The following sections describe three mechanistic strategies that generate hydrostatic pressure in phloem of source organs. It is important to note that these are mechanisms, not necessarily descriptions of a given plant's entire export strategy. As we will see, some plants use more than one loading mechanism and continuing work is likely to reveal additional interactions and complexities that we are blind to at present.

Before detailing mechanisms of phloem loading, it is worthwhile discussing solute in the pre-phloem pathway. Schulz (2015) recently reviewed this subject for all loading strategies across a diversity of plant lineages and only general considerations are discussed here. The distribution of mesophyll and vascular tissue in the lamina of arabidopsis (*Arabidopsis thaliana*) suggests that Suc needs to travel at most six cells before reaching the phloem of a minor vein (Haritatos et al. 2000a). Plasmodesmatal coupling between mesophyll cells and between mesophyll cells and bundle sheath cells is high. In immature leaves, green fluorescent protein moves freely through the primary plasmodesmata of these interfaces, but becomes more restricted during the sink-to-source transition when secondary plasmodesmata become more prevalent (Oparka et al. 1999). Nonetheless, these plasmodes-

mata still allow efficient movement of Suc-sized solutes, as indicated by the spread of photo-activated fluorescein (Liesche and Schulz 2012a, b). In addition, a primarily symplastic route for solute exchange is implied by a narrow concentration window of external solute that causes plasmolysis across spongy and palisade mesophyll cells, indicating a consistent internal solute potential (Beebe and Evert 1992; Turgeon and Medville 1998).

The term phloem loading has historically been used for the energized accumulation of solute into the companion cell/sieve element (CC/SE) complex to generate hydrostatic pressure exceeding that of the surrounding tissues. As the term implies, energy is consumed to "pack" solute into the phloem against a concentration gradient. Interestingly, Münch's original hypothesis did not include this energized phloem-loading step but instead proposed that the highest solute concentration is at the site of photoassimilation in mesophyll cells. Later analysis, mostly in herbaceous crop plants, showed that the Suc in phloem sap is much more concentrated than in cytoplasm and energized loading became the mechanism *de rigueur* for driving long distance phloem transport (see Turgeon 2010a).

The discovery of active phloem loading, resulting in exceptionally high sugar concentrations and osmotic potential in the sieve tubes, was seen to augment the Münch pressure-flow hypothesis and led to the widely-accepted view that active loading evolved to drive long-distance transport. That teleological scheme has been called into question by the realization that phloem loading in many trees, where transport distances are longest, is passive, with a downhill gradient of Suc from mesophyll cells to sieve tubes (see below). According to this concept, the adaptive advantage of active loading is not to power transport *per se* but to allow the mesophyll cells to lower their sugar content, freeing up carbohydrate to drive rapid growth (Turgeon 2010a).

III. Apoplastic Loading

Phloem loading of Suc from the apoplast was first proposed in the 1960s (see reviews by Geiger 1976; Giaquinta 1983). In this model, Suc in mesophyll cells moves toward vascular tissues through plasmodesmata and enters the apoplast in the immediate vicinity of the phloem: most likely from phloem parenchyma or bundle sheath cells that are in direct contact with the CC/SE complex (Fig. 3.2). From the apoplast, Suc is then loaded into the CC/SE complex by energized co-transport with protons (Giaquinta 1977, 1979). It is noteworthy that this model, which is now well proven, was established only with physiological evidence and without the benefit of molecular biology and fluorescent tracers. Indeed, the technical advances of the last 40 years have confirmed the model's accuracy rather than introduce unexpected steps. Examination of plant lineages that deviate from this model led to the description of completely alternative loading pathways rather than modest tweaking of the apoplastic loading hypothesis (see below).

Efflux from the mesophyll symplast is the first step in apoplastic phloem transport (Fig. 3.2). Because of the favorable drop in sugar concentration from the mesophyll symplast to the apoplast, passive transport would be sufficient for efflux. Although this step clearly occurs, molecular characterization of the mechanism remained enigmatic and led to broad speculation of which proteins were involved (see, for example, discussion in Ayre 2011). SWEET (Sugar Will Eventually be Exported Transporters) proteins were recently identified by Förster resonance energy transfer (FRET)-based sugar sensors as a novel class of transporter, initially for glucose and then for Suc (Chen et al. 2010, 2012). Research on SWEETs is in its infancy, but is expanding rapidly (Chen, 2014; Eom et al. 2015). There are 17 SWEET members in arabidopsis and 21 in rice, falling into four clades, and these are involved in

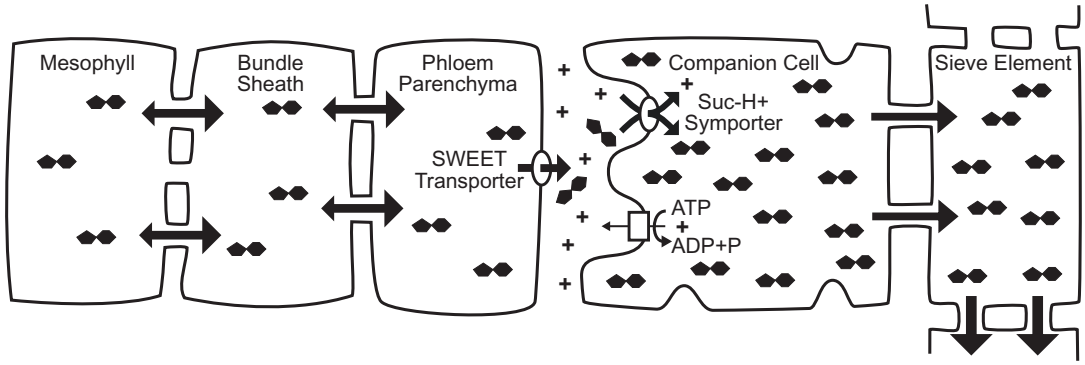

Fig. 3.2. **Phloem loading sucrose from the apoplast.** Sucrose produced in mesophyll cells moves cell-to-cell through regular plasmodesmata. From the phloem parenchyma cells, sucrose enters the apoplast through SWEET proteins down a favorable concentration gradient. From the apoplast, sucrose is taken up, against a steep concentration gradient, into the companion cell / sieve element complex via sucrose transporters energized by the proton motive force. ATP is hydrolyzed by P-type ATPases to provide the proton motive force. Other solutes intended for transport similarly need to undergo efflux to the apoplast and uptake to the companion cell / sieve element complex, or need to be synthesized in the companion cells. Some species have transfer cell morphology – invaginations of the plasma membrane presumably to accommodate large quantities of transmembrane transport systems – on companion cells, phloem parenchyma, or both. Companion cells of apoplastic loaders do have relatively uncommon plasmodesmata with phloem parenchyma and bundle sheath cells (not shown), but their role is not clear. If they are functionally open, they may provide solute exchange, but would also be conduits for wastefully loss of accumulated sucrose. Water and some ions are provided from the xylem (not shown). The size and direction of the arrows indicate potential solute movement. General movement toward sieve elements is assumed, but symplastically connected cells in the pre-phloem pathway maintain near equilibrium

a diversity of transport processes throughout the plant. Facilitated transport of Suc from phloem parenchyma cells to the apoplast of the CC/SE complex is mediated by members of clade III. Functional redundancy is high: Clade III in arabidopsis has seven members, paralogs *AtSWEET11* and *AtSWEET12* share 88% similarity, and both express in phloem parenchyma cells. Single mutations have minimal phenotype while the double mutant accumulates starch and ^{14}C in $^{14}CO_2$ feeding studies. However, even these do not accumulate as much carbohydrate as *Atsuc2* mutants (see below), suggesting that other paralogs, or alternative mechanisms, operate to facilitate efflux in preparation for phloem loading. *AtSWEET13*, also in clade III, is upregulated approximately 16-fold in the *Atsweet11/Atsweet12* double mutant, suggesting further redundancy (Chen et al. 2012).

The second step of apoplastic phloem loading is energized uptake into the CC/SE complex of minor veins (Fig. 3.2). This is mediated by Suc/proton symporters called either SUTs or SUCs (Suc Uptake Transporters or Suc Uptake Carriers, respectively) and is energized by the proton motive force. *SUTs* constitute a small multigene family ranging from as few as three members (Solanaceae) to nine (arabidopsis) that group into three to five clades, depending on whether the major branches are kept together or subdivided. Monocots and dicots have independent clades for high affinity uptake across the plasma membrane (such as phloem loading) and all plants share a clade that localizes to the tonoplast and another clade that is poorly characterized but may catalyze low-affinity uptake or act as Suc sensors. SUTs are involved in a diversity of Suc transport processes throughout the plant and have been studied and reviewed extensively from biochemical, genetic, physiological and evolutionary perspectives (Bush 1993; Braun and Slewinski 2009; Ayre 2011; Reinders et al. 2012).

Because of their significance in whole plant carbon partitioning and productivity, SUTs participating in phloem loading are the best studied. Because uptake is energized by the proton motive force, Suc can accumulate to high levels such that the total solute concentration in source phloem can exceed 1 Osm. Sugar alcohols (polyols) are prominent transport sugars in some species, and appear to be loaded into the phloem from the apoplast by proton symporters in a mechanism equivalent to Suc loading from the apoplast (Noiraud et al. 2001; Gao et al. 2003; Reidel et al. 2009).

Many, but not all, species that phloem load from the apoplast have anatomical modifications for efficient movement of nutrients to and from the apoplast compartment (Fig. 3.2). These "transfer cells" have secondary wall ingrowths and amplified plasma membrane enriched in transport proteins. Transfer cells are found in other locations in plants at interfaces where high-efficiency solute transfer between symplast and apoplast are required (Offler et al. 2003; McCurdy and Hueros 2014). Identification of CCs with extensive transfer cell morphology contributed significantly to early models of apoplastic phloem loading (Gunning et al. 1968). Pea plants have CCs with transfer cell morphology, presumably for efficient loading (Wimmers and Turgeon 1991) while arabidopsis has phloem parenchyma cells with transfer cell morphology for efficient solute release (Haritatos et al. 2000a). *Senecio vulgaris* (old-man-in-the-spring) has transfer cell morphology in both cell types (Pate and Gunning 1969).

IV. Symplastic Loading

Before the 1990s it was generally thought that apoplastic loading was universal in higher plants, although a crack in that sanguine image had already begun to form. Electron microscopy studies had shown that plasmodesmatal frequencies are very high at the interface between bundle sheath cells and CCs in the minor veins of *Cucurbita pepo* (Turgeon et al. 1975). On a much more comprehensive scale, Yuri Gamalei conducted electron microscopy studies on minor veins of over 1000 species, demonstrating that some have "open" (type 1) phloem, with many plasmodesmata leading from the mesophyll to the CC, while the phloem of many other plants is "closed" (type 2), with few such plasmodesmata (Gamalei 1989, 1991). This feature is notably uniform within plant families. Thirty percent of the dicotyledonous families examined by Gamalei (e.g., Tiliaceae and Salicaceae) have open phloem, which provides the plants with at least a potential route for symplastic loading. In contrast, 40% of the families studied, (e.g., Fabaceae and Brassicaceae) have "closed" phloem and are assumed to load from the apoplast. It should be noted that there are always some plasmodesmata leading into minor vein CC from surrounding tissue, even in plants with "closed" phloem. These plasmodesmata must play an important role because there is a severe downside to their presence: they are the avenues by which viruses enter the phloem to become systemic. The remaining 30% of dicot families (type 1-2a) fall into a middle category with moderate plasmodesmatal numbers.

If plasmodesmata provide open channels for flux of ions and compounds between cells, how is the high concentration of sugar in the minor vein phloem of type 1 plants established and maintained? Why does the Suc not leak back out toward the mesophyll? It appears that there are two answers to that question. The first is that in many plants, almost all of which are trees, the concentration of Suc in the cytosol of mesophyll cells is very high, even higher than in the phloem (Turgeon and Medville 1998; Rennie and Turgeon 2009; Fu et al. 2011). Therefore, Suc can move by diffusion, or a combination of diffusion and bulk flow (Voitsekhovskaja et al. 2006), into the phloem (passive phloem loading) (Fig. 3.3). The second answer is that

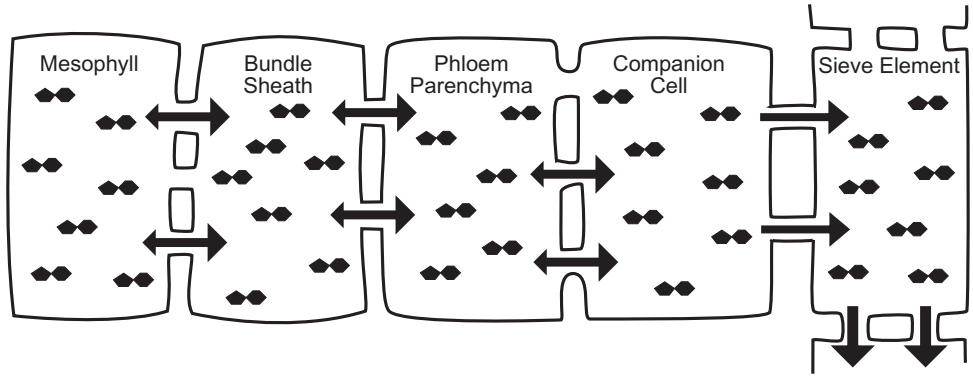

Fig. 3.3. **Passive loading through the symplast.** Species that use passive loading have regular plasmodesmata connecting mesophyll cells through to the sieve elements, which allow relatively free movement of small solutes. There is not an energized concentrating step into the companion cell / sieve element complex. The photosynthetic mesophyll cells have the highest solute concentration in the transport path, and consequently the highest hydrostatic pressure. The size and direction of the arrows indicate potential solute movement. General movement toward sieve elements is assumed along modest downhill concentration gradients

in other species, both trees and herbs, Suc is converted in the phloem into larger sugars that cannot pass back into the mesophyll simply because they are too large, thus vectoring flow out of the leaf. This is known as the polymer trap mechanism (Fig. 3.4). We will now look at these two symplastic phloem-loading processes.

V. Passive Symplastic Loading

The families that Gamalei characterized as having open phloem (type 1) fall into two distinct groups. Some are polymer trap plants (see below) while others are putative passive loaders. If the polymer trap families are removed from the type 1 category, almost all the remaining plants, in other words the putative passive loading species, are trees (Davidson et al. 2011).

If phloem loading is truly passive, the solute concentration must be very high in the cytosol of mesophyll cells (Fig. 3.3). Indeed, some species in Gamalei's typology with high plasmodesmatal frequencies have more than 50 times the foliar concentrations of transport sugar than species with sparse plasmodesmata (Rennie and Turgeon 2009), and when one considers that the Suc concentration in the cytosol is typically several times that in the vacuoles (Heineke et al. 1994; Winter et al. 1993, 1994), the sugar content could be very high (Fu et al. 2011). Interestingly, the trees that are likely to load passively, but not those that have the characteristics of apoplastic loading, contain small but significant amounts of raffinose and stachyose in the phloem sap (Slewinski et al. 2013). The role of these sugars is not known but it is possible that even a limited synthesis of these compounds from Suc in the phloem is sufficient to accelerate export in passive loading plants.

Passive loading also assumes that solute concentrations in the sieve tubes and associated CC are at least marginally lower than in the surrounding mesophyll cells. Solute concentration in cells can be estimated by plasmolysis: soaking tissue in solutions of graded osmolality and examining different cell types to see, in this case by electron microscopy, if the plasma membrane has pulled away from the cell wall (Geiger et al. 1973). Russin and Evert (1985) plasmolyzed leaf tissue of poplar (*Populus deltoides*), a species with abundant plasmodesmata leading into the minor

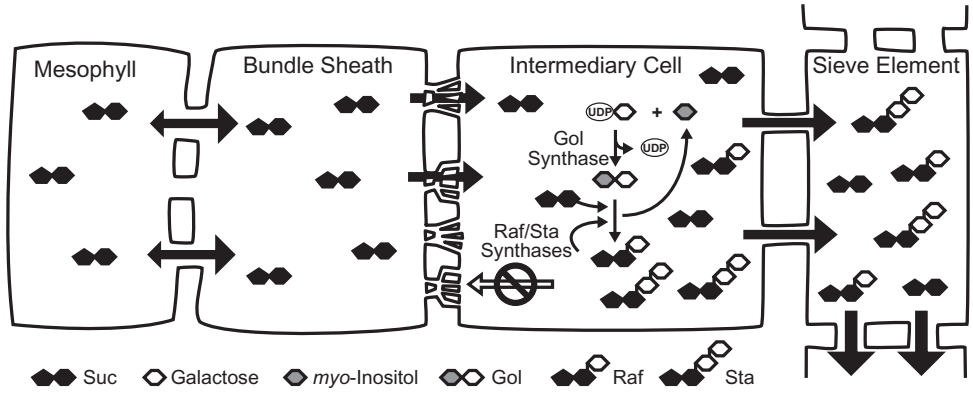

Fig. 3.4. **Loading through the symplast using the polymer-trap mechanism.** Regular plasmodesmata among mesophyll cells and bundle sheath allows relatively free solute movement. Sucrose from bundle sheath cells diffuses into intermediary cells (specialized companion cells) through fields of highly branched plasmodesmata found exclusively at this interface. In intermediary cells, a proportion of sucrose is converted to raffinose family oligosaccharides, raffinose (Raf) and stachyose (Sta) by the sequential action of raffinose synthase and stachyose synthase. In some species, higher-order oligosaccharides are created by additional enzymes. The galactosyl donor for raffinose family oligosaccharide synthesis is galactinol (Gol) created from UDP-galactose and myo-inositol by galactinol synthase. Conversion of Suc to raffinose family oligosaccharides favors the continued passive entry of sucrose, while raffinose family oligosaccharide accumulation generates hydrostatic pressure. The size and direction of the arrows indicate potential solute movement; note that the raffinose family oligosaccharides are thought to be too large to diffuse back toward the bundle sheath, and are thus trapped

vein phloem, and found that solute concentration in the CC is lower than in the mesophyll, which is in marked contrast to results obtained from apoplastic loaders in which the phloem has very high solute content. This is consistent with passive migration of solute from mesophyll to the CC. However, these authors postulated active loading from CC to SE because the solute concentration of the latter was slightly higher than that of the former (Russin and Evert 1985). While this could be true, the difference between the two was smaller than the concentration steps of mannitol used to plasmolyze the tissue, so it may not be real. In other words, there is no convincing evidence for an uphill solute gradient from the mesophyll into the minor vein sieve tubes in poplar. A similar study in willow, also in the Salicaceae (Turgeon and Medville 1998), failed to reveal any measurable solute concentration differences between the mesophyll, CC and SE.

Several predictions follow from the passive loading hypothesis. First, mesophyll cells should contain a considerable amount of photoassimilate, at least in the cytosol, in order to provide nearly equivalent levels to the SEs. Second, plasmodesmata connecting the phloem to surrounding cells should be abundant. Third, exogenously applied [^{14}C]Suc should not accumulate in minor veins as it does when loading is active. Available data on a range of woody and herbaceous species are consistent with these predictions. Woody species such as apple and poplar have much higher sugar levels in leaves than herbaceous species. They fall into Gamalei's type 1 category with open phloem, and when leaf tissue is provided with [^{14}C]Suc it does not accumulate in the minor vein network as it does in plants that load actively, either from the apoplast or by polymer trapping (Fu et al. 2011; Reidel et al. 2009; Rennie and Turgeon 2009).

In another test of the passive loading hypothesis, gray poplar (*Populus tremula X alba*) plants were genetically transformed to introduce invertase to the cell wall space, a protocol that severely inhibits phloem loading and growth in plants that load through

the apoplast. The transgenic poplar grew normally, without any carbohydrate accumulation in the leaves, consistent with transfer of Suc from the mesophyll into the phloem through plasmodesmata, not across the apoplast (Zhang et al. 2014). This suggests that Suc transporters are not directly involved in phloem loading in poplar.

Although available data are consistent with passive loading in many trees, this raises another question: is the pressure generated this way sufficient to drive transport by Münch pressure-flow over long distances? It is useful here to note that Münch did not invoke an active phloem-loading step in his transport model (Münch 1930). He assumed that mesophyll cells are the source of the pressure that drives phloem transport. Even though intuition suggests that translocation over the distances encountered in trees requires a larger pressure differential than in small plants, actual measurements (though plagued by experimental difficulties) have so far failed to reveal large, or even measurable, pressure differences between sources and sinks in trees (Knoblauch and Peters 2010; Turgeon 2010b). Although this is puzzling, it must be recognized that phloem transport efficiency is highest if the pressure differential is small (Thompson 2006). The required pressure difference between sources and sinks required to drive flow over long distances is difficult to model (Nikinmaa et al. 2014; Ryan and Asao 2014) but theory (Jensen et al. 2011, 2012) and empirical data from lab-on-a-chip devices (Jensen et al. 2009; Comtet et al. 2017a) are consistent with the conclusion that relatively small pressure differences are able to drive transport in trees.

VI. Active Symplastic Loading (Polymer Trapping)

Zimmermann (1957) was apparently the first to detect very high concentrations of raffinose-family oligosaccharides (RFOs; raffinose and stachyose) in phloem sap, in this case in ash (*Fraxinus americana*). Small quantities of mobile raffinose had been detected in other species, but the RFO concentration in the sap of the ash tree was much higher, exceeding that of Suc. Subsequent work demonstrated that this is a qualitative difference in the sense that it identifies a unique mechanism of phloem loading (Turgeon and Gowan 1990) in at least 15 families of dicots, including the Cucurbitaceae and Oleaceae (Turgeon et al. 2001).

RFO plants have other identifying phloem characteristics: the CCs in the minor veins are especially large, with dense cytoplasm and small vacuoles, and they are connected to bundle sheath cells by very abundant, asymmetrically-branched plasmodesmata (Turgeon et al. 1975; Fisher 1986; Hoffmann-Thoma et al. 2001). Indeed, these cells probably have the most abundant plasmodesmata at any interface in plants. These plasmodesmata are visibly narrowed on the bundle sheath side in *Coleus blumei* (Fisher 1986), but dye-coupling experiments have shown that they are open to small-molecule movement (Turgeon and Hepler 1989; Liesche and Schulz 2012a, b). The correlation of RFO transport and the presence of these unique plasmodesmata led to the concept of polymer trapping, a phloem loading mechanism in which Suc enters the CC through plasmodesmata, rather than the apoplast, and is converted into RFOs, which are too large to pass back though the plasmodesmata into the bundle sheath and mesophyll (Turgeon and Gowan 1990).

This proposed mechanism (Fig. 3.4) is consistent with several lines of evidence. RFO synthesis occurs in the specialized CCs in the minor veins, often called "intermediary cells," described above (Holthaus and Schmitz 1991; Beebe and Turgeon 1992; Haritatos et al. 2000b). The plasmodesmata that form the potential route for Suc influx are secondary and develop just as export of photoassimilate begins (Volk et al. 1996).

There is a perfect correlation between RFO transport and the presence of intermediary cells (Turgeon et al. 1993). This includes the interesting case of *Amborella trichopoda*, the sole extant member of the order Amborellales, which is apparently sister to all other flowering plants (Turgeon and Medville 2011). Inhibition of RFO synthesis inhibits phloem loading and transport (McCaskill and Turgeon 2007). Conversely, phloem loading in putative polymer trap plants is insensitive to either chemical (Weisberg et al. 1988; Turgeon and Gowan 1990; van Bel et al. 1992; Flora and Madore 1993) or genetic inhibition (Zhang and Turgeon 2009) of Suc transporter activity.

An interesting prediction that follows from the polymer trap model is that molecules the same size, or smaller, than Suc should be able to enter the intermediary cells passively and be exported, essentially hitchhiking in the assimilate flow powered by RFOs. This does appear to be true. When [^{14}C]Suc is applied to the abraded leaf tissue of polymer trap species that transport RFOs and mannitol, the minor veins accumulate ^{14}C to levels well above those in the mesophyll, as seen in autoradiographs. However, this is not true when leaf discs of the same plants are exposed to [^{14}C]mannitol, presumably because there is no mannitol-concentrating mechanism on the phloem membranes (Reidel et al. 2009; Rennie and Turgeon 2009; Fu et al. 2011). In contrast, radiolabeled [^{14}C]Suc and [^{14}C]sorbitol both accumulate to high levels in the minor veins of *Plantago major* (Reidel et al. 2009; Fu et al. 2011), a sucrose/sorbitol transporting species that loads via the apoplast. This result supports the concept that small molecules in mesophyll cells, such as sugar alcohols, have unrestricted access to the phloem via plasmodesmata in species that load symplastically.

The most contentious issue concerning the polymer trap hypothesis is the requirement for size discrimination between Suc and raffinose even though the dimensions of these molecules are not very different (Turgeon and Gowan 1990). Precise size discrimination by pores is theoretically possible but they must not be much wider than the smaller of the permeant molecules, and this reduces overall flux (Dechadilok and Deen 2006). It seems likely that the extreme density of plasmodesmata at the bundle sheath-intermediary cell interface is a response to this narrowing, making up for inefficiency with numbers. Nonetheless, it needs to be shown that the polymer trap mechanism is consistent with biophysical principles. On this point the jury is still in deliberation but leaning toward a positive verdict. Haritatos and Turgeon (1995) thought the mechanism feasible based on equivalent pore analysis. Liesche and Schulz (2013b) were less confident. They concluded that, "none of the configurations (of theoretical pore types) could enable a diffusion-driven Suc flux that matches the reported rates (of transport)..." Dölger et al. (2014) were somewhat more optimistic, concluding that the mechanism can in principle function but only by "pressing the theories for hindered transport to the limit of (or beyond) their validity." A more recent analysis by Comtet et al. (2017a, b) is more optimistic still: "…we find that the conditions required to provide segregation and gradient inversion lead to physiologically reasonable rates of export, if account is taken for the unusually high density of plasmodesmata in trapper species."

It should be emphasized that these *in silico* models, while instructive, are naive in important ways, assuming in most cases idealized shapes not only for the molecules in transit but also the pores. It seems likely that plasmodesmatal pores are not that simple, that they are constructed for specific purposes at specific interfaces – this much we can infer from the wealth of data on structure/function relationships of membrane pores. While the internal structure of plasmodesmata remains opaque to us due to their minute dimensions and the limitations of electron microscopy, it seems entirely feasi-

ble, in fact likely, that they are configured subtly to optimize discrimination of permeant molecules on the basis of shape and chemistry. This is a difficult subject; progress will require a considerably better understanding of plasmodesmatal substructure than is possible with current technology.

Why has polymer trapping evolved? There must be a selective advantage. It seems unlikely that the transport of raffinose and stachyose specifically confers some chemical bonus in sinks since these sugars are rapidly hydrolyzed as they are unloaded. A more feasible possibility is that the system allows small molecules, possibly of a protective nature, to load from the mesophyll to the phloem through plasmodesmata, without having to cross any plasma membrane. In this scenario, there would be no positive force to drive sucrose into the phloem because it would leak back toward the mesophyll through the abundant plasmodesmata, creating a futile transport-leak cycle. Mechanistically, the solution to this problem is to create oligomers from sucrose that are too large to back-diffuse; thus the "polymer trap." As an example of such a protective molecule, the iridoid glycoside catalpol is exported from the leaves of *Catalpa speciosa*, an RFO transporter (Turgeon and Medville 2004). Catalpol, which protects plants from insect herbivores, is approximately the size of sucrose.

However, there is an implicit assumption in this storyline that warrants discussion. The assumption is that back-diffusion of sucrose to the mesophyll must be prohibited to allow sugar to build up in the leaf phloem to drive long-distance transport. However, Comtet et al. (2017b) show that, counterintuitively, even higher rates of export can be achieved if polymers such as RFOs produced in the phloem are allowed to back diffuse. If that is the case, why does this not occur? Why is the mesophyll essentially devoid of RFOs in *Catalpa speciosa*, the cucurbits, and other polymer trap plants? As discussed above, a reasonable explanation is that inhibiting back-diffusion keeps the soluble carbohydrate level low in mesophyll cells which frees up carbon and increases growth potential (Turgeon 2010a).

VII. Heterogeneous Phloem Loading

Some plants appear to use more than one mechanism of phloem loading and it is possible that such heterogeneity, or "mixed" loading, is common in plants (Orlich et al. 1998; van Bel 1993; Slewinski et al. 2013). Polymer trap species are the most obvious example. In the cucurbits, the smallest veins are very simple, with two intermediary cells engaged in polymer trapping. But in some minor veins there is a third CC with the appearance of an "ordinary" CC, typical in apoplastic loading species and with relatively little symplastic connection to surrounding cells. Larger veins in all polymer trap plants have more of these ordinary CCs, and proportionately fewer intermediary cells (Turgeon et al. 1975; Fisher 1986; Gamalei et al. 1994; Voitsekhovskaja et al. 2009). This suggests that polymer trapping is complemented by apoplastic loading, a conclusion borne out by experimental evidence. First, polymer trap plants transport more Suc than expected if they only rely on polymer trapping (Mitchell et al. 1992; Turgeon 1996). Second, certain species have modified intermediary cells, with fewer plasmodesmata and they also have transfer cells in the minor veins (van Bel et al. 1992; Turgeon et al. 1993; Voitsekhovskaja et al. 2006). These plants transport mainly Suc with smaller amounts of RFOs. Third, when the RFO pathway is genetically downregulated (McCaskill and Turgeon 2007), plants grow slowly, but they still transport Suc. Fourth, infection of melon (*Cucumis melo*) plants with cucumber mosaic virus results in upregulation of Suc transporter (*SmSUT1*) gene expression and elevated Suc content in the phloem (Gil et al. 2011). It is also intriguing that presumed apoplastic loaders such as ara-

bidopsis are also able to grow and in some cases reproduce when the Suc transporter has been downregulated. Arabidopsis plants homozygous for the *AtSUC2–4* knockout allele are extremely stunted but still produce a small number of seeds, indicating that long-distance transport is occurring, albeit poorly (Srivastava et al. 2009a). It was suggested that other solutes such as K^+, amino acids, low RFO levels, and/or hexose may contribute sufficiently to an osmotic gradient to drive phloem long-distance transport. These plants also accumulate approximately 18-fold higher carbohydrate levels (soluble sugars and starch) in mature leaves and it is possible that these plants have a passive symplastic loading component, even though they are not anatomically optimized for this export strategy.

In some species, the loading mechanism is not clearly defined because of contradictory or ambiguous results, or because multiple mechanisms may be at work. Rice is an example. Rice was generally assumed to load from the apoplast because of early characterization of the *OsSUT* gene family (Aoki et al. 2003) and because other well studied monocots use apoplastic loading (Braun and Slewinski 2009). However, rice plants with silenced *OsSUT1* appeared normal in growth and vigor, suggesting that rice may use passive symplastic loading (Eom et al. 2012). This in turn led to a detailed review of the evidence for and against symplastic loading with the weight of evidence on the side of apoplastic loading (Braun et al. 2014). The detection of Suc synthase and proton pumping pyrophosphatases by immunohistochemistry in CC/SE complexes was interpreted as support for apoplastic loading (Regmi et al. 2016), but further research may be required to resolve this issue.

Additional evidence for heterogeneous phloem loading can be gleaned from Gamalei's (1989, 1991) data. Although almost all experiments on phloem loading have been conducted on model species that fall into the more extreme of his groups, as defined by plasmodesmatal numbers, many species have intermediate numbers of plasmodesmata between mesophyll cells and the minor vein phloem (type 1-2a), i.e., fewer than the cucurbits or poplar but more than known apoplastic loaders such as arabidopsis and tobacco. Many type 1-2a plants are woody. Perhaps these intermediate frequencies are simply averages that mask heterogeneity of cell types in a single vein, some CC loading through the apoplast, others through the symplast. Another possibility is that the plants load via the apoplast but possess a relatively high density of tightly regulated plasmodesmata for unknown functions. It is also possible that these species load by an undiscovered mechanism, or some marriage of known mechanisms in individual cells. Further analysis of phloem loading in these plants is warranted.

VIII. Control Mechanisms for Loading and Transport

Suc transport is a dynamic process that must be controlled relative to the environmental and physiological needs of the plant. Despite well-supported mechanistic descriptions of phloem loading and long-distance transport, and characterization of the genes involved, there is a void in our understanding of how these features contribute to the needs of the whole plant in natural environments. In the following sections, we describe what is known about regulation of phloem loading from a molecular perspective and then transition toward a more holistic understanding of regulation on the whole-plant scale. Our goal is to discuss the regulation of phloem loading, and we thus exclude a large body of work on the regulation of various sugar transporters unless a direct role in photoassimilate export from the leaf is shown or implied.

Loading and export initiate during the leaf sink-to-source transition and the maturation of the minor veins. This is seen as changes in

vein ultrastructure (Turgeon and Webb 1976) and in expression of the genes catalyzing the phloem loading process. SUT genes with a clear role in phloem loading are induced in the minor veins in a pattern consistent with the sink-to-source transition: expression initiates at the distal tip and progresses toward the base as the leaf matures (Riesmeier et al. 1993; Truernit and Sauer 1995; Aoki et al. 1999). Activation of *AtSWEET11* and *12* are not reported directly, but expression is lower in "young rosettes" than "developed rosettes" and, based on microarray data, show high co-expression with *AtSUC2* (Chen et al. 2012). Considering the paired function of SWEETS and SUTs, co-activation is reasonably expected. In plants that load by the polymer trap mechanism, *GALACTINOL SYNTHASE* similarly shows expression correlating with the sink-to-source transition in *Cucumis melo* and *CmGAS1* promoter activity follows the same pattern in transgenic arabidopsis (Haritatos et al. 2000b; Volk et al. 2003). Changes in gene expression among plants employing "passive loading" have not been addressed.

In addition to the leaf reaching a certain age to undergo the sink-to-source transition, the *AtSUC2* promoter requires light stimulation for this induction in minor veins, as determined in transgenic tobacco plants (Wright et al. 2003). It is reasonable to speculate that the endogenous *NtSUT1* does also. In addition, *ZmSUT1*, *StSUT1*, and all three SUTs from tomato (*SlSUT1*, *2*, and *4*) are diurnally regulated (Kühn et al. 1997; Aoki et al. 1999; Chincinska et al. 2008). Meta-analysis of microarray experiments similarly shows modest (~2 fold) circadian oscillations for *AtSUC2* (Haydon et al. 2011). Moderate diurnal and/or circadian oscillations are not surprising. Photosynthesis occurs during the day and Suc needs to be exported, but transport is also needed during the dark period when Suc is derived from starch. Thus, on the face of it, expression of the major *SUTs* involved in phloem loading track Suc levels during the diurnal cycle: arabidopsis leaves maintain consistent levels of Suc in leaves throughout a 24-hr period, with at most a two-fold difference between day and night. It is not known if similar diurnal or sugar-tracking regulation occurs in species loading by polymer trapping. In passive loading species, sugar tracking is expected since the gradients between mesophyll cells and the sink tissues directly drive transport, but even here, regulation of plasmodesmata gating cannot be excluded.

Post-transcriptional regulation of SUT activity has also been described. SUT proteins undergo rapid turnover, with half-life estimates of less than two hours (Kühn et al. 1997; Vaughn et al. 2002). This, combined with the fact that the *AtSUC2* promoter is among the strongest companion-cell specific promoters (Srivastava et al. 2008, 2009b), implies that protein levels can be quickly altered in response to stimuli. There is also evidence for SUT1 protein cycling between plasma membrane and internal vesicles to influence activity. Liesche et al. (2010) propose that SUT1 localization to sterol-enriched membrane rafts promotes actin-dependent endocytosis, after which SUT-containing vesicles may be targeted to the lytic vesicle for degradation or recycled to the plasma membrane.

SUT protein activity may be modulated through interactions with other cellular components (Krugel and Kühn 2013). SUT proteins show a potential to form homo- and heterodimers in yeast (Reinders et al. 2002; Schulze et al. 2003) and StSUT1 homodimers *in planta* were confirmed through biochemical and imaging techniques (Krügel et al. 2008, 2009). In both plants and yeast, an oxidative environment increases the proportion of dimer relative to monomer (Krügel et al. 2008, 2009). However, the significance of SUT oligomerization is yet to be fully appreciated.

Examination of SUT regulation for clues into the regulation of export from leaves is intuitive but there is also evidence that SUT activity and transport regulation are not

tightly coupled. *Atsuc2* knockout mutations have a clear transport-deficient phenotype of very stunted growth and extreme carbohydrate accumulation (Gottwald et al. 2000; Srivastava et al. 2009a). Knockout plants can be rescued by *AtSUC2* cDNA fused to several different CC-specific promoters including 2 kb of the *AtSUC2* promoter, and promoters derived from Commelina Yellow Mottle Virus and the *RolC* gene of *Agrobacterium tumefactions* (Srivastava et al. 2008, 2009b). Similarly, some, but not all, cDNAs encoding SUTs from different plant lineages can rescue the phenotype (Sivitz et al. 2005; Wippel and Sauer 2012; Dasgupta et al. 2014). Although CC specific, the promoters respond to different stimuli and it is reasonable to assume that the SUT proteins do as well, indicating that fine-tuning *SUT* expression and activity is not essential for transport (Dasgupta et al. 2014). Most significantly, overexpression of *AtSUC2* cDNA from the *AtSUC2* promoter in wild type plants (i.e., two copies of endogenous *AtSUC2* and two copies of *AtSUC2* cDNA) led to more phloem transport of Suc, but also led to a distinct stunting phenotype that was attributed to a disruption in carbon/phosphate homeostasis. From the standpoint of regulating phloem transport, this indicates that gene copy number overcomes any coordinated regulation of transcription or protein activity (Dasgupta et al. 2014; Yadav et al. 2015).

Researchers have also over-expressed genes involved in the polymer trap mechanism in CC to study symplastic loading. This was done in the CC of arabidopsis and potato, where Suc should have been prevalent, but levels of raffinose and stachyose transported through the phloem were minimal (Hannah et al. 2006; Cao et al. 2013). Failure to recapitulate synthesis and transport of raffinose and stachyose in the phloem of apoplastic loaders can be viewed as evidence that many basic aspects of the regulation and biochemistry of symplastic loading have yet to be understood (see Yadav et al. 2015 for further discussion).

If increased accumulation of solute is a mechanism to regulate export from the leaf, it can be achieved by several methods other than direct regulation of the genes or proteins involved in the loading mechanism. For example, it was recently demonstrated that CC-specific over-expression of a proton-pumping pyrophosphatase enhanced phloem transport of Suc, but did not result in the stunted phenotype observed from *SUT* over-expression (Pizzio et al. 2015; Khadilkar et al. 2016). In the proposed mechanism, the proton-pumping pyrophosphatase localizes to the CC plasma membrane and synthesizes pyrophosphate rather than hydrolyzing it. More pyrophosphate stimulates Suc oxidation, leading to more ATP and more proton motive force via the action of the P-type ATPases (Gaxiola et al. 2012). Although there was increased Suc transport, *SUT* and *SWEET* expression was not altered, suggesting that a more energized phloem can enhance flux through existing carriers. Furthermore, it was suggested that stunting was not observed because, unlike the situation with *SUT* over-expression, in which only Suc transport is enhanced, more proton motive force would have energized all the transporters and led to a richer and more balanced transport stream (Khadilkar et al. 2016).

In numerous species, transfer cell morphology will accentuate in phloem parenchyma cells, CCs, or both cell types under high light conditions compared to low light conditions (Wimmers and Turgeon 1991; Amiard et al. 2005, 2007; Adams et al. 2014). These transfer cell ingrowths provide more plasma membrane surface area to, presumably, accommodate more transport proteins, and their formation may impart some level of control over maximal export rates. However, it is not clear how widespread this response is: spinach, for example, does not alter transfer cell invaginations with increased photosynthetic photon flux density, nor does it

increase vein density (Amiard et al. 2005). In addition, increases in the number and size of phloem cells correlates strongly with higher light conditions and increases in layers of palisade cells, particularly in winter annuals, presumably to accommodate the greater quantities of photoassimilate to be transported (Cohu et al. 2014).

To this point, we have focused on potential regulation at the sites of solute accumulation, but regulation could also occur in the symplastic connections in the pre-phloem pathway, between the CC and SE, or along the sieve tubes. Several mutations in maize have characteristics of blocked transport, including reduced stature, carbohydrate hyper-accumulation in leaves, and anthocyanin increases correlating with elevated carbohydrates. These are *Tie-dyed1*, *Tie-dyed2*, and *Psychedelic* (Braun et al. 2006; Baker and Braun 2008; Slewinski and Braun 2010). *Tie-dyed2* encodes a callose synthase. The mutant showed incomplete vascular differentiation and implied a defect in symplastic solute movement between CC and SE (Slewinski et al. 2012). Callose deposition at sieve plates has long been known to block phloem transport in wounded sieve tubes, and callose homeostasis is also now well recognized to modulate trafficking through plasmodesmata (De Storme and Geelen 2014). Another maize mutation, *sucrose export defective1*, showed accumulation of starch and anthocyanin in the tips of mature leaves that resulted from a block to photoassimilate export. This mutation caused aberrant plasmodesmatal structure at the critical interface between bundle sheath and vascular parenchyma, indicating a block in the symplastic flow of Suc and other nutrients in the pre-phloem pathway upstream of release to the apoplast (Russin et al. 1996). Both of these mutants are significant as they demonstrate that export from the leaf can be disrupted, and thus possibly regulated, at any step along the path: before, during, or after the phloem loading step. Although best characterized in model systems for loading from the apoplast, this same principle applies to all loading mechanisms.

IX. Integration of Whole-Plant Carbon Partitioning

Ultimately, photoassimilate loading and export from the leaf must be regulated relative to the needs of the entire plant. A physiological trigger regulating Suc loading appears to be hydrostatic pressure. For example, incubating a test system in hypertonic solutions of sorbitol to draw out water and reduce internal pressure enhances Suc uptake and acidification of the bathing solution, suggesting that both SUTs and ATPases are stimulated (Smith and Milburn 1980; Aloni et al. 1986; Daie 1996). Turgor-regulated SUT activity is also consistent with findings that drought stress sufficient to impact photosynthesis has relatively little effect on translocation since osmotic adjustment maintains pressure and transport (Sung and Krieg 1979). Microarray experiments show modest increases (~2-fold) in *AtSUC2* expression in response to drought, abscisic acid (a drought-induced hormone), or turgor stimulation (Grennan 2006). Also, more effective transport during drought is suggested as an effective drought tolerance mechanism in drought resistant bean cultivars (Cuellar-Ortiz et al. 2008).

Sugar signaling in combination with changes in hydrostatic pressure may provide a whole-plant read-out of carbon status. Exogenous Suc represses *AtSUC2* and *BvSUT1* in the leaves of arabidopsis and sugar beet (*Beta vulgaris*) leaves, respectively (Chiou and Bush 1998; Vaughn et al. 2002; Dasgupta et al. 2014) and *PsSUT1* in pea cotyledons (Zhang et al. 2007). *AtSUC2* is also repressed by external glucose (Wingenter et al. 2010). These are specific responses to indicators of carbon status since *AtSUC2* is not repressed by external solute

such as sorbitol or NaCl (Dasgupta et al. 2014).

Bush and colleagues proposed a model in which Suc levels and *SUT* expression in phloem integrates the needs of sink tissues with output capacity of source leaves (Chiou and Bush 1998; Vaughn et al. 2002). Specifically, changes in sink strength alter the rate of Suc unloading and impact phloem Suc content, which in turn modulates *AtSUC2* expression. If sink strength is low, Suc levels increase in the phloem and represses *SUT* expression in CC. Repressed *SUT* expression in CC then leads to Suc accumulation in the leaf apoplast and carbohydrate in mesophyll cells, and ultimately to the well-documented feedback inhibition on photosynthesis (Stitt et al. 2010). High sink strength and reduced Suc in the phloem would have the inverse effect.

Patrick and colleagues proposed that Suc levels and associated changes in turgor regulate nutrient release and uptake in developing pea seeds to function as the link between the input needs of the embryo and the output capacity of the maternal seed coat (Zhang et al. 2007; Zhou et al. 2009). Suc uptake into embryo cotyledons of pea correlates with *PsSUT1* expression, implying that it is the SUT primarily involved in uptake. *PsSUT1* expression is inhibited by increasing internal Suc levels suggesting that intracellular Suc is the signal for metabolic demand and controls *PsSUT1* transcription (Zhou et al. 2009). The resulting Suc uptake by the cotyledons in turn controls efflux from the seed coat by a turgor-gated mechanism (Zhang et al. 2007). In this component of the model, lower Suc in the apoplast increases turgor pressure in the cells of the seed coat (*i.e.,* the solute potential in the apoplast increases, making more water available for uptake into cells) and activates carriers involved in nutrient release. As nutrients are released, the apoplastic solute potential decreases causing turgor pressure in the seed coat to be relieved, and the carriers to be deactivated. Pressure in the seed coat thus acts as a feedback-regulated homeostat to control nutrient release to embryos (Zhou et al. 2009). SWEET proteins for Suc are shown to be important in the seed coat and endosperm of arabidopsis to feed the embryo (Chen et al. 2015), and a SWEET for hexose is important for seed filling in maize and rice (Sosso et al. 2015). It will be most interesting to explore the regulation of these genes in relation to sink / source dynamics.

In the models of Bush (Chiou and Bush 1998; Vaughn et al. 2002) and Patrick (Zhang et al. 2007; Zhou et al. 2009), the basic mechanisms for Suc-mediated control of influx and efflux in developing seeds and control of phloem loading are analogous. Since turgor is proposed to control efflux from the seed-coat symplast, it is reasonable to speculate that turgor may similarly influence Suc release in source leaves via the SWEET carriers. Combining turgor-gated control and Suc-mediated signaling provides a model for whole-plant carbon partitioning, in which the Suc needs of the filial symplast is transmitted through the phloem to the source leaf mesophyll symplast via the activation or repression of Suc transporters and facilitators (Fig. 3.5).

X. Conclusions

Photoassimilate export in the phloem is one link in an integrated demand/supply system, the function of which is to convert raw materials (C and other elements) into finished products in leaves and transport them to regions of need for storage and growth. In the past, it has been commonplace to regard agricultural yield as sink dominated, i.e., to be driven exclusively by the unloading and metabolism of photoassimilate in growth and storage tissues rather than by source (photosynthetic) activity. This emphasis on sink regulation may have stifled investigation into signaling systems that coordinate

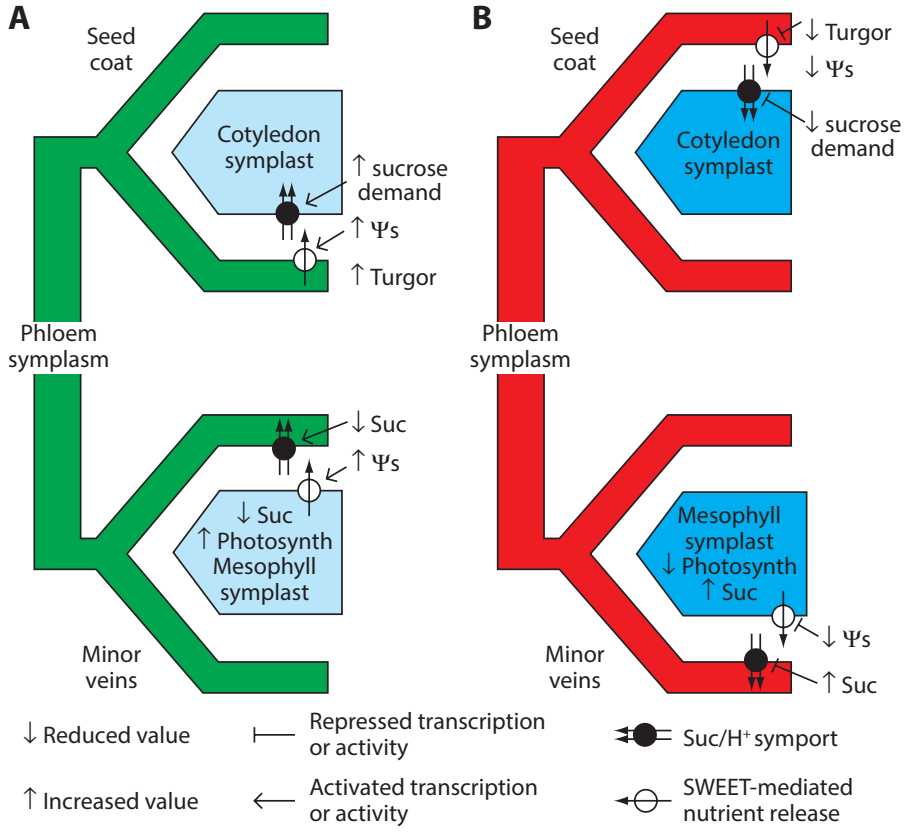

Fig. 3.5. **Potential control of phloem transport by integrating sink demand with photosynthesis in source leaves.** (**a**) Strong sink demand in the cotyledons of developing seeds stimulates Suc transporter activity at the cotyledon surface (top of diagram; blue color density represents relative levels of Suc and other nutrients). Suc uptake increases the apoplast solute potential (Ψs), allowing water to be pulled away to the seed-coat symplast. Increased hydrostatic turgor pressure in the seed coat activates nutrient release. Lower levels of Suc or reduced hydrostatic pressure in the phloem (green) activates Suc transporters in the transport and loading phloem of source leaves. Suc removal from the leaf apoplast by phloem loading increases apoplast solute potential, and the increased pressure differential between phloem parenchyma and the apoplast, or Suc levels directly, may activate nutrient release from the leaf symplast analogous to that described for the seed coat. Efficient phloem loading and export from the leaf stimulates photosynthesis. (**b**) Low sink demand in the seed cotyledons permits Suc to accumulate which represses the Suc transporters at the cotyledon surface. Suc in the apoplast is not loaded into the cotyledons and consequently the apoplast solute potential is low. Water is removed from the seed coat symplast, reducing the hydrostatic turgor pressure and reducing nutrient release. Along the transport and loading phloem of source leaves (red), increased Suc levels or hydrostatic pressure reduces Suc transporter activity and phloem loading. Suc in the apoplast may reduce nutrient release from leaf symplast, and accumulation of Suc in mesophyll cells represses photosynthesis

photosynthesis and demand. There is now clear evidence that developing, and even mature, leaves respond to changes in sink activity in a number of species-specific ways, both anatomical and physiological, to increase C fixation and carbohydrate delivery (Adams et al. 2018). In the future, it may be possible to manipulate this supply chain selectively and productively but to do so we need a more detailed understanding of phloem export mechanisms and how they are regulated.

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Chapter 4

Leaf Water Transport: A Core System in the Evolution and Physiology of Photosynthesis

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Summary

In most terrestrial ecosystems, water availability is the principal governor of primary productivity. Vascular plants can only sustain high rates of photosynthetic activity by transporting enormous quantities of water from reserves in the soil to the sites of gas exchange in leaves to prevent desiccation of photosynthetic tissues. This demand for water requires plants to invest in a vascular system that begins as a simple pipe system in roots and branches and terminates in a sophisticated network of veins in the leaf. This chapter will examine the tight linkage between photosynthesis and the efficiency of water transport in leaves, explaining how plants use a non-living network of xylem to deliver water under high tension to evapo-

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rating cells. We explore how plants achieve high efficiency in water delivery by developing an intricately branched system of leaf veins as a means of piping water close to the stomatal layer, and how evolution has shaped the venation of higher plant species as densely reticulated networks.

I. Transporting Water for Carbon – Principles of Cohesion-Tension Theory and the Link Between Water Transport and Photosynthetic Capacity in Leaves

The oxygenic process of photosynthesis that characterizes terrestrial plants evolved in aquatic bacteria 2–3 billion years ago. Subsequent evolution has carried the sites of photosynthesis far away from the aquatic realm, but a singular dependence on water remains. Water is the only source of electrons used in the process of CO_2 assimilation by plants, while at the same time it provides the liquid medium that bathes the membranous photosynthetic machinery. The leaves of modern plants represent a solution to bringing the aquatic process of photosynthesis onto dry land. They house photosynthetic cells in a transparent compartment, enveloped by a cuticular film that allows light to enter but prevents rapid desiccation. The evolutionary drive to become more prolific than competitors places considerable selective pressure on the ability to aggressively grow vegetative and reproductive tissue. However, plant growth is limited by the rate at which CO_2 can be fixed and turned into new cells. For this reason, the photosynthetic surfaces of plants must be highly porous to the atmospheric CO_2 that acts as the source of carbon skeletons used in plant growth. Ideally, plants require a cuticle that allows CO_2 to enter, while preventing water from escaping the leaf. Sadly, biological evolution has never yielded such a material; instead the cuticle of leaves is punctured by millions of stomatal pores that open when conditions are suitable for photosynthesis to allow CO_2 to enter the leaf while water diffuses rapidly out of the leaf. Hence plants are forced to pay a very hefty price for CO_2, in terms of water vapor exiting from stomata along a parallel pathway (Fig. 4.1). This water must be replaced or leaves rapidly desiccate (Raven 1977).

Water is sometimes relatively abundant in soils, but the earliest land plants lacked a system for transporting water to the sites of photosynthesis and were likely limited to photosynthesis during periods of very high humidity, after which leaves dried out until rainfall and humidity allowed cells to hydrate and photosynthesis to recommence (Ligrone et al. 2012). The predominance of desiccation-tolerance in the relatives of early land plants and the very early evolution of desiccation tolerance genes (Komatsu et al. 2013) bears testimony to the opportunistic nature of early terrestrial photosynthesis. Sometime in the Lower Devonian period, after the emergence of plants onto land, specialized tubular xylem cells with reinforced walls evolved (Ligrone et al. 2002; Boyce et al. 2003; Edwards 2003; Carafa et al. 2005) allowing plants to transport water from the soil to replace water transpired during photosynthesis. This saw the beginning of a trajectory towards internal water transport and away from opportunistic desiccation tolerance. The transport of water in these vascular systems is a passive process of flow between the water potential present in humid soil, to the lower water potential present in partially desiccated leaves. This effortless means of lifting water from the soil was explained more than 120 years ago (Dixon and Joly 1895) and remains strongly supported by modern tools of plant physiology (Angeles et al. 2004). Water is effectively pulled from the soil by

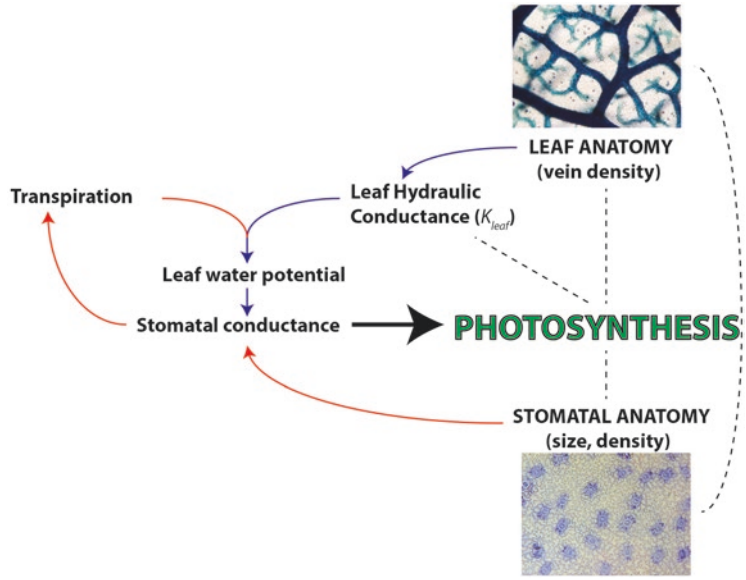

Fig. 4.1. Mechanistic and correlational interactions between leaf hydraulic and physiological parameters in well-watered plants. Direct interactions between the supply of water (blue arrows) and demand for water (red arrows) are shown by unbroken arrows, while indirect interactions are shown by dashed arrows

evaporation, enabled by the cohesive properties of water that prevent the water column in the plant from "breaking" (unless certain thresholds are exceeded – see Section V).

The evolution of vascular plants saw improvements in the capacity of the vascular system to supply water to leaves, but a dependence on water cohesion and tension in the xylem remains as the sole driver of bulk water transport during evapotranspiration. One of the key innovations in vascular plants that enabled sustained photosynthesis was a spatial and functional association between stomatal valves and photosynthetic tissue. Unlike the first stomata that evolved in bryophyte ancestors and appear to be associated with reproductive tissue (Boyce 2008; Pressel et al. 2014), the stomata in early vascular plants were found on leaves, and evidence from the living relatives of these early plants indicates that the action of these stomata clearly regulates the supply of CO_2 for photosynthesis and the loss of water in tandem (McAdam and Brodribb 2012).

The connection between stomata and photosynthesis was a major turning point in plant evolution because it led to a dramatic improvement in the efficiency of water use during photosynthesis, but also a degree of convergence among C^{13} plants in the ratio of water exchanged per unit CO_2 fixed. This is evidenced by the relatively conservative range of ^{13}C isotope discrimination found in terrestrial plants (Körner et al. 1988; Diefendorf et al. 2010). The ability of plants to produce high densities of stomata on leaves allows potential maximum rates of CO_2 uptake for photosynthesis to approach that of free diffusion (Lehmann and Or 2015). However, the fact that rates of water vapor efflux from leaves are typically 1000 times higher than inward CO_2 fluxes raises the possibility that maximum stomatal conductances to CO_2 might ultimately be limited by the ability of plants to transport the large volumes of water lost during maximum assimilation from the soil to the photosynthetic tissue (Brodribb and Feild 2000; Hubbard et al. 2001). The existence of a "hydraulic limitation" on maximum photosynthesis is most clearly evidenced by the strong stomatal sensitivity exhibited by most

plant species to changes in the evaporative gradient between the leaf and the atmosphere. Stomata typically close when the evaporative demand increases (Darwin 1898) allowing plants to maintain an optimal ratio of water loss to carbon gain (Cowan and Farquhar 1977), but the trigger for this closure is likely to be transient leaf desiccation caused by insufficient hydraulic supply (Raschke and Resemann 1986; Saliendra et al. 1995). Analyses of the xylem sap flow of many species demonstrate a general pattern of stomatal closure during high midday evaporative demand due to limitations in the supply of liquid water from the xylem (Oren et al. 1999). Leaf desiccation is translated into stomatal closure either hydraulically (Brodribb and McAdam 2011) or via the synthesis of abscisic acid (McAdam and Brodribb 2015), thus preventing further decline in leaf water content.

A. Linking Hydraulics and Photosynthesis

A theoretical link between the photosynthetic output of leaves and the hydraulic capacity of the xylem to deliver water arises due to the action of the stomata (Fig. 4.1). Beyond providing a rapid pathway for CO_2 diffusion into leaves, the clearest adaptive benefit conferred by stomata is that they can close to conserve water during times of limited water supply, thereby reducing the risk of damage to xylem or photosynthetic tissues (Jones and Sutherland 1991; Cochard et al. 2002; Brodribb and Holbrook 2003). However, as the common entry and exit point for CO_2 and water, the stomata co-regulate both photosynthesis and transpiration. This means that reductions in the rate of CO_2 uptake under dry atmospheric or soil conditions are a collateral cost associated with stomatal closure in response to falling leaf water content. Whenever stomatal closure occurs under conditions of hydrated soil but high evaporative demand (high leaf temperature and/or low air humidity), it can be considered that the assimilation rate is limited by the capacity of the hydraulic system to supply sufficient water to keep stomata open.

According to the cohesion-tension theory, the flux rate of water (F) from the roots to the evaporating tissue in the leaf is determined by the product of the hydraulic efficiency of water transport system (defined as a hydraulic conductance K) and the water potential gradient across the plant ($\Delta\psi$). Equation 4.1:

$$F = K \cdot \Delta\psi \tag{4.1}$$

This latter term is constrained by the function of stomata that prevents leaf water potential in actively transpiring leaves from falling much below −2 MPa before stomata close (as evidenced by the fact that leaves typically lose turgor in the range − 1.5 to −2.5 MPa; (Bartlett et al. 2012). The result is that most plants are limited to a rather conservative range of $\Delta\psi$ to drive water transport (a maximum of less than 2 MPa). This scenario means that improvements in the supply of water for photosynthesis are unlikely to be achieved by modifying $\Delta\psi$, but rather by changing xylem anatomy or plant allometry to increase K (Fig. 4.1). Thus, it might be expected that maximum stomatal conductance and consequently assimilation (Wong et al. 1979) must be limited by whole plant hydraulic conductance. Advantages in producing highly conductive roots, stems and leaves is thus apparent for supplying high rates of productivity, but what about at the low end of the productivity spectrum where photosynthesis is limited by something other than water availability? In this case, the substantial cost of producing the xylem (Murphy et al. 2016) would mean that a plant adapted to very low evaporative demand or low photosynthetic rates could be expected to have low K to maintain an optimal allocation of resources (Givnish 1986). It thus follows that the maximum stomatal conductance of a leaf or species should be proportional to the hydraulic conductance of the xylem supply system. This hypothesized linkage between the efficiency of the xylem water transport

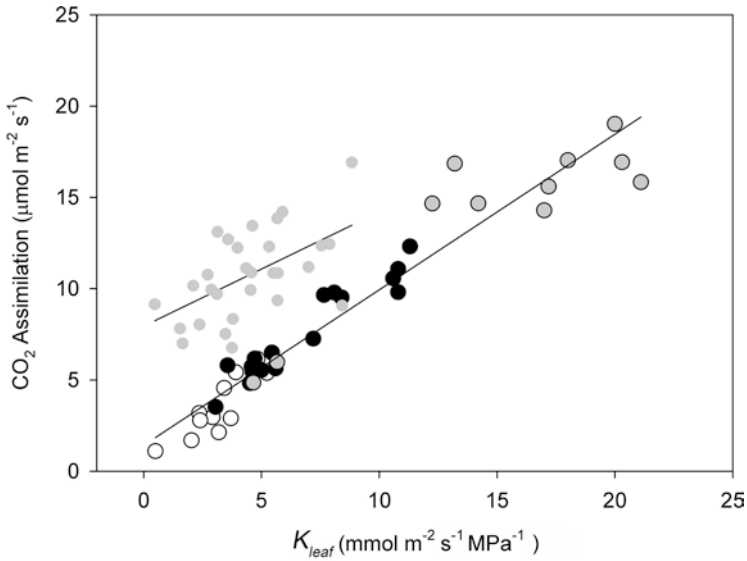

Fig. 4.2. A strong correlation between CO_2 uptake and leaf hydraulic conductance of ferns (open circles), conifers (black circles), and angiosperms (grey, black border) sampled in the field (data from Brodribb et al. 2007). Data from *Vibernum* species (grey circles) grown in a common garden (data from Scoffoni et al. 2016) showed a similar slope in the rate of CO_2 uptake vs K_{leaf} regression, but much higher rates of CO_2 uptake. This may be accounted for by non-standard conditions of humidity and temperature in the different studies, and fact that the *Vibernum* plants were fertilized while the other sample was made on trees growing under lower nutrient conditions in natural forest

system and the rate of photosynthesis (Jones and Sutherland 1991; Sperry et al. 1998) was first observed in sugar cane (Meinzer and Grantz 1990) and later found to be a general pattern in the stems of diverse rainforest species (Andrade et al. 1998; Brodribb and Feild 2000). More recently this connection was observed in leaves, where strong correlations between leaf hydraulic conductance and maximum photosynthetic capacity were documented (Brodribb et al. 2005; Franks 2006). Very strong links between leaf hydraulic conductance (K_{leaf}), stomatal conductance and photosynthesis (Fig. 4.2) have been observed both across the diversity of vascular plants (Brodribb et al. 2007) as well as within angiosperm clades (Scoffoni et al. 2016). These strong correlations arise due to the fact that leaves represent disproportionately large resistors in the hydraulic system (Sack and Holbrook 2006), and thus exert a large influence on the hydraulic character of the whole plant.

A significant body of research now identifies the coordination of xylem, stomata and photosynthesis as a fundamental aspect of the evolution of vascular land plants (see reviews (Sack and Holbrook 2006; Brodribb 2009; Sack and Scoffoni 2013). The origins, anatomy, function and ecology of this interesting co-evolution of tissues is discussed in the following sections.

II. Measuring and Modeling K_{leaf}

Water transport through most of the plant body occurs via the non-living xylem tubing, and is thus relatively easy to understand and measure based either on Poiseuille's Law for one-dimensional laminar flow, or Darcy's Law for pressure-driven flow through a porous medium. Leaves and roots pose additional complications for water transport because they are three dimensional, and water must flow from the non-living xylem

tissue into living tissue with various compartments having different hydration status and water transport properties (Tyree et al. 1981; Zwieniecki et al. 2007). This transfer may incur very large hydraulic resistances because when water leaves the relatively large, open pipes of the xylem to reach its destination in the mesophyll tissue, it must flow either through apoplastic pathways in the walls of living cells, across cellular membranes with limited hydraulic conductivity or in the vapour phase through intercellular airspaces. For this reason, the hydraulic properties of the leaf have a disproportionate influence on whole-plant K, constituting >30% of the hydraulic resistance to water flow in the entire plant (Sack and Holbrook 2006). The relative contributions of the xylem and outside-xylem pathways in leaves vary greatly across species, but the outside-xylem pathways contribute about 50% of leaf resistance on average (Sack et al. 2005). Quantifying these resistances and their variation across species and environmental conditions is challenging and there are multiple methods for measuring the components of K_{leaf}. These include pushing or sucking water into leaves by exogenous pressures (Tyree et al. 1993; Tyree et al. 1999) or measuring vapour (Nardini et al. 2001) or liquid fluxes produced by natural water potential gradients (Blackman and Brodribb 2011) or water potential dynamics in the leaf (Brodribb and Holbrook 2003). Different methods can yield different results (Brodribb and Holbrook 2006b) because there is no straightforward experimental method to determine the water potential at the end of the water transport pathways in leaves (i.e., the stomatal pores), so that all studies of K_{leaf} are defined operationally in terms of equilibrated bulk leaf water potential. Thus, reported values of outside-xylem hydraulic conductance represent flow restrictions from the xylem to some point, probably in the mesophyll, where the water potential during active transpiration is similar to that of an excised and equilibrated leaf. If it were possible to include more distal pathways in these measurements, the outside-xylem portion of K_{leaf} would appear to contribute an even larger proportion of leaf resistance.

Studies of different leaf processes require understanding of different pathways – for example, to understand effects of dehydration on xylem, photosynthesis or stomatal function requires estimates of xylem, mesophyll or epidermal water potential, respectively. Deeper analysis of such effects therefore requires some way to probe the functioning of leaf water transport in greater detail than possible with current experimental technology. Modeling can help in this regard by using biophysical constraints to limit the range of possible water potential distributions in relation to measurable features of anatomy and environmental conditions. For example, (Sack et al. 2004) used an electrical analog model to quantify the contributions of different vein orders to the total resistance through the leaf xylem network, and (Cochard et al. 2004b) combined a model of the leaf xylem network based on Poiseuille's Law with pressure probe measurements to show most xylem resistance arose not from simple longitudinal friction but rather from movement between conduits. The latter model was later used to explore effects of venation architecture on hydraulic vulnerability (Scoffoni et al. 2011). More recent work has highlighted another powerful outcome of formal modeling, revealing non-intuitive or even counter-intuitive implications of hypotheses. For example, (Peak and Mott 2011) used a novel model that incorporated water vapor transport between the mesophyll and epidermis to show that some unexplained features of stomatal function could be explained by the hypothesis that guard cells are tightly coupled to air in the stomatal pore by water vapor transport. Similarly, (Rockwell et al. 2014) and (Buckley 2015) explored coupled liquid and vapor phase transport in a spatially explicit model of the outside-xylem compartment and concluded that vertical vapor transport

from warm layers of illuminated mesophyll can contribute greatly to water activity and thus water potential in tissues closer to the stomata. This means that measurements of K_{leaf} based on water potentials of leaves excised during illuminated transpiration actually include a vapor phase component.

Modeling is no silver bullet for leaf hydraulics research, however, and several critical questions limit the confidence and accuracy of current models. Notably, the physics of water flow around cells through the apoplast remains unclear because apoplastic nanopores are within 1–2 orders of magnitude of the effective size of water molecules, which means continuum-based modeling (e.g., using Poiseuille's Law or Darcy's Law) may be unsuitable. The distribution of membrane water permeabilities also remains very poorly known for leaf tissue, and particularly for mesophyll cells. Nevertheless, models can help to constrain the range of possibilities and thereby direct experimental research by focusing questions.

III. Adaptation and Regulation of K_{leaf}

A. *Vein Density*

As the transpiration stream flows from xylem tissue to the sites of evaporation it encounters a major hydraulic resistance (the outside-xylem compartment). The resistance of this pathway is proportional to the length of the flow path between the vein endings and the stomata (Brodribb et al. 2007). For this reason, the branching density of the water-transporting leaf venation is the primary anatomical determinant of the hydraulic conductance of any leaf. Increased vein branching reduces the length of this final pathway that water must flow outside xylem to evaporative sites (see below), and has been shown to be strongly correlated with K_{leaf} and assimilation rates across a diversity of plant clades (Brodribb et al. 2007). Leaf vein density is a highly plastic trait within species (see below), and shows important evolutionary trends (see below) making it one of the most useful and informative leaf anatomical traits (Fig. 4.1). Recent authors have suggested that the co-influence of leaf vein density on both leaf morphology and gas exchange gives this key trait a central role in the overall leaf economics spectrum (Reich 2014), even placing vein density at the center of the leaf economic trade-off (Blonder et al. 2011; Blonder et al. 2013). This final suggestion has been the subject of debate, with some support from studies within plant families (Brodribb et al. 2013), but not from broader phylogenetic sampling (Li et al. 2015).

It should be mentioned, especially in the context of Chapters 2 and 3, that vein density may influence assimilation not only via its effect on water transport, as discussed here, but also by influencing the rate of export of photosynthates from the leaf (Adams et al. 2007). Associations between the abundance of phloem tissue in minor veins and maximum assimilation rates in some herbaceous species (Muller et al. 2014) suggest that under conditions of light and CO_2 saturation, the density of phloem transport tissue in the venation can become limiting on photosynthetic rate. This phloem limitation probably represents a photosynthetic bottleneck only under experimental conditions of CO_2 saturation, while limitation of photosynthesis by water supply may be the more regular scenario under field conditions of CO_2 and humidity.

B. *Vein Xylem and Leaf Anatomy*

Due to the simple structure and flow in stem vasculature, the constraints on hydraulic conductance in stems are largely determined by the size and structure of xylem conduits and the pit membranes that connect them (Tyree and Zimmermann 2002). Compared with stems, leaves present the added complexity of a highly-branched venation system

that is joined to living tissue, making it more difficult to determine the relationship between xylem anatomy and hydraulic conductance. Nevertheless, weak correlations between xylem anatomy and K_{leaf} have been noted including vessel size in major veins (Maherali et al. 2008), petiole size (Sack and Frole 2006), and midrib xylem area (Aasamaa et al. 2005). Conduit sizes in the minor leaf veins do not appear to be correlated with K_{leaf} (Blackman et al. 2010). In minor veins, it has been proposed that the connections between leaf vessels play a more important role in limiting K_{leaf} than vessel size (Feild and Brodribb 2013) see below).

The strong influence of outside-xylem resistances on water transport inside the leaf means that anatomical characteristics influencing water transport in the mesophyll should be important in determining K_{leaf}. Features such as lignified sclereids that make hydraulic "short-cuts" through the mesophyll have been shown to improve K_{leaf} (Brodribb et al. 2007), while bundle sheath extensions that connect veins and the epidermis appear to also improve overall water conduction (Buckley et al. 2011). Mesophyll features that influence the length of the outside-xylem pathway such as mesophyll thickness (Aasamaa et al. 2001) also affect K_{leaf}, but the interaction with leaf thickness is uncertain. Theoretically, it would be expected that leaf thickness and vein density should co-vary (Noblin et al. 2008), a notion supported by a study of 48 species of Proteaceae (Brodribb et al. 2013), but not in other studies (Sack et al. 2013).

C. Xylem-Stomatal Tissue Coordination

The direct connection between delivery of liquid water by leaf veins and the loss of water vapor through stomata in leaves suggests that some degree of coordination between these tissues might be expected. Spacing between stomata and veins appears to be highly conserved within leaves with veins observed to branch and loop in a fashion that preserves a constant mean distance between xylem and stomatal cells (Fiorin et al. 2015). Assuming that significant costs are involved in the production of xylem tissue (Carins-Murphy et al. 2016) and the maintenance of stomata (Franks et al. 2012), an optimal allocation of costs must result from a balance between the density of veins (the primary anatomical determinant of K_{leaf}) and the associated density of stomata (which determines maximum vapor loss from leaves). In support of this concept, strong coordination between vein and stomatal densities has been observed within species (Brodribb and Jordan 2011), among species (Wan et al. 2004; Heinen et al. 2009; Carins-Murphy et al. 2016), and even within leaves (Li et al. 2013), suggesting that this coordination represents a fundamental feature of leaf development. The mechanism behind tissue coordination between veins and stomata in leaves appears to be a common dependence on cell size in the leaf (Carins Murphy et al. 2016). Using epidermal cell size as a reference, it has been shown that the densities of veins and stomata are both similarly "diluted" by the size of epidermal cells. Thus the shade leaves of many species have larger epidermal cells than sun-leaves, resulting in lower densities of both veins and stomata (Heinen et al. 2009). This type of acclimation makes sense in terms of the lower energy available for both photosynthesis and evaporation in the shade, such that fewer stomata are required for gas exchange, and thus fewer veins for water delivery.

D. Regulation of K_{leaf}

Physiological regulation of K_{leaf} has been suggested as an additional means of coordination between the demand for water and the supply of water in leaves. Regulation of tissue conductance by living cells in the leaf transpiration flow path might be expected in a similar way to the aquaporin dependence of root hydraulic conductance (Henzler et al. 1999). Initial reports that K_{leaf} was enhanced

when leaves were moved from darkness to light were thus attributed to aquaporin expression (Cochard et al. 2007). These measurements were conducted with a pressure injection technique used to measure K_{leaf}. This method requires liquid water to flow out of stomatal pores, and it has been argued that the observed changes in K_{leaf} may have been due to stomatal restrictions to liquid water flow upon closure in the dark (Sack et al. 2002). Confirmation of a light effect on K_{leaf} in some species was made by employing multiple methods, one of which was independent of stomatal aperture (Zhu et al. 2005). This study suggested that a light effect was commonly associated with leaves bearing bundle sheath extensions to the epidermis (heterobaric leaves). The action of mercuric blockers of aquaporin activity also seem to support an enhancement of K_{leaf} by these proteins (Nardini et al. 2005), although no significant change in K_{leaf} was observed when *Arabidopsis thaliana* antisense plants for a number of key aquaporin proteins were examined (Parent et al. 2009). The balance of evidence suggests that aquaporins play multiple roles in leaves, one of which is an enhancement of outside-xylem water flow. The importance of this role remains to be fully understood, but it is unlikely that the dynamic influence of aquaporins in leaves is as fundamental as that demonstrated in roots (Heinen et al. 2009).

More recently it has been suggested that the stomatal-closing phytohormone abscisic acid (ABA) plays a role in down-regulating K_{leaf} (Pantin et al. 2013) in *A. thaliana*. Down-regulation of aquaporin expression was again suggested as the cause of decreased K_{leaf} after exposure to ABA, although the opposite has been observed in roots, where ABA increases the expression of aquaporin proteins (Zhu et al. 2005) resulting in increased hydraulic conductance of root cells (Wan et al. 2004) and whole plants (Parent et al. 2009). Down-regulation of K_{leaf} by ABA could have the effect of increasing stomatal sensitivity to declining leaf water potential under water stress, but data from other species do not yet provide support for this as a general mechanism in land plants (McAdam and Brodribb, 2015).

IV. Evolution of Modern Vein Networks

A common feature of angiosperm leaves is a reticulated vein network, typically arranged in a regular branching hierarchy. These loopy hierarchical topologies are a recurrent theme in laminate leaf evolution (Boyce 2005) and represent an efficient means of delivering water in a system prone to damage (Katifori et al. 2010). Although there seems to be nothing special about the topology of angiosperm veins, the branching density of veins found in most clades of angiosperms is unprecedented in evolution. Ferns, gymnosperms and even early-branching angiosperms appear only able to produce leaves with vein densities up to a maximum of approximately 5 mm vein length mm^{-2} leaf area. A major shift during the early evolution of angiosperms saw the sudden appearance of leaves with vein densities in a much higher range, up to maxima around 25 mm mm^{-2} (Boyce et al. 2009). This transformation is evident both in ancestral state reconstructions using living ancestors (Brodribb and Feild 2010) as well as in fossil floras that span the Cretaceous period (Feild et al. 2011), and represents a clear change in the capacity of leaves to transport water and export photosynthate. The innovation of high vein density in angiosperms appears to have been a critical transition for the group, enabling the high rates of photosynthesis and productivity associated with increased water transport efficiency. The subsequent increase in potential productivity afforded by greater vein density is hypothesised to have been critical in allowing angiosperm evolution to progress from its origins in shady cloud forest (Feild et al. 2004) to canopy dominance.

The connection between vein evolution and the ecological rise of the angiosperms required firstly an anatomical change in angiosperms that allowed them to produce high vein density, and secondly a shift in atmospheric CO_2 to provide the suitable selective environment for the evolution of high rates of gas exchange (Boyce and Zwieniecki 2012). Reconstructions of paleo CO_2 concentrations suggest that CO_2 levels were falling at the time of vein density evolution in angiosperms, apparently satisfying this first criterion. In terms of an evolutionary change in vascular anatomy, it has been proposed that the occurrence of primary leaf xylem with highly porous (simple) perforation plates between vessel elements is restricted to derived clades of angiosperms, and that this may have been the trigger for vein density evolution in these clades (Feild and Brodribb 2013). Highly porous xylem is likely to be a prerequisite for high vein density because of the vascular miniaturization required when packing high densities of veins into the leaf lamina. It has been argued that reductions in xylem conductance resulting from reduced lumen size in vein xylem have been partially recovered by the increase in conductance between vessel elements by the almost complete removal of inter-conduit perforation plates (Feild and Brodribb 2013).

V. Stress and Failure in the Leaf Hydraulic System

Leaves are located at the end of the hydraulic continuum in the plant body and are thus more exposed to desiccation than any other plant tissues. While photosynthetic and other metabolic processes in leaves appear quite resilient to desiccation or osmotic stress (e.g., mangroves and desiccation tolerance in many moss and fern species), the leaf vascular system appears much more vulnerable to damage under water stress (Choat et al. 2012).

Stem xylem is known to be vulnerable to cavitation, leading to air blockages (air-embolisms) in xylem cells when water tension exceeds certain limits (Tyree and Sperry 1989). These limits can be determined in stems by using different techniques including bench drying, centrifugation, and air pressurization to simulate water stress and induce embolisms, the effects of which can be quantified in terms of hydraulic conductance. By using these techniques, it has been shown that the pressure or tension required to cavitate 50% of the water transport capacity of the xylem is species specific, and is related to the ability of plants to grow under different soil moisture regimes (Brodribb and Hill 1999).

Given the importance of leaves for photosynthate supply, and the particular exposure of leaves to water stress, there has been a recent focus on the hydraulic vulnerability of leaves to water stress. Traditionally it has been suggested that leaves may function like fuses in the hydraulic circuit, embolizing early to protect more costly upstream tissues such as stems (Zimmermann 1983; Nardini and Salleo 2000). This idea has generally been supported by studies of leaf vulnerability in woody plants (Choat et al. 2005; Hao et al. 2007; Nolf et al. 2015), but not in herbs (Skelton et al. 2017). Questions about the timing of leaf cavitation, and in particular the possibility of recovery or repair of K_{leaf} after water stress, remains hotly debated.

Complexities in the technical and interpretive approaches to measuring K_{leaf} in drying leaves have led to uncertainty as to whether K_{leaf} is (1) highly vulnerable to water stress, failing even before stomata close or (2) stable until thresholds for significant vein embolization are reached, after stomatal closure. The implications for scenario (1) are that veins are diurnally cavitated and repaired, or that resistances outside the xylem are highly dynamic; while scenario (2) suggests K_{leaf} remains static until leaves become exposed to extremes of water stress causing non-repairable cavitation in leaf xylem. New methods have recently clarified the timing of air-embolization using an opti-

cal technique (Fig. 4.3) to show conclusively that the decline of K_{leaf} by cavitation under water stress occurs after stomatal closure (Brodribb et al. 2016; Skelton et al. 2017). These studies clearly support scenario (2) in terms of leaf vein embolism being a permanent injury under severe stress. Repair of embolisms in this case requires rehydration aided by the development of positive root pressure (Knipfer et al. 2015). The possibility of reversible K_{leaf} depression caused by something other than embolism still remains open.

One possible mechanism that could lead to reversible changes in K_{leaf} (without embolism) is through the deformation of tissues under water stress. This has been clearly demonstrated in the conifer needles of several species where collapse of tracheids or other water conducting tissues has been directly observed (Cochard et al. 2004a; Brodribb and Holbrook 2005; Zhang et al. 2014). In angiosperms, it has been suggested that changes in the turgor of bundle sheath or mesophyll tissues may explain correlations between declining K_{leaf} and the turgor loss point of leaves (Brodribb and Holbrook 2006b; Kim and Steudle 2007). Physical shrinkage of the leaf prior to turgor loss point has also been associated with declines in K_{leaf} that would precede embolism formation (Scoffoni et al. 2014) and could potentially be rapidly reversed. There is conflicting evidence about the sensitivity of the outside xylem pathway to water stress. Some authors conclude that strong declines in K_{leaf} can be observed prior to stomatal closure (Scoffoni et al. 2017), suggesting that this action should induce more effective stomatal closure. It is unclear how such a response would improve the rapidity or effectiveness of stomatal closure given that declining K_{leaf} could amplify the rate of decline in leaf water potential during evaporation, potentially

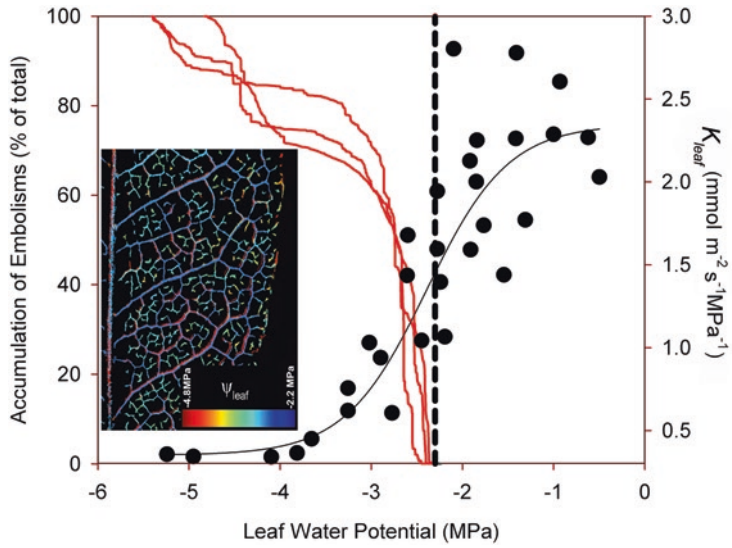

Fig. 4.3. A vulnerability curve of leaf hydraulic conductance in the rainforest tree *Eucryphia moorei* (data from Brodribb et al. 2016). As leaf water potential drops during imposed water stress, losses in K_{leaf} (closed circles) typically follow a sigmoidal decline commencing slightly before or at the turgor loss point (dotted vertical line). The accumulation of embolisms (red lines from three leaves) recorded optically (insert from Brodribb et al., 2016) approximately follows the measured loss of K_{leaf}. Embolism is not generally observed until after stomatal closure (represented here by leaf turgor loss). A map of embolism formation in a single leaf of *E. moorei* shows the timing of embolism formation. A general progression of embolism from large veins to minor veins can be seen from the colour scale (in the case of multiple embolisms in a vein the first embolism is shown on top)

reducing the effectiveness of stomatal control by causing wrong-way stomatal responses (Buckley 2005).

Resolving the sensitivity of the outside xylem hydraulic pathway to water stress will require careful experimentation, preferably with techniques that are independent of transpiration rate, because changes in K_{leaf} that are measured as a reduction in transpiration rate suffer the complication of uncertain changes in the pressure gradients between compartments in the leaf (Zwieniecki et al. 2007). Results from non-transpiring leaves show little evidence of declining K_{leaf} until cavitation is initiated, while calculations from transpiring leaves suggest that K_{leaf} rises to a maximum as water potential approaches zero (Brodribb and Holbrook 2006a).

VI. Conclusions

The leaf vascular system is required to satisfy complex demands of transporting water evenly and efficiently to an evaporating surface while remaining robust to water stress and other damage. Satisfying these demands makes the leaf hydraulic network the most complicated end of the plant water transport system. The goal of understanding this complex system is motivated by the clear and consistent relationship between K_{leaf} and CO_2 assimilation, a relationship that emphasizes the central role played by leaf vascular systems in governing plant production. Patterns of leaf hydraulic evolution in angiosperms provide a striking example of the potential leaf hydraulics hold for improving the performance of crop species. Future developments in methodology will clarify questions about the influence of dynamic agents such as ABA levels and leaf turgor on the efficiency of leaf water transport, and this will be greatly assisted once we can evolve towards using more experimentally friendly model species than *A. thaliana*.

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Chapter 5

Leaf Anatomy and Function

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Summary

Plant leaves provide the following main functions: (1) light interception and utilization of light energy for photosynthesis. This includes efficient light absorption under low and moderate light, while reducing excess light absorption under high light. (2) Incorporating CO_2 as the substrate of photosynthesis, while limiting the amount of water lost. (3) Maintaining a stable internal environment for physiological processes by modulating leaf temperature. (4) Maintaining structural integrity that allows leaves to photosynthesize under various mechanical stresses such as gravity, wind, rainfall and herbivory. (5) Transporting water, photosynthates, and nutrients to realize efficient functioning of the plant and the leaf.

Subjected to both anatomical and environmental constraints, natural selection has resulted in an intricate leaf anatomy that balances the above functions and allows plants to grow and produce progeny. As a result, plants coordinate size, number, shape, and arrangement of cells, adjust the thickness and chemical composition of cell walls, and utilize physical phenomena such as water evaporation, refraction, and reflection of light.

In the present chapter, common features of leaf anatomy are described and the current knowledge related to its functions is summarized. Special emphasis will be given to leaf optical properties, gas diffusion, water transport, and mechanical properties. Acclimation and adaptation of leaf anatomy in response to environmental conditions will be reviewed. In addition, we will discuss the physiological mechanisms and ecological significance of these responses.

I. Introduction

Leaf morphology varies greatly across species, and a closer look, for example by using an optical microscope, reveals finer-scale differences in cell and tissue structure between different species and between plants grown in different environments (Fig. 5.1). Leaves are typically composed of an epidermis, parenchymatous tissue called mesophyll, and a vascular system (Fig. 5.1a). Many mesophyll cells contain small green organelles, the chloroplasts. These chloroplasts play a vital role in the process of photosynthesis, which sustains almost all life on earth. Leaf anatomy facilitates this primary function of plants: accommodating the biochemical and biophysical processes of photosynthesis that provide carbohydrates to the whole plant.

The photosynthetic machinery uses light energy to power the biochemical reactions leading to the fixation of CO_2. Chlorophyll and possibly other pigments in the chloroplasts absorb light energy that is utilized by the photosystems to drive electron transfer in the photosynthetic machinery located in thylakoid membranes. Oxygen and chemical energy (adenosine triphosphate and nicotinamide adenine dinucleotide phosphate) are produced by this machinery, and the chemical energy is used to assimilate CO_2 by enzymatic reactions (Calvin-Benson cycle) in the stroma of chloroplasts. CO_2 molecules diffuse from the atmosphere into the chloroplasts through the stomata and the porous leaf structure. Assimilated carbon is partly stored as starch in the chloroplasts and the rest is transported to other parts of the plant in a form of sucrose (plus raffinose-family sugars and sugar alcohols) via the phloem (see Chap. 8 for details on the photosynthetic mechanism and Chap. 3 for photosynthate transport). The biochemical and photochemical reactions of the photosynthetic process are sensitive to environmental factors such as irradiance, CO_2 concentration, temperature, water availability, and nutrient availability. All these process are facilitated, and sometimes even constrained by the structural

organization of plant leaves. For example, light absorption by the leaf depends on the anatomy because the structure of the leaf tissue affects the light path via reflection and refraction. The diffusion rate of CO_2 depends on the porosity and tortuosity within leaves as well as other anatomical and biochemical processes. The transport of water and photosynthates depend on the mesophyll anatomy and vascular system.

Leaves emerge from the shoot apical meristem of stems or branches. Cell divisions in many dicot leaves finish at an early stage of leaf expansion. After that, cell expansion is chiefly responsible for the growth of the leaf blade. Monocot leaves and some dicot leaves keep their elongation zone limited to the base of leaves even at later stages. In parallel with leaf expansion, the photosynthetic machinery in the chloroplasts develops and starts photosynthesizing. In most cases, the maturation of cell walls marks the end of the leaf expansion. Active photosynthesis continues until the end of the leaf lifespan unless environmental stresses limit the activity. Nutrients are then reallocated from the senescing leaf for recycling.

In this chapter, functional aspects of leaf anatomy will be reviewed, focusing on the plasticity of leaf anatomy in response to environmental variation and interspecific differences. We will see that the mesophyll anatomy affects light absorption, gas-exchange, and water transport. The vasculature provides mechanical support and a continuous water supply from the veins, which is indispensable for metabolic processes. A shortage of water may interfere with biochemical processes such as photosynthesis, but the loss of turgor will also affect the structure and morphology of a leaf and inhibit its ability to receive sunlight efficiently. The leaf surface not only controls and regulates the light environment inside the leaf, CO_2 exchange, and water evaporation, but also maintains leaf structure and acts as a barrier against physical damage and biotic stresses. The control of transpiration by the stomata also plays a role in regulating leaf temperature and preventing over-heating under strong irradiance. As a consequence, the inner mesophyll tissues are protected, which allows for a more stable environment for biochemical processes such as photosynthesis.

II. Types of Leaves and Their Anatomy

Leaves can be described from an ontogenetic or anatomical perspective. The existing terminology for both fields is often confused and incorrectly or inconsistently applied (Augsten et al. 1971). Because this confusion persists even in recent literature, we will describe leaf types from both viewpoints, and give clear definitions of the most commonly used terms.

From an ontogenetic perspective, leaves and tissues are distinguished based on the meristematic origin of the cells. In an early stage of leaf formation, the leaf meristem closest to the shoot apical meristem (the adaxial side) differentiates from that on the other (abaxial) side (Nakata and Okada 2013; Fukushima and Hasebe 2014). Most flat leaves are so-called bifacial leaves, the adaxial side of the leaf primordium becomes the morphological upper side of the leaf, and the abaxial side forms the lower side (Fig. 5.1a). However, in rare cases (i.e., resupinate leaves: Fig. 5.2c, d), the petiole or leaf lamina may twist, and the abaxial side becomes the morphological upper side of the leaf. Layers of tightly linked cells, called epidermis, develop from the adaxial and abaxial meristems and form the two distinct sides of a bifacial leaf. Bifacial leaves are typically flat, but more or less cylindrical (terete), or even spherical leaves (e.g., *Senecio rowleyanus*) are also known (Fukushima and Hasebe 2014). Parenchyma cells located between the two epidermal layers are called mesophyll and this tissue develops from both the adaxial and abaxial meristems. Vascular bundles

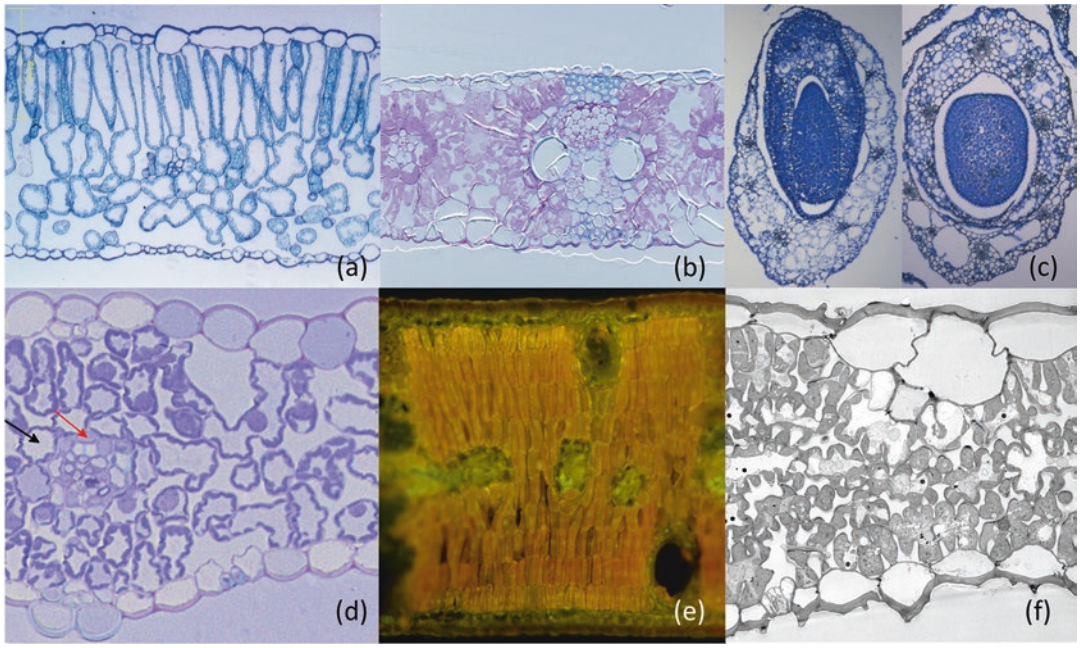

Fig. 5.1. Cross-sectional photographs of (**a**) a typical C_3 leaf (dorsiventral, bifacial, and homobaric) of *Nicotiana tabacum*, (**b**) a typical C_4 leaf of *Sorghum bicolor*, (**c**) unifacial leaves of *Juncus prismatocarpus* (left) and *Juncus torreyi* (right), (**d**) a homogenous isolateral leaf of *Triticum aestivum*, (**e**) an isolateral leaf from *Eucalyptus pauciflora*, and (**f**) a leaf with lobed mesophyll cells from *Oryza sativa*. In (**d**), red arrow indicates the mestome sheath and black arrow indicates the parenchymatous bundle sheath. Photographs were kindly provided by Dr. Shinichi Miyazawa (**a**), Dr. Chieko Saito and Dr. Youshi Tazoe (**b**), Dr. Xiaofeng Yin (**c**), Ms. Elinor Goodman and Dr. Margaret Barbour (**d**) and Dr. Shinya Wada (**f**)

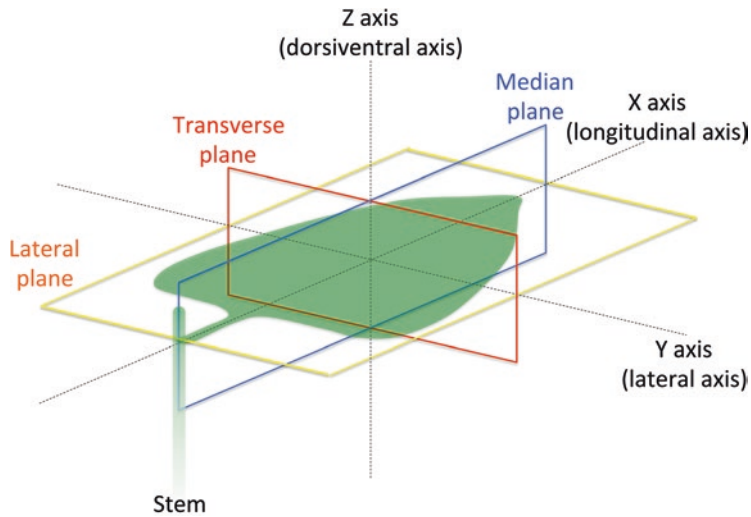

Fig. 5.2. Anatomical axes and planes in the leaf

are embedded in the mesophyll, with xylem developing adaxially and the phloem abaxially (Nakata and Okada 2013).

Some monocots develop so-called unifacial leaf blades (Fig. 5.1c), where the development of the adaxial meristem is suppressed, and the epidermis and underlying mesophyll are typically formed by the abaxial meristem alone (Kaplan 1975; Yamaguchi et al. 2010). Such leaves can be tubular or cylindrical (terete), with vascular bundles arranged in a ring (e.g., *Juncus* and *Allium* species with rounded leaves). Alternatively, the leaves are flattened, such as the ensiform (i.e., sword-shaped, medially flattened) leaves of *Acorus gramineus* and *Iris* spp. Such leaves often have two layers of vascular bundles (i.e., the vasculature remains radially symmetrical), with the phloem part of the vascular bundle located closest to the nearest leaf surface (Yamaguchi et al. 2010; Fukushima and Hasebe 2014; Mashayekhi and Columbus 2014). Occasionally the two layers cannot be clearly distinguished and the orientation of the vascular bundles appears alternating (Imamura and Hida 1956; Mashayekhi and Columbus 2014).

Leaf tissues and cells can also be distinguished based on their shapes and arrangement (i.e., from a purely anatomical perspective). In many species, cells in some or all layers of the mesophyll can differentiate into elongated, regularly distributed cells. This elongation can be in the longitudinal direction (e.g., *Elodea canadensis, Galanthus nivalis, Leucojum vernum*, see also Fig. 5.2), medio-laterally (members of the Iridaceae, *Erythronium dens-canis*), or more or less perpendicular to the leaf surface (dorsiventrally; Haberlandt 1914). Only in the latter case is this type of tissue typically called palisade tissue (Haberlandt 1914; Esau 1965; c.f. Fig. 5.1d). Cells that are more variably shaped and irregularly distributed are called spongy tissue cells (Haberlandt 1914; Esau 1965). Although these cells often appear to be isodiametric (roughly spherical) in 2D transverse sections, the shape can be variable, from spherical to multi-lobed.

If mesophyll cells have multiple protruding lobes or have a gear-like shape as a result of cell-wall invaginations (ingrowths of the cell wall), they are typically called armed mesophyll (Chatelet et al. 2013). Examples can be seen in the Ranunculaceae, Pooideae, Bambusoideae, *Oryza* spp., *Sambucus* spp., *Viburnum* spp., *Pinus* spp., and *Adiantum* spp. (Haberlandt 1914; Carolin et al. 1973; Chonan 1978; Sage and Sage 2009). Sometimes the individual lobes are predominantly arranged perpendicular to the leaf surface, just like regular palisade parenchyma, and these can be called "armed palisade" (Haberlandt 1914; Chatelet et al. 2013).

Though both palisade and spongy tissues can be loosely arranged, with airspaces between individual cells, the relative amount of airspace and the size of the individual pores are larger in the spongy tissue (Terashima et al. 2001; Lehmeier et al. 2017). In addition to the typical elongated palisade cells and irregularly shaped spongy cells, a layer of trapezoid cells (funnel cells) can be observed in some species between the palisade and spongy tissues (e.g., *Ficus elastica, Pulmonaria officinalis*) or instead of the palisade layer (e.g., *Begonia* spp.) (Haberlandt 1914; Sheue et al. 2012).

A few types of special mesophyll cells deserve mention here. In many legumes (Fabaceae), one or occasionally two layers of the mesophyll are anatomically distinct, and consist of laterally stretched and lobed parenchyma cells between the minor veins of the leaf (Fig 5.3a, Metcalfe and Chalk 1950; Brubaker and Lersten 1995). The occurrence of this so-called paraveinal mesophyll is mostly restricted to members of the Fabaceae, but Brubaker and Lersten (1995) suggest that it may also be present in *Populus deltoides* and *Solidago canadensis*. A second type of unusual mesophyll cell is found in some grasses and many members of the Bambusoideae, where distinct colorless cells

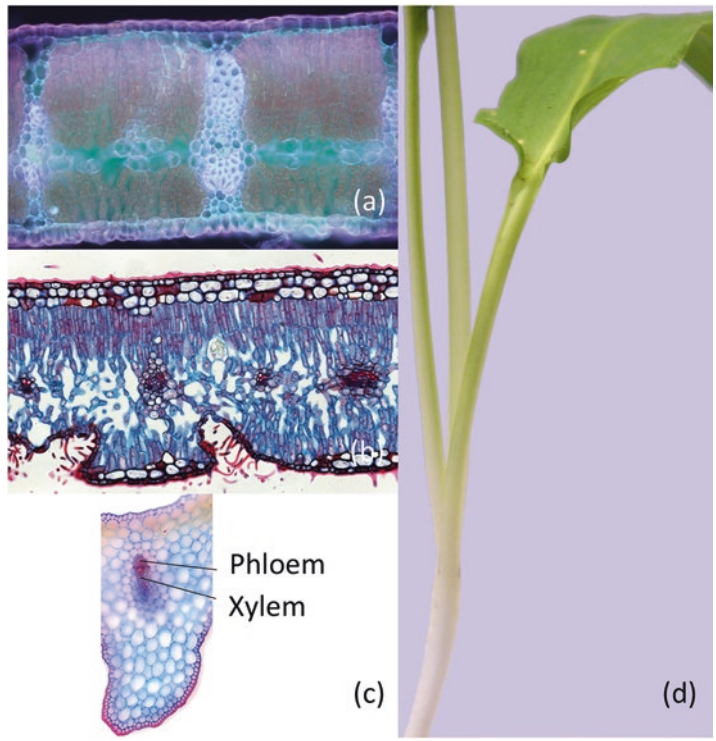

Fig. 5.3. Cross-sectional photographs of (**a**) a heterobaric leaf from *Erythrina crista-galli* and (**b**) a leaf with a multi-layed epidermis and crypts from *Nerium oleander*. (**c**) and (**d**) show the upside-down positioning of phloem and xylem and the twisting petiole of a resupinate leaf from *Allium ursinum*, respectively

are visible between the veins. These cells are called fusoid cells because they appear to have a pyriform or fusiform shape (i.e., pear or spindle-shaped) when viewed in transverse sections (Metcalfe 1956; March and Clark 2011). The cells are elongated in medio-lateral and longitudinal directions (see Fig. 5.2) and thus have more plate-like shapes when viewed in three dimensions (Metcalfe 1956). Large intercellular spaces, which may result from partial collapse of some of the cells, are often found between two consecutive fusoid cells (March and Clark 2011). The development of fusoid cells seems to be dependent on shade conditions (March and Clark 2011).

If a clear differentiation in cell shape can be observed between both sides of a leaf (e.g., palisade and spongy cells at the adaxial and abaxial side, respectively), a leaf is called dorsiventral. Although the developmental origin of a cell may put some constraints on the function, it has been shown that the functional and morphological fate of a cell is mainly determined by the location of cells within the organism (Poethig 1987). Thus, both bifacial and unifacial leaves can develop a dorsiventral anatomy (e.g., Imamura 1931; Mashayekhi and Columbus 2014). In addition, some leaves develop palisade tissue both ad- and abaxially, giving rise to the so-called heterogeneous isolateral (or isobilateral) leaf structure (Fig. 5.1e, DeLucia et al. 1991; Smith et al. 1997). Often, in particular for many grasses, no clear differentiation of the mesophyll can be observed, and such leaves are called homogeneous, homogeneous isolateral or equifacial (Fig. 5.1d, Metcalfe and Chalk 1950; Pyankov et al. 1999).

A typical dicotyledonous leaf is dorsiventral, with palisade tissue forming near the

light-exposed surface of the leaf (usually the adaxial side) and spongy tissue developing towards the shaded (usually abaxial) side (Metcalfe and Chalk 1950). In resupinate leaves, or leaves that adhere closely to the stem with the adaxial surface, the abaxial side is exposed to light and these leaves often show an inverse dorsiventral anatomy, with palisade tissue developing on the abaxial side (Haberlandt 1914; Bredenkamp and Van Wyk 2001; Hofreiter and Lyshede 2006).

Not only the mesophyll, but also the epidermal cells often show a typical dorsiventrality, with differences in the density of stomata, cell sizes and trichome (hair) density (Metcalfe and Chalk 1950). Although stomata can occur on both sides of leaves with a dorsiventral mesophyll (amphistomatic or amphistomatous), it is more common they are exclusively found on the abaxial (usually shaded) side (hypostomatic or hypostomatous; Wilkinson 1979; Muir 2015). In floating leaves of aquatic plants, resupinate leaves, or leaves that closely adhere to soil, stem, or other leaves (e.g., some *Saxifraga* species), stomata may develop only on the adaxial (usually light-exposed) side (epistomatic or epistomatous, sometimes also called hyperstomatic) (Metcalfe and Chalk 1950; Bredenkamp and Van Wyk 2001; Muir 2018).

The epidermis is covered with a thin layer called cuticle. The cuticle consists of wax layers, a cuticular membrane composed of hydrophobic compounds, and cellulose encrusted with cutin (Martin and Juniper 1970). The cuticle is an important barrier that limits water loss from leaves (Burghardt and Riederer 2007), reflects or filters ultraviolet light (Krauss et al. 1997), and mechanically protects the leaf from external stresses such as rubbing, abrasion, and biotic threats (e.g., pathogens or herbivory) (Onoda et al. 2012).

When a leaf is sectioned paradermally (i.e., in a plane parallel to the epidermis), we can observe veins extending throughout the mesophyll. These veins include the xylem and phloem, but only rarely a vascular cambium (Esau 1965). Water moves through the xylem from roots to the cells of the leaves (Chap. 4) and carbohydrates are transported by the phloem from mesophyll cells to the whole plant (Chap. 3).

These veins are usually separated from the mesophyll by a bundle sheath: a tightly connected, radial file of parenchymatous or sclerenchymatous cells, sometimes with a few airspaces, that wholly or partially surrounds the vascular bundle (Carolin et al. 1973; Leegood 2008). Cells not directly connected to the vasculature, but structurally similar have sometimes been included in this definition (see Carolin et al. 1973). Examples of the latter can be seen in some C_4 grasses (e.g., *Arundinella nepalensis, Triodia scariosa*), where bundle-sheath-like cells are not always immediately adjacent to the vasculature (Carolin et al. 1973; Dengler et al. 1994; Lundgren et al. 2014). Bundle-sheath cells are not lobed or dorsiventrally elongated like many mesophyll cells, but may be elongated centrifugally or in the direction parallel to the adjacent veins. Cell walls between the bundle-sheath cells are sometimes impregnated with lignin (and possibly suberin), and in these cases the bundle sheath has been referred to as an endodermis (Lersten 1997; Liesche et al. 2011). In many grasses, a suberized layer of cells called the mestome sheath surrounds the vasculature, but a second, parenchymatous bundle sheath is also present (Carolin et al. 1973; Canny 1990; Dengler et al. 1994; Dengler and Nelson 1999). Mestome and bundle-sheath cells generally contain few chloroplasts (Leegood 2008), except in leaves featuring the Kranz anatomy associated with C_4-metabolism (Fig. 5.1b, Dengler et al. 1994; Dengler and Nelson 1999; Muhaidat et al. 2007). In gymnosperms, the bundles are embedded in a so-called transfusion tissue consisting of tracheids and parenchymatous cells. The transfusion tissue is separated from the mesophyll by an endodermis-like bundle sheath (Esau 1965; Liesche et al. 2011).

Bundle-sheath extensions (BSE) are groups of cells extending from the bundle sheath to the epidermis (Wylie 1952). These cells contain no chloroplasts and are often sclerified. If many veins possess such BSEs, the resulting compartmentalization may restrict gas diffusion in the leaf; such an anatomy is called heterobaric (Fig. 5.3a). On the other hand, if BSEs are absent, the leaves are called homobaric (Neger 1912, 1918; Terashima 1992).

III. Leaf Anatomy and Its Major Functions

A. Light Absorption – Leaf Optics

Chlorophyll pigments absorb light of wavelengths between 400 nm and 700 nm (the photosynthetically active radiation, PAR; McCree 1972; Clark and Lister 1975a,b), which is in the same range as the light visible to the human eye. A leaf typically absorbs ca. 90% of the available PAR (Evans and Poorter 2001). Figure 5.4a shows the absorptance spectrum of a spinach leaf, indicating that leaves absorb blue (400–500 nm) and red (600–700 nm) light well. The absorptance between 500 nm and 600 nm (green) is 10 to 15% lower. As a result, the reflectance and transmittance of leaves peak around 550 nm, leading to the perception that leaves are green.

Similar to the light gradients observed in a forest canopy, there is a light gradient inside a single leaf (Terashima and Saeki 1983; Vogelmann and Björn 1984). Under sunny conditions, chloroplasts located close to the light-exposed surface of a leaf receive strong collimated light while chloroplasts located close to the shaded side of a thick leaf experience lower light intensities (Fig. 5.4b). The photosynthetic rates of individual chloroplasts increase with light absorption, but this response levels off at high light intensity (light saturation). If the absorbed light energy is greater than that can be utilized by photosynthesis, photosynthetic light use efficiency decreases and such an excess of energy may result in chronic photodamage to the photosystems (Aro et al. 1993; Long et al. 1994). Therefore, for chloroplasts near the light-exposed side, reducing light absorption is important. On the other hand, for the chloroplasts near the shaded side, maximizing light absorption is important so that the carbon-gain by photosynthesis is sufficient to cover the cost of their construction and maintenance (Terashima and Hikosaka 1995). The light gradient within a leaf is affected not only by the amount of light-absorbing pigments in a leaf but also by its anatomy and distribution

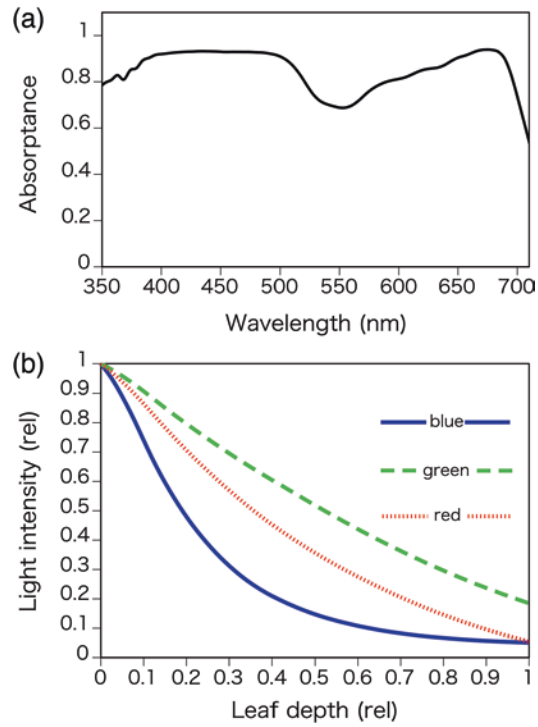

Fig. 5.4. (**a**) Absorptance spectrum of a *Spinacia oleracea* leaf and (**b**) light intensity gradients for different wavelengths of light (blue = 450 nm, half bandwidth of 50 nm; green = 550 nm, half bandwidth of 50 nm; red = 650 nm, half bandwidth of 10 nm) through a leaf (from the upper surface = 0 to the lower surface = 1.0) measured by chlorophyll fluorescence emission from cross-sections of spinach leaves. The data for (**b**) was kindly provided by Dr. John R. Evans. (Re-calculated from Vogelmann and Evans 2002)

of chloroplasts (Vogelmann 1993; Terashima et al. 2009).

The shape of mesophyll cells affects the light gradient in a leaf. When the light is collimated, the columnar and vertically-aligned palisade cells allow a considerable portion of the light to penetrate deeper into the leaf (Terashima and Saeki 1983; Vogelmann and Martin 1993; Vogelmann and Evans 2002), especially when chloroplasts are arranged along the anticlinal (vertical) cell walls (Gorton et al. 1999). The light intensity becomes weaker with increasing depth in the leaf, and the irregular shaped spongy cells alternating with air spaces increase reflection and refraction of light, which increases the optical path length through a leaf and thus increases light absorption. This increase in the optical path length through a leaf due to mesophyll anatomy is called the détour effect (Vogelmann 1993; Terashima et al. 2009). Reflection and refraction are caused by the difference in refractive indexes of leaf components (e.g., cells have a higher refractive index than air spaces), and thus the détour effect is influenced by cell shape. Terashima and Saeki (1983) sliced leaves of *Camellia* paradermally and showed a greater capacity for light absorption per unit chlorophyll in spongy tissue than in palisade tissue (Fig. 5.5). They also examined the effect of infiltration with olive oil (with a refractive index similar to that of the cell) into the intercellular air space on the apparent extinction coefficient of chlorophyll in order to examine the effect of the détour effect. Infiltration significantly reduced the extinction coefficient, especially in spongy tissue, which supported the hypothesis that the elongation of light path length increased light absorption. Similar results were obtained by Vogelmann and Evans (2002), who showed that the path-lengthening effect in the spongy tissue is especially strong for green light.

In a leaf, absorption of PAR occurs in discrete units (the chloroplasts), whereas the rest of the leaf is relatively transparent. This non-uniform distribution of pigments decreases the possibility of photons encountering pigments and results in less light absorption compared to a hypothetical leaf with pigments homogeneously distributed. This is called the sieve effect, and this effect is larger for strongly absorbed light (e.g., blue versus green). The sieve effect may seem to be a disadvantage for leaves, but it allows for a better control of light absorption. For example, in many plant species, chloroplasts change their shape and position in response to environmental factors such as light intensity (Fig. 5.6; Wada 2013). Under high light, chloroplasts adhere along cell walls parallel to the light direction (*profile position*: Fig. 5.6b), whereas under low light

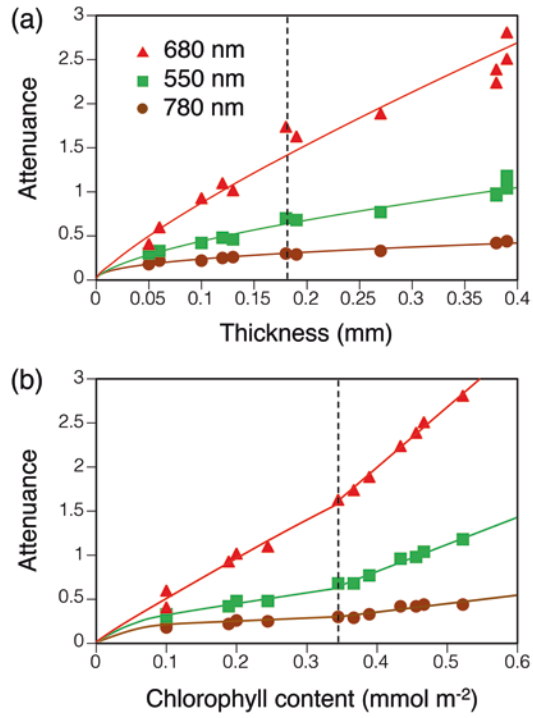

Fig. 5.5. Attenuance of monochromatic light in paradermal sections of a *Camellia japonica* leaf as a function of (**a**) thickness and (**b**) chlorophyll content. All paradermal sections had an upper epidermis and a collimated light source was used to illuminate the section. The dotted lines indicate the boundary between the palisade tissue and the spongy tissue. (The figure was redrawn from data originally published by Terashima and Saeki 1983)

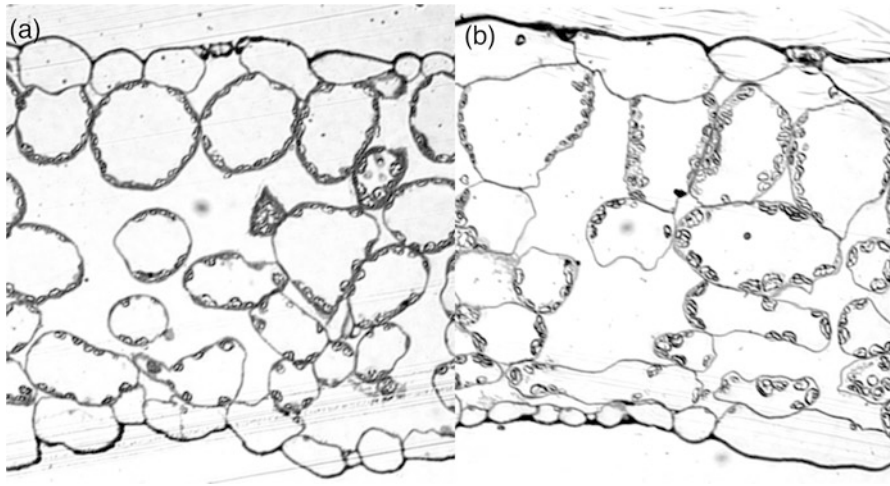

Fig. 5.6. Cross-sections of *Arabidopsis thaliana* leaves. Chloroplasts are arranged in (**a**) a typical face position under blue-filtered light and (**b**) in a profile position under high white light. Light micrographs of leaf sections were taken at 200 times magnification. (See Tholen et al. 2008 for experimental conditions)

they accumulate along walls perpendicular to the light (*face position*: Fig. 5.6a). The profile position allows a larger part of the light to pass through the leaf (Gorton et al. 1999) and limits the exposure to high light to those chloroplasts close to the illuminated side of the leaf. Thus, the profile position can reduce the amount of photodamage resulting from strong light (Kasahara et al. 2002; Sztatelman et al. 2010). In addition, the profile position allows more light to penetrate deeper in the leaf, resulting in a more uniform light gradient throughout the leaf, allowing chloroplasts deeper in the leaf to contribute efficiently to whole-leaf photosynthesis (Brugnoli and Björkman 1992; Terashima and Hikosaka 1995; Gorton et al. 1999). This would also allow photodamage to be shared over the leaf, which may prevent a decrease in the photosynthetic potential (Davis and Hangarter 2012). By contrast, the face position limits transmittance and may increase photosynthetic efficiency under low light intensities (Zurzycki 1955). In shade leaves, which often have large mesophyll cells, chloroplasts may move more freely, resulting in relatively large differences (>20%, Haupt and Scheuerlein 1990) in light absorption between low and high light environments. Chloroplast movement may be more restricted in sun leaves, with narrower palisade cells, resulting in only small differences in optical properties (Davis et al. 2011; Higa and Wada 2016).

BSEs, which are composed of tightly packed transparent cells (containing few or no chloroplasts), would allow light to penetrate deeper into the leaf (Karabourniotis et al. 2000; Nikolopoulos et al. 2002; Xiao et al. 2016). Karabourniotis et al. (2000) showed that some BSEs behave as transparent windows and can decrease the slope of the light gradient in the leaf.

Biominerals such as calcium carbonate or oxalate crystals in the mesophyll or epidermis are frequently observed in many plant families (Metcalfe and Chalk 1950). These biominerals also allow light to scatter and penetrate deeper, resulting in a reduced light gradient within a leaf (Gal et al. 2012). Under high light intensities, this allows more light to reach the deeper parts of the leaf, possibly increasing photosynthesis.

The fusoid cells of basal grasses and bamboo have also been related to light absorption. While such cells are always present in shade leaves of these species, March and Clark (2011) showed that sun leaves of three

bamboo species lacked fusoid cells. Infusion of leaves containing fusoid cells with mineral oil changed their optical properties, suggesting that such cells may play a role in absorbing light more efficiently in shade leaves (March and Clark 2011).

Because light absorption by chlorophyll pigments strongly depends on the color of the light, the light gradient through a leaf also changes dependent on color. Using a chlorophyll fluorescence imaging technique, originally developed by Takahashi et al. (1994), Vogelmann and colleagues showed significant differences in the light gradient through a leaf among blue, green, and red light (Fig. 5.4b, Vogelmann and Evans 2002; Brodersen and Vogelmann 2010). Since there is less attenuation of green than of blue or red light as light penetrates through a leaf (Fig. 5.4b), any additional green light absorbed by chloroplasts deep in the leaf increases leaf photosynthesis to a greater extent than additional red or blue light (Terashima et al. 2009). If leaves were black and absorbed all wavelengths equally, light absorption of chloroplasts near the irradiated surface would increase, but absorption of light by chloroplasts deeper in the leaf would decrease. Light use efficiency (photosynthetic rate per unit illuminated light) in such black leaves would be lower compared to that of green leaves (Terashima et al. 2009).

With the exception of the guard cells of the stomata, the epidermal cells of angiosperm plant species rarely contain chloroplasts (but see Joel and Gepstein 1985). Chloroplast-containing epidermal cells are much more common in ferns, bryophytes, and submerged or aquatic higher plants (Nasrulhaqboyce and Duckett 1991; Sakurai et al. 2005; Sheue et al. 2007; Mommer et al. 2006). Nevertheless, the epidermis may still play a role in light absorption. Many species, especially herbaceous plants, have a papillary or bumpy leaf surface consisting of convex epidermal cells that may affect the light intensity in the underlying mesophyll tissue (Haberlandt 1914). The leaves of some understory species, such as *Begonia erythrophylla*, *Colocasia esculenta*, and *Impatiens velvetea*, have extremely bumpy surfaces (Brodersen and Vogelmann 2007). It has been suggested that such a rough leaf surface lowers reflectance and increases the absorptance of diffuse light (Haberlandt 1914; Bone et al. 1985; Vogelmann 1993) because a smooth cuticular layer has been shown to result in a more mirror-like (specular) reflectance of light (Woolley 1971). However, Brodersen and Vogelmann (2007) found no relation between the convexity of epidermal cells and the absorptance of diffuse light. An alternative view holds that convex shaped cells may focus light on specific chloroplasts in the mesophyll (Vogelmann 1993; Vogelmann et al. 1996). Interestingly, this focusing effect is greater for direct light compared to diffuse light and is also present in leaves of sun plants adapted to high light intensities such as *Trifolium repens* and *Medicago sativa* (Martin et al. 1989; Vogelmann 1994). It remains unclear whether such light focusing by epidermal cells has an advantage for photosynthesis. Turgor pressure of the epidermal cells may inevitably lead to some level of bumpiness at the leaf surface. Moreover, it has been argued that convex cells may also make the leaf surface more water repellent (Neinhuis and Barthlott 1997; Wagner et al. 2003; Bhushan and Jung 2006). Water droplets or films on the surface of leaves can induce stomatal closure and reduce photosynthesis (Ishibashi and Terashima 1995; Terashima et al. 1995). This would provide leaves with convex shaped epidermal cells with an advantage under wet conditions.

B. CO_2 Diffusion and Assimilation

The supply of carbon dioxide (CO_2) affects the rate of photosynthesis. Atmospheric CO_2 diffuses through stomata into intercellular airspaces, dissolves in apoplastic water layers, and subsequently diffuses through a number of cellular components, including

cell walls, membranes, cytosol, chloroplast envelopes, and chloroplast stroma (Evans et al. 2009). Finally, CO_2 reaches the site of carboxylation, where it is fixed by ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco). The ease with which CO_2 diffuses through a leaf is called conductance (the reciprocal of resistance). The conductance of CO_2 through the leaf is determined not only by the physical and chemical properties of the conducting medium (air, cell material), but also by the arrangement of the tissues, cells, and cellular components, i.e., by the leaf anatomy (Parkhurst 1994; Evans et al. 2009). It is common to divide the leaf CO_2 diffusion conductances into two main components: the stomatal conductance (g_s) describing the conductance between the atmosphere and substomatal cavity and the mesophyll conductance (g_m) describing the conductance between the substomatal cavity and the site of carboxylation. When stomata are open, the magnitudes of these conductances are roughly comparable (Warren 2008; Flexas et al. 2008). Both g_s and g_m are sensitive to environmental conditions and other physiological processes, which are further explained in Chaps. 6 and 7. Here we focus on the relationship between leaf anatomy and mesophyll conductance.

1. Diffusion Through Intercellular Airspaces

Diffusion of CO_2 through the gas phase is about four orders of magnitude faster compared to diffusion through the liquid phase (Nobel 2009). However, the diffusion-path through airspaces inside leaves is often more than two orders of magnitude longer compared to the liquid-phase path through mesophyll cells into the chloroplasts, especially in thicker, hypostomatic leaves (Parkhurst 1994). For example, estimates of airspace resistance based on leaf anatomy in sun leaves are generally higher compared to those in shade leaves (Syvertsen et al. 1995; Piel et al. 2002; Ivanova et al. 2006). Direct measurements of gas space conductance are rare and rely on comparing assimilation rates between leaves in air and in helox (air with nitrogen replaced by helium); since CO_2 diffuses faster in helox than in air, airspace resistance can be estimated from the difference in assimilation rates. Parkhurst and Mott (1990) found that assimilation rates increased significantly under helox in several hypostomatic leaves such as *Cissus rhombifolia*, *Nerium oleander*, *Brassaia actinophylla*, and *Plectranthus australis*, calculating that diffusion through air accounted for about 20% of the total diffusion resistance. Other authors (Piel 2002; Piel et al. 2002) found no measurable resistance by airspace for hypostomatic leaves of *Rosa rubignosa* and *Nerium oleander*, although in *Quercus ilex* resistance through air accounted for more than 30% of the total diffusion resistance. It must be noted that these measurements depend on an accurate estimate of the CO_2 concentration in the substomatal cavity, which is commonly determined based on the evaporation of water from the mesophyll. Thus these measurements also rely on assumptions with respect to the location of water evaporation from the mesophyll cells. If transpiration occurs deeper in the mesophyll (Boyer 1985; Parkhurst and Mott 1990; Buckley et al. 2015), it would result in an underestimation of the airspace conductance, because estimates of stomatal conductance would already include a sizable portion of conductance through the airspaces (Piel 2002).

In models, diffusion through the intercellular airspaces can be described using the porous medium approximation, allowing the effect of leaf anatomy on gas diffusion to be analyzed using a simple model based on a few leaf anatomical characteristics (Syvertsen et al. 1995; Piel 2002; Niinemets and Reichstein 2003). The conductance through intercellular airspaces (g_{ias}; [mol m^{-2} s^{-1} bar^{-1}]) can be described by:

$$g_{ias} = sD(\rho/\tau)/(aL) \tag{5.1}$$

where s is the solubility of CO_2 in water [mol m^{-3} bar^{-1}], D is the diffusion coefficient of CO_2 through air [m s^{-1}], ρ is the porosity [m^3 m^{-3}] of the leaf and τ is the tortuosity factor [m^2 m^{-2}]. τ is defined as the squared ratio between the actual length of the diffusion path between two points and a straight-line distance between these points (Epstein 1989). L is the mesophyll thickness [m]. a is a dimensionless correction factor to account for the fact that the mesophyll thickness is not equal to the effective distance between stomata and chloroplasts because of the spacing of stomata on the leaf surface and the presence of CO_2 sinks along the diffusion path (Parkhurst 1994). These effects are often taken into account in reaction-diffusion models of the diffusion process (Parkhurst 1994; Terashima et al. 2001; Aalto and Juurola 2002; Ho et al. 2016). Terashima et al. (2001) accounted for the effect of sinks along the diffusion path in a one-dimensional model and showed that the diffusion path length becomes dependent on the assimilation rate of the leaf. From their results, it can be inferred that for many thinner hypostomatic leaves, the effective path length can be approximated by $a = 1/3$. Assuming the path length is halved when CO_2 can enter a flattened leaf from both sides, $a = 1/6$ may be used for amphistomatous leaves.

In reality, the diffusion through a leaf is not one-dimensional, and most CO_2 enters the leaf at discrete positions (through the stomata), which would increase the effective path length to some extent (Parkhurst 1994). It has been suggested that amphistomaty is an adaptive feature of thick leaves, allowing faster diffusion of CO_2 to chloroplasts in such leaves (Parkhurst 1978; Mott et al. 1982; Muir 2015). Amphistomaty is correlated with higher stomatal conductance (Beerling and Kelly 1996), but it is unclear whether the same is true for mesophyll conductance. If amphistomaty indeed allows for a higher g_{ias}, this may explain the greater water-use efficiency observed for amphistomatous species (Bucher et al. 2017) and the scarcity of hypostomatic leaves in dryer habitats (Parkhurst 1978). Amphistomaty is indeed associated with thicker leaves across hundreds of species, but the correlation is weak (Muir 2015). Amphistomaty was most closely associated with herbaceous plants with short life cycles and with plants from low precipitation environments (Muir 2015). In addition, amphistomaty was linked to high light environments, suggesting that amphistomaty is favored when CO_2 limits photosynthesis (Muir 2018). The prevalence of hypostomy suggests, however, that a disadvantage of amphistomaty exists, perhaps related to increased pathogen susceptibility (Muir 2015, 2018). It is tempting to conclude from the above that diffusion through the intercellular airspace is limiting photosynthesis in fast growing herbs that have high photosynthetic rates. Since stomata on the adaxial side and the abaxial side respond differently to environmental stimuli (Pospíšilová and Solárová 1980; Driscoll et al. 2006; Wang et al. 2008), the diffusion path length through airspaces is variable, and may result in a dynamic g_{ias} (Morison and Lawson 2007). A change in the path length for CO_2 diffusion through the mesophyll as a result of inhomogeneous (i.e., depending on the leaf side or patchy) stomatal closure may contribute to the often observed covariation between g_m and g_s (Flexas et al. 2008; Warren 2008).

In leaves of many xeromorphic or sclerophyllous (hard leaf) species, stomata are located in epidermal depressions called crypts (e.g., *Nerium oleander*, Fig 5.3b, Metcalfe 1979). Such crypts have traditionally been thought to reduce water loss through increasing boundary layer resistance, but recent modeling suggested that crypts have negligible effects on the transpiration rate (Roth-Nebelsick et al. 2009). Crypts reduce the distance between stomata and chloroplasts, and this may lower the diffusion resistance through the gas spaces in the leaf (Piel 2002; Hassiotou et al. 2009).

Equation 5.1 suggests that mesophyll porosity and tortuosity are important factors

affecting airspace conductance. Porosity can be estimated by infiltration with a water volume or by measuring the air volume in microscopy sections of the leaf. Slaton and Smith (2002) analyzed a wide range of species showing variations in porosity between 4 and 51%. Generally, shade leaves are more porous compared to sun leaves (Slaton and Smith 2002; Piel et al. 2002; Sack et al. 2003; Ivanova et al. 2006; Onoda et al. 2008; but see Syvertsen et al. 1995). Tortuosity is more difficult to assess compared to porosity, and depends on the shape and size of the pores in the mesophyll. Using a 2D model that accounted for the effect of distributed CO_2 sinks throughout the leaf, Morison et al. (2005) estimated the effective diffusion through airspaces. Their model was parameterized by measurements of CO_2 gradients surrounding small patches of grease on the leaf surface. Assuming the reduction in the diffusion coefficient relative to that in air was due to the effective porosity (ρ / τ) of the mesophyll, a tortuosity factor of τ = 2.9 for heterobaric *Phaseolus vulgaris*, and τ = 1.9 for homobaric *Commelina communis* can be calculated. These results may suggest that the lack of bundle-sheath extensions lowers tortuosity and allows for more rapid lateral diffusion in a leaf, which may be beneficial under conditions where the light intensity on the leaf surface is heterogeneous (Morison et al. 2005; Morison and Lawson 2007). Tortuosity can also be estimated by simulating a diffusion process using images of the anatomy of a porous medium (Nakashima and Kamiya 2007). However, to our knowledge, this method has not yet been applied for leaves (but see Table 5.1). A recent study investigating the effect of manipulating genes related to cell division patterning in *Arabidopsis* used a different image-analysis with a network theory and found that a reduced mesophyll tortuosity was linked to a lower g_m (Lehmeier et al. 2017).

Because the estimation methods of tortuosity described above require non-easily accessible techniques, an estimated factor of 1.57 has been used occasionally (Niinemets and Reichstein 2003; Peguero-Pina et al. 2012). However, since tortuosity generally scales negatively with porosity, it would be better to estimate tortuosity with estimations that take this into account. A commonly used method is based on the so-called Bruggeman relation: $\tau = \rho^{-\alpha}$, where α is a material specific constant that depends on the morphology of the porous material (Tjaden et al. 2016). It is important to note that the Bruggeman relation is valid only for materials in which the insulating phase is present in a low volume fraction and characterized by isotropically arranged shapes (Tjaden et al. 2016). Neither of these assumptions is valid for many leaves, which may have strongly anisotropic tissues (e.g., palisade paren-

Table 5.1. Comparison between the estimate of a mesophyll tortuosity factor (τ) based on porosity (ρ) alone ($\tau = \rho^{-0.55}$; Syvertsen et al. 1995) with experimental methods

Species	Porosity (%)	$\tau = \rho^{-0.55}$	Experimentally estimated τ	Method and citation
Phaseolus vulgaris (heterobaric)	36.4	1.7	2.9	Chlorophyll fluorescence imaging in combination with a lateral diffusion model (Morison et al. 2005)
Commelina communis (homobaric)	40.9	1.6	1.9	Chlorophyll fluorescence imaging in combination with a lateral diffusion model (Morison et al. 2005)
Arabidopsis thaliana (homobaric)	23.0	2.2	1.6	Network analysis of 3D images (Lehmeier et al. 2017)
Arabidopsis thaliana (homobaric)	23.0	2.2	2.3	Random walk method (Nakashima and Kamiya 2007) using microscope images (Tholen et al. 2008, D. Tholen, unpub. results)

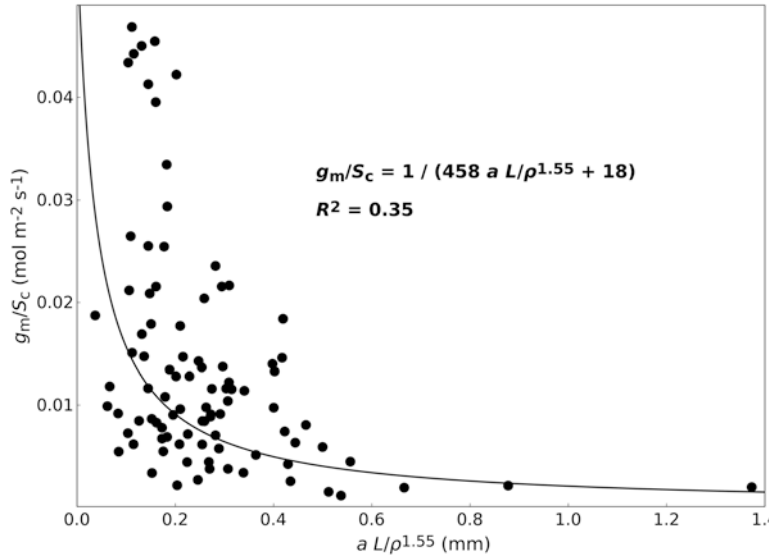

Fig. 5.7. Mesophyll conductance per unit chloroplast surface area (g_m/S_c) plotted against estimated effective distance of CO_2 diffusion in intercellular air space ($aL/\rho^{1.55}$). To account for the fact that the tortuosity estimates are inaccurate for leaves with very low amounts of airspace (Tjaden et al. 2016), leaves with $\rho < 0.2$ were excluded from the analysis. Data are from Evans et al. (1994), Syvertsen et al. (1995), Hanba et al. (2002), Kogami et al. (2001), Vyas et al. (2007), Giuliani et al. (2013), Tomás et al. (2013, 2014), Muir et al. (2014), Tosens et al. (2016), and Peguero-Pina et al. (2017). Leaf thickness was used as an estimate of mesophyll thickness (which was not reported in most cases). The line indicates the best fit through the data. Mesophyll conductance can be expressed as follows: $g_m = 1/(r_{ias} + r_{liq}) = 1/(\text{effective distance}/(Ds) + r_{liq})$, where r_{ias} is the resistance of CO_2 to diffusion in the intercellular air space, r_{liq} is the resistance of CO_2 to diffusion in mesophyll liquid phase, D is the diffusion coefficient of CO_2 in air, and s is CO_2 solubility in apoplastic water. Therefore, a line with the equation of $y = 1/(ax + b)$ was fitted to the data

chyma) and very low amounts of airspace (the dataset used for Fig. 5.7 had $0.07<\rho<0.68$). For isotropically arranged spheres, $\alpha = 0.5$, whereas for isotropically arranged cylinders $\alpha = 1$ should be used (Tjaden et al. 2016). Syvertsen et al. (1995) used $\alpha = 0.55$ to estimate the effective porosity of several hypostomatic leaves, simplifying Eq. 5.1 to:

$$g_{ias} = sD\left(\rho / \rho^{-0.55}\right) / L = sD\rho^{1.55} / (aL) \quad (5.2)$$

In Table 5.1 the relation by Syvertsen et al. (1995) is compared with the few available experimental estimates. The results suggest that this relation somewhat underestimates tortuosity, especially for heterobaric leaves.

If the gas-phase conductance is limiting the total mesophyll conductance, a correlation between g_m and the anatomical features described above should be evident. We collected published data from several studies that investigated g_m and leaf anatomy and tested whether g_m showed negative correlation with the effective distance of CO_2 diffusion through intercellular air spaces: $(aL)/\rho^{1.55}$ (Fig. 5.7). Since thicker leaves generally possess more chloroplast surface area facing intercellular spaces per unit leaf area (S_c) and since a larger surface area for diffusion allows for more liquid phase diffusion (Evans and Loreto 2000; Terashima et al. 2006), we expressed g_m per unit S_c. The negative correlation between the effective distance and g_m ($P < 0.001$) suggests that g_{ias} explains a significant amount of the variation observed in g_m. However, the diffusion coefficient estimated from the fit in Fig. 5.7 is about 3 times higher than the expected value

(Nobel 2009). Therefore, we may have overestimated the length of the diffusion path in the calculations above, but it is also possible that g_{ias} covaries with the thickness of cell walls or other properties that affect $g_{m.}$

2. *Diffusion Through the Mesophyll Cells*

At the interface between intercellular air and apoplastic water, the gas and liquid phases are commonly assumed to be in equilibrium. The concentration of CO_2 in solution can then be calculated using Henry's law. CO_2 subsequently diffuses through apoplastic water and the cell wall into the mesophyll cells. Mesophyll conductance per unit of exposed chloroplast surface area correlates negatively with wall thickness (Evans et al. 2009; Terashima et al. 2011) and the cell wall is thought to be responsible for up to half of the total diffusion resistance (Evans et al. 2009; Terashima et al. 2011).

After diffusion through the plasmalemma, CO_2 enters the cytosol. In C_3 plants, chloroplasts are generally located immediately adjacent to cell walls facing the intercellular space, and S_c correlates well with g_m (Evans and Loreto 2000; Terashima et al. 2006, 2011). In C_4 plants, CO_2 is initially fixed by phosphoenolpyruvate carboxylase, which is located in the mesophyll cytosol. Chloroplasts in these cells may be located farther away from the cell wall than is the case in C_3 plants (Stata et al. 2014). Recent measurements found no large differences in mesophyll conductance between C_3 and C_4 species (Barbour et al. 2016).

An increase in chloroplast thickness or number without a corresponding increase in the exposed chloroplast surface area would increase the diffusion path through the liquid phase and lower g_m (Terashima et al. 2006, 2011). A high g_m can be achieved by increasing S_c, which can result from either increasing leaf thickness or surface-to-volume ratios. This in turn can be achieved by the construction of smaller cells or elongation of mesophyll cells. However, smaller cells require more cell divisions, which could slow leaf growth, and thicker leaves may result in increased nitrogen costs, increased airspace resistance, and lower light-use efficiency. These issues may explain the occurrence of lobed mesophyll cells in many taxa (Haberlandt 1914). For example, rice mesophyll is heavily lobed, and chloroplasts with stroma-filled protrusions cover nearly the entire periphery of the small mesophyll cells (Fig. 5.1f, Chonan 1978; Sage and Sage 2009). Adachi et al. (2013) observed that the density of these mesophyll lobes was linked to a lower resistance to CO_2 diffusion and increased photosynthetic rates. However, Giuliani et al. (2013) found no strong effect of cell lobing on the photosynthetic rate in an analysis of structural leaf traits in rice and its wild relatives.

Mitochondria are typically located towards the center of mesophyll cells, with chloroplasts closer to the periphery (Sage and Sage 2009; Hatakeyama and Ueno 2017). This suggests increasing S_c may not only increase the surface area available for CO_2 diffusion, but may also partially block and re-assimilate the respiratory and photorespiratory CO_2 released by mitochondria that would otherwise escape to the atmosphere. More re-assimilation of (photo) respiratory CO_2 would increase the apparent g_m and potentially increase photosynthetic rates (Tholen et al. 2012b; Busch et al. 2013).

It has been hypothesized that chloroplast arrangement may also affect mesophyll conductance (Terashima and Hikosaka 1995). However, no significant effects on mesophyll conductance were found in *Alocasia brisbanensis* (Gorton et al. 2003). In *Arabidopsis thaliana*, the chloroplast avoidance response to high light resulted in a small decrease in S_c coupled to a corresponding decrease in g_m (Tholen et al. 2008).

C. *Temperature Modulation*

Photosynthesis is a chemical reaction catalyzed by enzymes and the rate of photosyn-

thesis therefore depends on leaf temperature. As a result of increased membrane fluidity and higher enzymatic activities at warmer temperatures, photosynthetic rate increases up to an optimum temperature (Lambers et al. 1998). When the temperature exceeds this optimum, the photosynthetic rate decreases due to 1) deactivation of enzymes, especially Rubisco and Rubisco activase and 2) an increase in the rate of photorespiration for C_3 plants (Salvucci and Crafts-Brandner 2004; Sage and Kubien 2007) (see Chap. 8 for details). Leaf temperature is influenced by atmospheric temperature, irradiance, water/humidity, wind, air pressure, and leaf angle, shape, and transpiration (Medina et al. 1978; He et al. 1996). Light energy increases leaf temperature because of light absorption by leaf pigments, as part of the light energy is converted into thermal energy (Gates 1980; Jones 1983). Leaf temperature can be decreased by transpirational cooling: latent heat is taken from the leaf when the vapor pressure difference between intercellular space and atmosphere causes evaporation of apoplastic water. The control of stomatal aperture is thus an important mechanism to control leaf temperature (Vogel 2009). Because the details of stomatal control are reviewed in Chap. 6, we focus on the anatomical aspects of leaf temperature homeostasis here.

Leaf size can affect temperature homeostasis. An increase in leaf area increases the thickness of the boundary layer, a thin layer of stagnant air surrounding the leaf (Nobel 2009): The boundary layer can be estimated as $\delta = c\sqrt{(vd/u)}$, where δ is boundary layer thickness [m], u is wind speed [m s^{-1}], d is leaf width [m] measured in the direction of the wind, v is the kinematic viscosity of air (1.53×10^{-5} m^2 s^{-1} at 25 °C), and c is a dimensionless empirical coefficient, which varies from 1.03 to 1.72 (Nobel 2009; Monteith and Unsworth 2013). If the thickness of the boundary layer increases, the resistance to water and CO_2 diffusion also increases (Nobel 2009). For example, for 20 cm wide leaves (e.g., *Aesculus* and *Platanus* leaves), the water vapor conductance of the boundary layer ($= D_v/\delta$, where D_v is the diffusion coefficient of vapor) at 25 °C becomes 0.6–1.0 mmol m^{-2} s^{-1} at a wind speed of 3 m s^{-1} and 0.3–0.6 mmol m^{-2} s^{-1} at a wind speed of 1 m s^{-1}. In the case of plants that possess gigantic leaves larger than 1 m, like banana (*Musa* spp.) and *Alocasia*, the boundary layer conductance becomes 0.3–0.4 mmol m^{-2} s^{-1} and 0.2–0.3 mmol m^{-2} s^{-1} at wind speeds of 3 m s^{-1} and 1 m s^{-1}, respectively. These values are comparable with the typical stomatal conductance of tree species and herbal species (0.25 mmol m^{-2} s^{-1} and 0.5 mmol m^{-2} s^{-1}; Nobel 2009). Therefore, a larger leaf area may result in slower evaporation rates and CO_2 exchange between leaf and atmosphere (Parkhurst and Loucks 1972), especially when the wind speed is low. A decrease in evaporation increases leaf temperature, and a decrease in CO_2 diffusion results in a lower CO_2 concentration inside the leaf (C_i).

Okajima et al. (2011) developed a model that estimates the photosynthetic rate of leaves in relation to leaf size, temperature, and wind speeds. The model showed that an increase in leaf area increases leaf temperature in the daytime, which could lead to a higher photosynthetic rate below the optimum temperature for photosynthesis. However, a larger leaf area also lowers C_i due to slower diffusion of CO_2 through the boundary layer, which in turn limits the rate of photosynthesis. Accordingly, at cooler temperatures, there is an optimum leaf area that balances the effects of boundary layer resistance and leaf temperature on the rate of CO_2 exchange. On the other hand, at warmer temperatures, the increase in leaf temperature does not lead to an increase in photosynthetic rates, and a smaller leaf size therefore seems more advantageous in terms of efficient photosynthesis. However, when temperatures fluctuate during the day, the larger surface area may allow the leaves to heat up more quickly to temperatures suitable for

photosynthesis, leading to higher photosynthetic returns during cool mornings (Michaletz et al. 2016). At the shoot level, large leaved species require less investment in stems and the reduced supporting cost per unit leaf area may lead to a growth advantage (Pickup et al. 2005). These considerations may explain why many tropical species have large leaves (Wright et al. 2017). It is also suggested that, in cold regions, larger leaves may experience greater negative consequences from freezing temperatures at nighttime. Radiative cooling leads to leaf temperatures that are lower than air temperature and slower heat diffusion from the surrounding air may increase the possibility of freezing (Wright et al. 2017).

Increases in leaf thickness and/or leaf water content increases the heat capacity, which can prevent a rapid rise in leaf temperature under high light conditions especially at slow wind speeds (< 0.1 m s^{-1}). Thus, increased leaf thickness and/or increased leaf water content are likely to be important for decreasing the incidence of extreme heat stress (Leigh et al. 2012; Schymanski et al. 2013). For example, Li et al. (2011) reported that higher heat tolerance in polyploid plants of *Lonicera japonica* compared to diploid plants was a result of the greater epidermal and palisade tissue thickness in the former. It is also reported that plants adapted to an active geothermal field (e.g., *Cistus salviifolius* and *Agrostis castellana*) develop a thicker epidermis and cuticle, higher palisade and spongy tissue density, and longer palisade cells (Bartoli et al. 2014, 2015).

D. Anatomy and Water Transport

Stomata provide an opening in the cuticle and epidermis through which CO_2 can diffuse into the leaf, but they also allow water to escape to the atmosphere. The transpiration of water has an important function in regulating leaf temperature (Vogel 2009), but also carries with it a risk of dehydration of the mesophyll cells, which could impair leaf biochemistry (Cornic and Massacci 1996). Without an efficient pathway for water to reach the site of evapotranspiration, stomata would need to close rapidly under conditions where transpiration rates suddenly increase, and this will negatively affect CO_2 assimilation rates. Leaves have developed an extensive vascular system for water transport and the hydraulic conductance through a leaf is affected by structural properties of the vasculature, such as minor and major vein length per area, conduit numbers and size, and vein topology (Cochard et al. 2004; Brodribb et al. 2007; Caringella et al. 2015; Sack et al. 2015; Xiong et al. 2015). For example, veins with large diameters can transport disproportionally greater amounts of water than those with small diameters (see Chap. 4, Hagen-Poiseuille flow), but larger veins are more susceptible to cavitation (formation of air bubbles, which interfere with water transport). Finer networks of leaf veins are associated with higher photosynthetic capacity across phylogenetically diverse species (Brodribb et al. 2007).

In many species, a layer of bundle-sheath cells surrounds at least a part of the vascular bundle and may even enclose the terminal tracheids (Leegood 2008). This bundle sheath conducts the flow of water between xylem and mesophyll. Water and dissolved substances may move easily through cell walls, but suberin or lignin deposits inside the wall could block this pathway, forcing the transpiration stream through the symplast (Sack and Holbrook 2006; Leegood 2008). In *Rhododendron* species sampled along an altitudinal gradient, lignified primary walls in the bundle sheath were associated with a lower leaf hydraulic conductance (Taneda et al. 2016). Ohtsuka et al. (2018), in a study with 11 angiosperm tree species, also showed that species with a lignified bundle sheath surrounding minor veins have higher resistance to apoplastic water movement. Such an impediment to the apoplastic pathway across the bundle sheath would allow for regulation of the transpiration

stream through water transport across cells by transporting membrane proteins such as aquaporins. Despite the observation that extensive suberin deposits are uncommon in leaves (Lersten 1997), experiments with fluorescent dye tracers suggested that the water stream indeed enters the symplast close to the xylem elements (Canny 1990). More recently, Shatil-Cohen et al. (2011) showed that the bundle sheath in *Arabidopsis thaliana* had decreased water permeability in response to drought or abscisic acid treatment. A similar and reversible effect was seen after treatment with an inhibitor of aquaporin action. Knocking down aquaporin expression in the vein or bundle sheath results in reduced leaf hydraulic conductance (Prado et al. 2013; Secchi and Zwieniecki 2014). These results support the view that the bundle sheath plays an important role in regulating water transport. A recent model of water transport in the leaf suggested that the surface area of the bundle sheath per unit leaf area (which mainly depends on vein length per area) is a strong determinant of the outside-xylem hydraulic conductance, but that the contribution of the bundle sheath to the total resistance is relatively small (Buckley et al. 2015).

The BSEs of heterobaric leaves connect the vasculature with the epidermis. Wylie (1952) hypothesized that the well-connected BSE cells would act as a more efficient water pathway compared to the sparsely connected mesophyll cells. Some earlier experiments with fluorescent dyes supported this view, although the use of dyes for tracing water transport beyond the bundle sheath is questionable (Canny 1990). More recently, Scoffoni et al. (2008) found that changes in hydraulic conductance in response to short-term differences in light intensity are stronger in heterobaric species, suggesting BSEs do play a role in facilitating water transport. Moreover, a tomato mutant without BSEs has reduced leaf hydraulic conductance (Zsögön et al. 2015). Additional evidence for a role of BSEs in water transport came from studying the response of stomata to changes in water availability. Before stomata close as a result of a rapid increase in evaporative demand or reduced water supply, they tend to open briefly as a result of a loss of turgor in the surrounding tissues that normally provide back pressure to the guard cells. Buckley et al. (2011) showed that such "wrong-way responses" are much stronger in leaves with BSEs, and further modeling suggested that hydraulic conductance was between 4 and 16 times higher in heterobaric leaves. The higher hydraulic conductance in heterobaric leaves could also be a result of other anatomical traits influencing hydraulic conductance, such as the size of mesophyll cells, which covary with presence of BSEs (Buckley et al. 2015).

The hydraulic resistance of the pathway through the mesophyll is substantial, but the effect of anatomy on this part of the pathway is still poorly understood (Sack et al. 2015). If most of the water evaporates from mesophyll cells that are close to the stomata, connectivity between mesophyll cells would be an important factor affecting hydraulic conductance, as the lost water is replenished by well-connected cells. It can also be expected that hydraulic conductance correlates negatively with the distance between veins and the epidermis. On the other hand, if most of the water evaporates from mesophyll cells that are close to the bundle sheath, water transport through the gaseous space would be more significant and hydraulic conductance would be expected to also correlate with leaf porosity (Buckley 2015; Buckley et al. 2015). Some studies have indeed found a negative correlation between leaf thickness and hydraulic conductance (Brodribb et al. 2007), but the opposite has also been reported (Aasamaa et al. 2001; Sack et al. 2003; Sack and Frole 2006). Buckley (2015) and Buckley et al. (2015) developed an analytical framework to study water transport outside of the bundle sheath and highlighted the importance of the gas phase for water transport. Mesophyll porosity and cell connectiv-

ity were key parameters influencing water conductance (Buckley et al. 2015). In addition to water transport via BSEs and between parenchymatous mesophyll cells, lignified cells outside the xylem occur in many species and are hypothesized to play an additional role in water transport between veins and epidermis (Brodribb et al. 2010). The paraveinal mesophyll of the Fabaceae (legumes) could also play a role in water transport. This tissue consists mainly of mesophyll cells, but sometimes partially or even completely of bundle-sheath cells (Kevekordes et al. 1988; Brubaker and Lersten 1995; Leegood 2008). In addition, at least in *Glycine max*, there is substantial evidence that paraveinal mesophyll plays a role in photosynthate transport, nitrogen metabolism, and protein storage (Brubaker and Lersten 1995).

Recent work suggests that the leaf water status itself serves as a feedback on hydraulic conductance (Scoffoni et al. 2014). Leaf dehydration is known to result in tissue shrinkage and mesophyll cells in dehydrated leaves are often deformed (Fellows and Boyer 1978; Canny and Huang 2006; Sancho-Knapik et al. 2011; Canny et al. 2012); such changes are likely to alter mesophyll porosity and cell connectivity resulting in an effect on leaf hydraulic conductance (Scoffoni et al. 2014).

E. Mechanical Function

Given the primary function of many leaves, it may seem that leaf anatomy has been exclusively optimized for photosynthesis. However, leaves allocate significant mass to non-photosynthetic structural components, such as cell walls, that account for between 10 and 70% of the leaf dry mass (see Chap. 17; Onoda et al. 2011). Such high allocation of dry mass to structural components indicates that mechanical stability is an important function of leaves. In this section, we describe how leaf anatomy is important for leaf mechanical functions.

Leaves are typically thin, flat, and often maintained horizontally, which is ideal to maximize light interception per unit leaf mass and facilitates the diffusion of CO_2 to the chloroplasts as discussed previously. However, such a structure increases the risk of mechanical failure because the horizontal alignment maximizes the bending moment of a leaf with its own weight and also because thin leaves are more vulnerable to external forces, such as wind, rainfall, abrasion, pathogen attacks, herbivory, and trampling. Leaves must therefore be mechanically "tough" in order to survive physical disturbance. Long-lived leaves (e.g., evergreen leaves) often have a tough structure and allocate relatively more leaf mass to structural components. Such an increase in allocation to structural components would increase leaf longevity, which in turn allows for more carbon gained per unit of carbon originally invested in the leaf over the leaf lifespan. However, higher investment in structural components often constitutes a trade-off with instantaneous photosynthetic efficiency because of lower resource allocation to the photosynthetic apparatus and lower CO_2 diffusion conductance due to thicker cell walls (See section IIIB2, Chap. 16 and Onoda et al. 2017).

Different types of mechanical properties are important to withstand or avoid various types of mechanical stresses (Niklas 1992; Wright and Vincent 1996). For example, bending stiffness is important to maintain leaf structure against gravity or under moderate wind (e.g., Onoda et al. 2015), shear strength is important to protect against insect herbivores that feed on leaf tissues by chewing (e.g., Sanson 2006), tensile strength may avoid damage resulting from mammalian grazers who forage leaves by pulling (e.g., Vincent 1982), and surface hardness is important for defense against the initial attacks of herbivores (e.g., Grubb 1986) and to avoid damage related to rubbing between leaves (Wilson 1984). Finally, the cellular bulk modulus of elasticity determines to

what extent cells are deformed under desiccation stress (e.g., Tyree and Hammel 1972). These different mechanical properties may be achieved by differences in the amount and quality of cell walls, leaf thickness, density, cell size, the amount and arrangement of particular tissues (sclerenchyma), and so on (Grubb 1986; Wright and Illius 1995; Wright and Vincent 1996). There are also leaves that have special mechanical properties, such as spines, thorns, prickles, and silica accumulation, which have been reviewed elsewhere (Raven 1983; Read and Stokes 2006).

Thick cell walls are often found in the epidermis, vascular tissues, and BSEs, whereas palisade and spongy tissues have relatively thin cell walls. Cell wall thicknesses of these thick-walled tissues are often 5 to 10 times thicker than those in mesophyll tissues, e.g., 0.5–10 μm for epidermal cell walls (Onoda et al. 2015) compared to 0.1–1 μm for mesophyll cell walls (Terashima et al. 2011; Tosens et al. 2016). The external cell wall of the epidermis is thicker than the internal wall. Cuticle layers are deposited on top of the external wall, thereby hardening the leaf surface (Wiedemann and Neinhuis 1998; Onoda et al. 2012).

Vascular tissues need to have rigid cell walls to resist negative water potentials. Cell wall thickness per unit conduit diameter is tightly associated with embolism resistance (Hacke et al. 2001). Furthermore, although the main function of veins is related to water and nutrient transport, major veins (and petioles) also play a role as scaffold in maintaining leaf orientation and structure (Roth-Nebelsick et al. 2001). In addition, the veins provide a higher shear resistance than leaf lamina, which reduces insect herbivory often targeting soft leaf lamina tissues (Lucas et al. 1991). The thin cell wall of the spongy and palisade tissue facilitates diffusion of CO_2 into the cells (see section IIIB). However, given the substantial variation in mesophyll cell wall thickness, thicker walls may have advantages under some conditions even at the cost of lower CO_2 diffusion rates. For example, thicker walls are thought to prevent excessive shrinkage of leaves under drought that would negatively affect hydraulic conductance (Scoffoni et al. 2014). In addition, thicker walls could serve as a better defense against herbivores, especially leaf miners that selectively feed on mesophyll tissue.

In addition to the mechanical function of each individual tissue, the combination of multiple tissues affects the mechanical properties of the whole leaf. As described earlier, in flattened leaves, the leaf lamina is composed of two epidermal layers with mesophyll tissue in between. This anatomy is analogous to the "sandwich structure" used in engineering applications, such as airplane wings and surfboards (Gibson et al. 1988). A typical sandwich structure is composed of stiff outer surfaces and a lightweight core, which greatly increases specific stiffness (stiffness per unit mass) for bending. High bending stiffness with a minimum leaf mass would be ideal to increase the capacity to maintain large flat leaves horizontally and thus maximize light capture per unit dry mass (Gibson et al. 1988; Niinemets and Fleck 2002). A recent study showed that leaves across a range of species have indeed stiff epidermises and soft mesophyll tissues, forming efficient sandwich structures (Fig. 5.8, Onoda et al. 2015).

BSEs in heterobaric leaves and turgor pressure in homobaric leaves keep the two epidermal layers some distance apart, helping to maintain the lamina bending stiffness and preventing local buckling, because bending stiffness is proportional to the third power of lamina thickness (Gere and Timoschenko 1999). In addition, turgor pressure also serves to "control" lamina thickness such that a 20% reduction in leaf water content resulted in similar (~20%) or even greater (35%) reduction in lamina thickness in a deciduous woody species (Sancho-Knapik et al. 2011). If leaf thickness decreases by 20%, bending stiffness would decrease by 49% ($(1 - 0.2)^3 = 0.51$). Therefore, a small

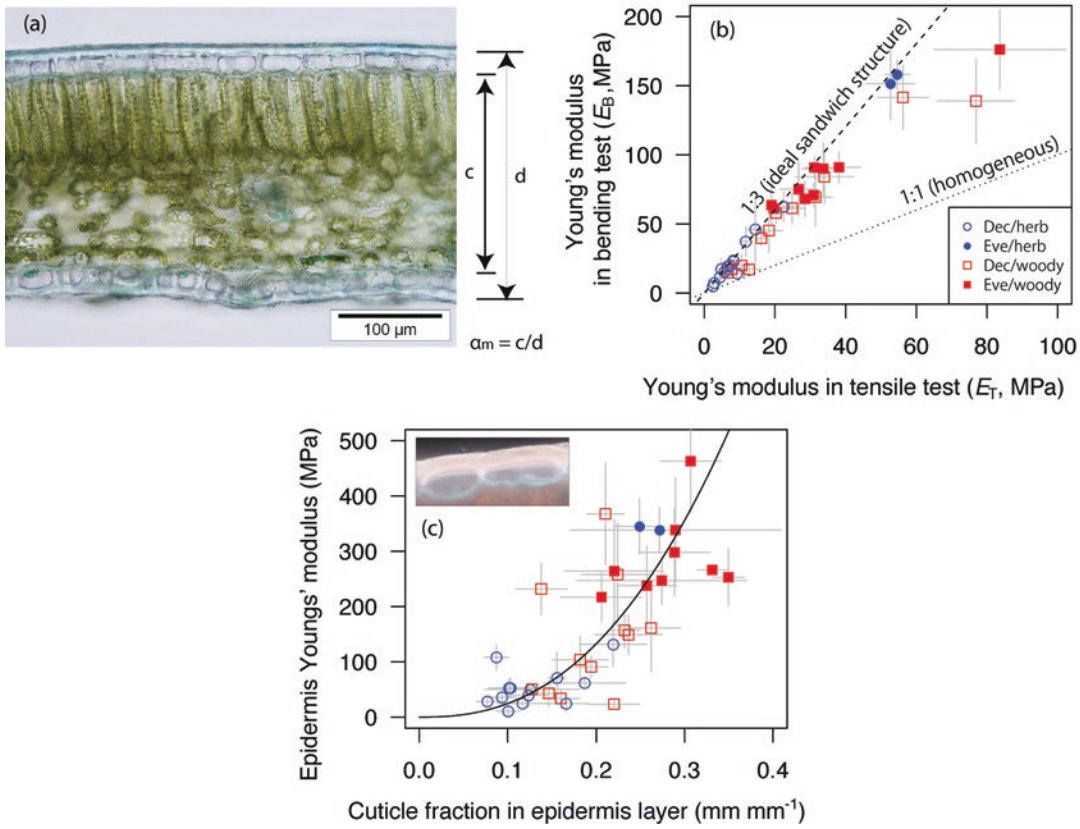

Fig. 5.8. The leaf "sandwich" structure and its importance to mechanical functions. (**a**) Typical leaf cross-section. The leaf lamina consists of an upper and lower epidermis with mesophyll tissue in-between. This resembles a composite material used in engineering construction (e.g., in airplane wings), where high bending stiffness in combination with minimum weight is important. The Young's modulus of the lamina can be expressed as the products of the Young's modulus of the epidermis (E_f) and that of the mesophyll (E_c) weighted by the thickness fraction of the mesophyll (α_m). The Young's modulus measured by a tensile test (E_T) can be expressed as $E_T = (1 - \alpha_m)E_f + \alpha_m E_c$ and that measured by a bending test (E_B) can be expressed as $E_B = (1 - \alpha_m^3)E_f + \alpha_m^3 E_c$. From these two equations, E_B/E_T can be expressed as $\frac{E_B}{E_T} = \frac{1-\alpha_m^3(1-\beta)}{1-\alpha_m(1-\beta)}$ where β is E_c/E_f. If the epidermis is much stiffer than the mesophyll (i.e., $\beta \sim 0$), $E_B / E_T \sim 1 + \alpha_m + \alpha_m^2$, which approaches 3 for leaves with a thin epidermis. (**b**) The relationship between E_B and E_T was examined for 36 angiosperm species. Many species aligned closely to the 1:3 line, indicating that many leaves have an efficient sandwich structure. (c) The variation in epidermis stiffness correlates with the fraction of the leaf comprised of cuticle and outer cell walls of the epidermis, suggesting that leaf cuticles are made of stiff material. (Figures are redrawn from Onoda et al. 2015)

change in leaf water content greatly affects the leaf bending stiffness when leaf thickness is controlled by turgor pressure. Such a reduced stiffness allows leaves to wilt and avoid strong light under water stressed conditions (Zhang et al. 2010).

Intercellular airspace can also contribute to bending stiffness by increasing the lamina thickness per unit dry mass, i.e., leaves with more airspace can be thicker for a given amount of leaf mass (Onoda et al. 2008). A higher volume fraction of intercellular airspace in shade leaves compared to sun leaves can be explained by mechanical requirements rather than by photosynthesis requirements as described above, because improved CO_2 diffusion conductance is not a limiting factor in shade leaves (Parkhurst 1977;

Terashima et al. 2001). The volume fraction of intercellular airspace in the mesophyll of shade leaves can be 10–30% more than in sun leaves (Piel et al. 2002; Onoda et al. 2008). Following a similar logic as for turgor-controlled leaf thickness, a 20% greater lamina thickness due to increased intercellular space can translate into a 73% higher lamina bending stiffness without adding biomass ($1.2^3 = 1.73$). This rough quantification suggests the importance of intercellular airspace in maintaining a larger light interception area for a given mass in shaded environments.

F. Functions of the Leaf Surface – The Role of Trichomes

1. Trichome Morphology

Outward growths of the shoot epidermis are often referred to as trichomes (Werker 2000). Trichomes are either unicellular or multicellular appendages, and can be found on various plant organs. Here, we will focus on leaf trichomes. There is an enormous diversity in trichome morphology, dimension, position, microstructure and capacity to secrete (Fig. 5.9). Trichomes can be unicellular and unbranched (a simple one-celled hair), while uni/multicellular branched trichomes are also common, such as the star-shaped hairs of *Arabidopsis thaliana*. Other common trichomes are the scale and peltate hairs, which are shield-shaped and usually attached to the plant surface by a short stalk. Some trichomes are capable of secretion (Wagner et al. 2004). These so-called glandular trichomes may secrete various kinds of chemicals such as terpenoids and phenylpropanoids depending on the species, and are important for plant-animal interactions (Wagner et al. 2004).

The density of trichomes varies extensively across and also within species and even within a single individual or organ. Plants growing in arid or sunny environments often show higher trichome densities, but many other environmental factors are thought to play a role (Johnson 1975; Pérez-Estrada et al. 2000; Gregoriou et al. 2007). It is important to note that such plastic changes in the density of trichomes could be strongly associated with an ontogenetic change in leaf area (Roy et al. 1999).

2. Trichome Functions

Many functions of leaf trichomes have been proposed, including but not limited to a role in increasing water use efficiency (Nobel 2009), light reflection (Ehleringer and Björkman 1978), temperature control (Ehleringer and Mooney 1978), water absorption (Schmitt et al. 1989), as water or dust repellent (Barthlott et al. 2010), as a defense against herbivory (Levin 1973; Dalin et al. 2008), or providing a more suitable environment for leaf-inhabiting arthropods (Voigt et al. 2007).

Trichomes, especially on the abaxial side of hypostomatic leaves, can increase the thickness of the leaf boundary layer, thus reducing the rate of water loss in water-limited habitats (Nobel 2009). For example, Ehleringer and Mooney (1978) estimated that a trichome layer thickness of 0.35 mm can increase the boundary layer thickness by up to 50% in a desert plant (*Encelia farinosa*). However, Benz and Martin (2006) analyzed 12 *Tillandsia* species whose trichome layer thickness varied between 0.052 and 0.5 mm and concluded that the contribution of trichomes to the leaf boundary layer thickness was at most 9.7%. The relative contribution of leaf trichomes to the total resistance for H_2O diffusion may be small or negligible because the boundary layer resistance is usually much smaller than stomatal resistance (Amada et al. 2017). Nonetheless, there are a number of studies that reported positive correlations between trichomes and water use efficiency (e.g,. Vitousek et al. 1992; Ichie et al. 2016). It is likely that several leaf characteristics such as low stomatal conductance, high nitrogen content, and

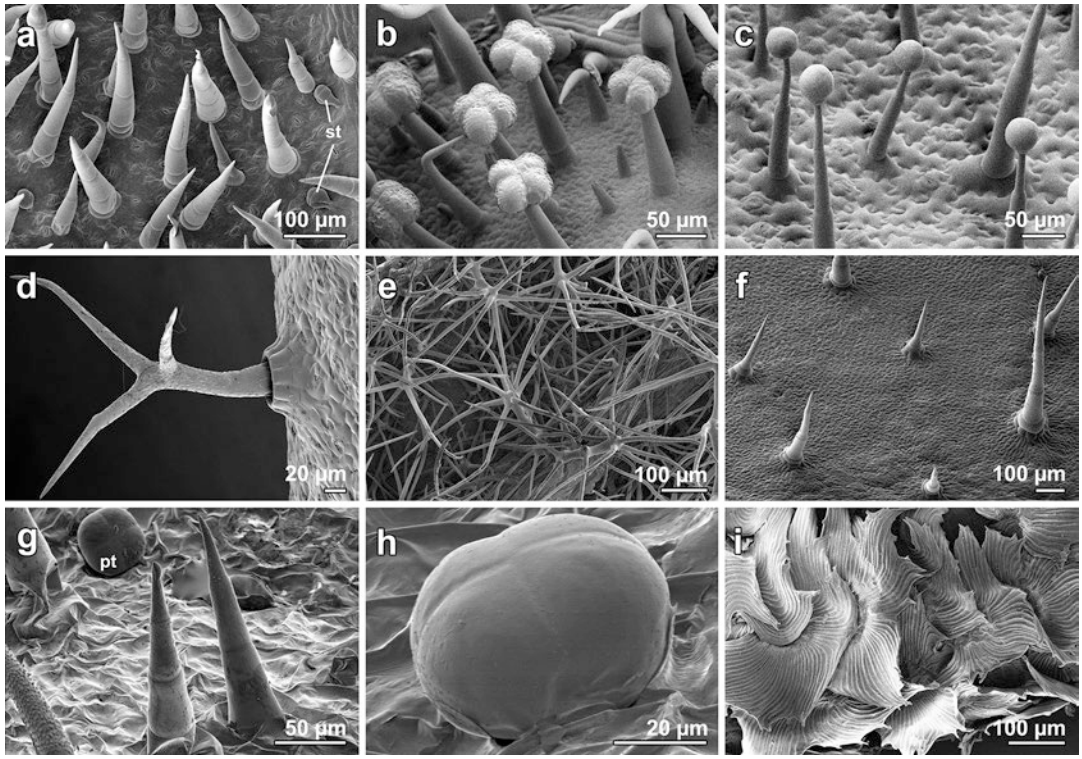

Fig. 5.9. Cryo-scanning electron micrograph images of pubescent abaxial leaf surfaces. (**a**) *Lagenaria siceraria* (Molina) Standl. densely covered with multicellular, uniseriate, tapered trichomes. (**b**) Capitate and cone-shaped trichomes on *Solanum lycopersicon* L. (**c**) Capitate, multicellular, uniseriate trichomes on *Nicotiana sylvetris* Speg. & Comes. (**d**) Unicellular, branched trichome of *Arabidopsis thaliana* L. (**e**) *Verbascum thapsus* L., densely covered with multicellular, branched trichomes. (**f**) *Cucumis sativus* L. bears multicelluar, uniseriate, tapered trichomes with multicellular socket and corrugated surface. (**g**, **h**) *Columnea sanguinea* (Pers.) Hanst. is equipped with cone-shaped, tapered, multicelluar and peltate trichomes; detail of a four-cellular peltate trichome in **h**. (**i**) A dense coverage of peltate trichomes found on *Tillandsia usneoides* L. (Courtesy of Dr. Dagmar Voigt, Technische Universität Dresden, Institute for Botany, Dresden, Germany)

thicker leaves may be coordinated with trichome density to increase water use efficiency in water-limited environments (see Cordell et al. 1999; Wright et al. 2003).

Some leaves have trichomes that reflect a certain fraction of solar irradiance. Reflecting strong irradiance may be important to prevent overexcitation of the photosynthetic apparatus, avoid over-heating of leaves, and maintain favorable leaf temperature for photosynthesis (Ehleringer and Björkman 1978; Skelton et al. 2012). Some trichomes can also effectively reflect or filter ultraviolet light (Liakopoulos et al. 2006).

Certain trichomes, particularly scale hairs, can absorb water. A notable example is found in *Tillandsia* species (and other related members of the Bromeliaceae) that utilize elaborate, peltate trichomes that transfer water from the leaf surface into the mesophyll tissue, while their root structures function solely as holdfasts incapable of water absorption (Schmitt et al. 1989). Similarly, it is known that trichomes can absorb nutrients as well as water (e.g., pineapple, Sakai and Sanford 1980). Konrad et al. (2015) proposed that trichomes can promote water condensation and increase humidity at the leaf surface. This would allow for stomata to remain open for longer periods during the day, thereby increasing

the length of the effective photosynthetic period.

The role of trichomes as a defense mechanism against herbivory and pathogen attacks is well-known (reviewed by Levin 1973; Dalin et al. 2008). Many studies found that an increased trichome density is related to a decrease in herbivory or tissue damage (Dalin et al. 2008).

IV. Acclimation and Adaptation

A. Responses of Leaf Anatomy to Light

As explained in the previous sections, leaves develop under a certain condition can have various anatomical functions. However, plants experience a constantly changing environment. Because plants are immobile, the ability to adjust their leaf anatomy to environmental differences is vital for survival and successful competition with other plants (e.g., Oguchi et al. 2006, 2017). The light environment in particular varies significantly: for example, the light intensity above the leaf canopy of a tropical rain forest can be more than 100 times higher than that in the understory (Chazdon and Fetcher 1984; Onoda et al. 2014). Sun-acclimated leaves are generally thicker and have a higher nitrogen content, photosynthetic protein content, and photosynthetic capacity per unit area compared to shade leaves. A simplified explanation of these differences is that chloroplasts, as the main site of the photosynthetic process, require large amounts of nitrogen for their construction (Terashima and Hikosaka 1995). Under low light conditions, it would be inefficient to have too many chloroplasts because this would result in many chloroplasts (especially those on the shaded side of the leaf) unable to receive enough light to cover their cost of construction and maintenance by photosynthesis. On the other hand, under high light conditions, if the number of chloroplasts per unit leaf area is insufficient, the leaves lose some proportion of the available energy. Sun leaves need to be thicker to achieve a large chloroplast surface area facing intercellular spaces per unit leaf area (S_c), high mesophyll conductance (g_m), and high photosynthetic capacity (see Terashima et al. 2001 and 2006 for details).

The formation of palisade tissue strongly depends on the light environment (Haberlandt 1914). Modeling has indicated that maximal photosynthetic rates at a given light intensity can be achieved with a specific ratio between palisade and spongy tissues (Tholen et al. 2012a). Yano and Terashima (2001) showed that the anatomy of developing leaves is not controlled by their own light environment, but by the light environment of already expanded leaves (Fig. 5.10). This long-distance control of leaf anatomy was also seen in C_4 plants (Jiang et al. 2011). Munekage et al. (2015) recently showed that locally perceived blue light influenced palisade elongation, but a long-distance signal (induced by high light and oxidative stress) in mature leaves increased palisade tissue cell densities in *Arabidopsis thaliana*. In isolateral and resupinate leaves, the development of palisade tissue does not depend on the adaxial origin of the tissue as it can also develop from the abaxial meristem (Hofreiter and Lyshede 2006, e.g., Fig. 5.1e). In addition, in the scale leaves of plagiotropic *Thuja plicata* shoots, palisade tissue can develop on any light-exposed side of the leaves irrespective of the ab- or adaxial origin of the tissue (Dörken 2013). Although the direction of light seems to be the most important signal for the elongation of mesophyll cells, temperature, CO_2 concentration, and gravity may also play a role (Imamura 1931; Leadley et al. 1987; Pino et al. 2008; Dumlao et al. 2012). In addition, genetic factors are also important in acclimation. For example, leaves of shade-tolerant plants generally have only a single layer of palisade tissue, exhibiting no propensity to develop multilayered palisade parenchyma even under

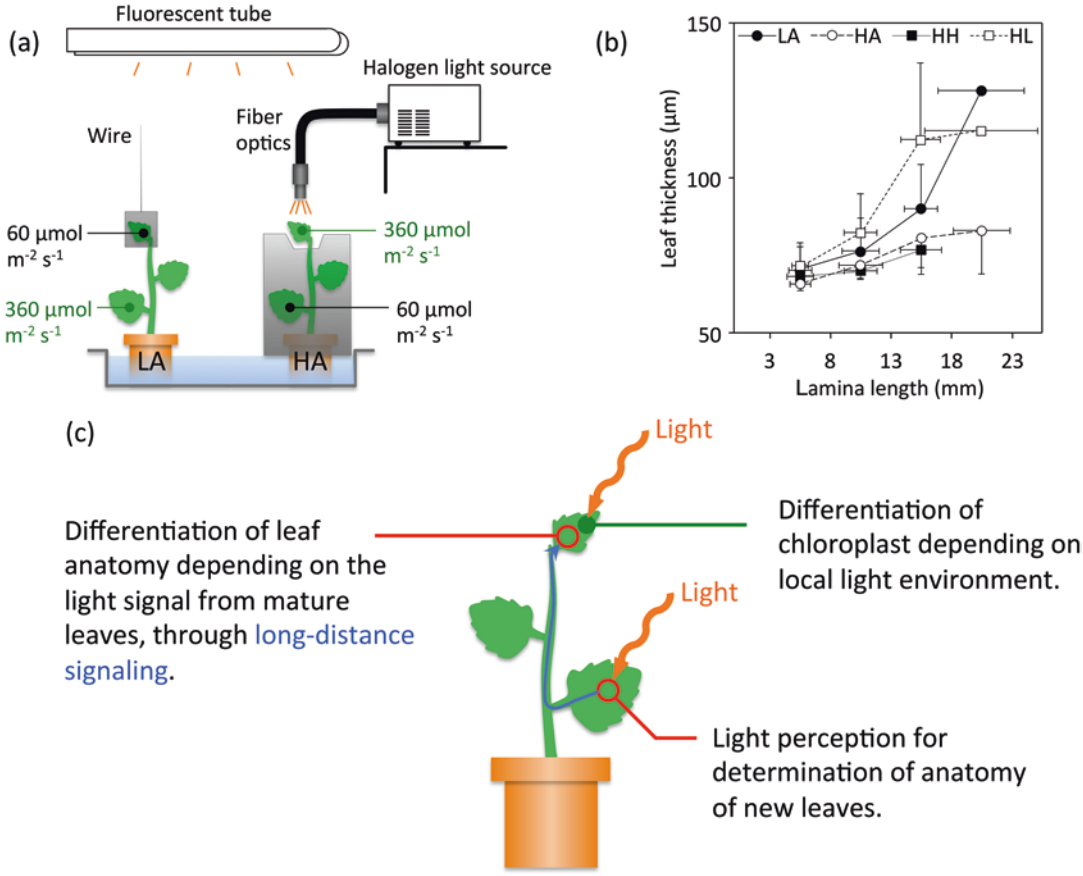

Fig. 5.10. (**a**) Light treatment design, (**b**) responses of leaf thickness to different light treatments, and (**c**) a model scheme of light sensing mechanisms for developing leaves following Yano and Terashima (2001). Low-light apex (LA) treatment and high-light apex (HA) treatment are left and right, respectively, in panel (**a**). In the LA treatment, only the shoot apex was covered by a shading screen. In the HA treatment, the shoot apex was illuminated with a halogen lamp through fiber optics, but the other parts of the plant were covered by a shading screen. In the HH treatment, whole plants were grown under high-light conditions (360 μmol photons m^{-2} s^{-1}). In the HL treatment, whole plants were transferred to low-light conditions (60 μmol photons m^{-2} s^{-1}). One month old high light grown plants were transferred to the above conditions and studied after 6 days. In panel (**b**), the vertical and horizontal bars indicate standard deviations (n = 1 to 8)

sunny conditions (Murchie and Horton 1997; Pons and Poorter 2014).

Chloroplasts also show sun/shade acclimation (Ballantine and Forde 1970; Skene 1974). Chloroplasts that developed under high light conditions had smaller granal stacks, larger stromal space, higher chlorophyll a/b ratios, higher Rubisco/chlorophyll ratios, and thus higher photosynthetic rates per unit chlorophyll at light saturation compared to chloroplasts that developed under low light conditions (Terashima and Inoue 1985; Pearcy and Seemann 1990; Nishio et al. 1993). On the other hand, shade-acclimated chloroplasts have a higher amount of chlorophyll per photosystem. These differences are observed even within a single leaf dependent on the internal light gradient (Terashima and Inoue 1984). Although some of the leaf anatomical traits described in sec-

tion IIIA reduce the steepness of the light gradient, a difference in light intensity within the leaf is inevitable. Therefore, chloroplasts near the light-exposed side and near the shaded side show sun and shade traits, respectively, matching their photosynthetic metabolism with light availability (Evans 1999).

Plant responses to light availability can be considered at different time-scales; seconds (sunflecks), hours (daily changes), months (seasonal changes), and years (gap formation in the canopy). For understory plants, disturbances of the canopy as a result of wind-damage, disease, herbivory, or logging cause a sudden increase in irradiance. In response to such changes in light intensity, various biochemical and anatomical traits are adjusted both in the existing fully developed and newly expanding leaves (Björkman 1981; Pearcy and Sims 1994). The acclimation mechanism of newly expanding leaves is almost the same as the plasticity between sun and shade leaves described above; however, the acclimation mechanism of already expanded leaves can be restricted by anatomical constraints. It has been shown that once leaves have developed in shade, transfer to a higher growth light environment does not generally result in an increase in their photosynthetic capacity to the level of sun leaves that developed under sunny conditions (Jurik et al. 1979; Sims and Pearcy 1992; Frak et al. 2001; but see Amiard et al. 2005). This restriction is mainly caused by the inability of mature leaves to change leaf thickness (Milthorpe and Newton 1963; Wilson 1966; Sims and Pearcy 1992). As described in section IIIB, chloroplasts are located near mesophyll cell walls to efficiently receive CO_2 from the intercellular airspace. When the mesophyll cell surface is fully occupied by chloroplasts, leaves exposed to higher light conditions do not increase the volume of chloroplasts and photosynthetic capacity does not increase (Fig. 5.11a, b; Oguchi et al. 2005). In contrast, when the mesophyll cell surface is not fully occupied by chloroplasts, leaves of some species are able to increase the volume of chloroplasts under high light and correspondingly achieve higher photosynthetic capacities (Fig. 5.11c, d, Oguchi

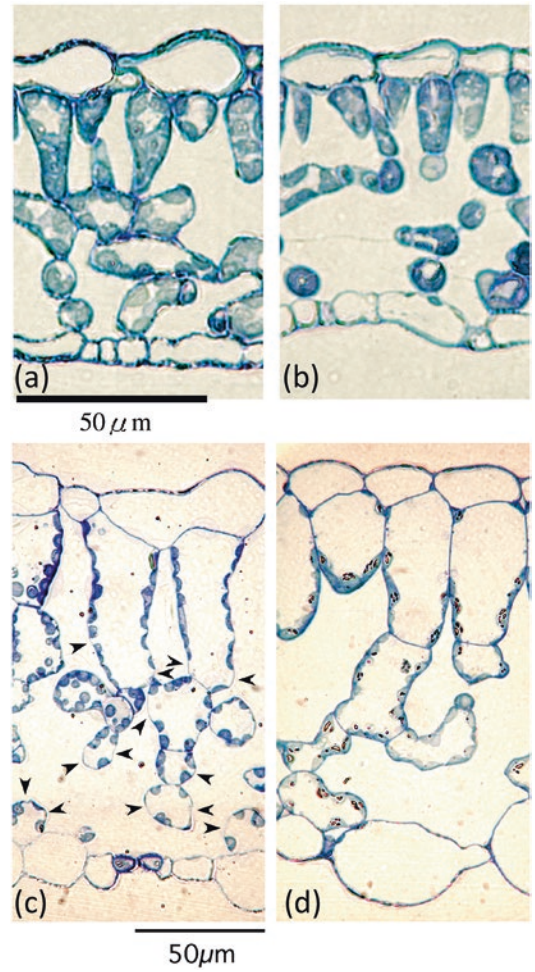

Fig. 5.11. Leaf cross-sections of mature leaves of *Fagus crenata* (**a**, **b**) and *Chenopodium album* (**c**, **d**) grown continuously in low light (LL = 70 μmol photons m^{-2} s^{-1}) (**a**, **c**) and transferred from low to high light conditions (LH = 350 μmol photons m^{-2} s^{-1} for *F. crenata* and 700 μmol photons m^{-2} s^{-1} for *C. album*) (**b**, **d**). Light micrograph magnification is 400 times. The depth of the cross-section was 0.8 μm. In *F. crenata* (**a**, **b**), because there was no vacant space near the mesophyll cell surface, the leaves that were transferred from low to high light were unable to increase the chloroplast volume per leaf area (Oguchi et al. 2005). In *C. album*, arrowheads show the available open space for chloroplasts, which allowed for an increase in chloroplasts per leaf area in response to an increase in irradiance. (Oguchi et al. 2003)

et al. 2003). Interestingly, in a small number of species, fully expanded leaves can still change leaf thickness or mesophyll cell length, resulting in an increased mesophyll surface area in response to a change in irradiance (Gauhl 1976; Bunce et al. 1977; Bauer and Thoni 1988; Kamaluddin and Grace 1992; Oguchi et al. 2005, 2006). Such species may sacrifice some traits, such as leaf toughness and thereby herbivory tolerance, but retain more flexible cell walls that allow for growth after maturation.

The light environment is perceived by a number of photoreceptors (light-receptors). So far, the following three photoreceptor categories have been discovered in plants: phytochrome is a pigment-protein activated by red light and deactivated by far-red light (Hendricks 1960; Smith 2000). Because sunlight transmitted through canopies is enriched with far-red light compared to red light, the phytochrome activation state enables plants to sense the level of shading. Cryptochrome is a blue light receptor identified by Cashmore and colleagues (Ahmad and Cashmore 1993; Cashmore et al. 1999) involved in the control of blue light inhibition of stem elongation and anthocyanin synthesis. Phototropins, blue-light receptors identified by Briggs and colleagues (Briggs and Huala 1999; Christie et al. 1999), are involved in the control of chloroplast movement (Wada 2013) and stomatal opening (Shimazaki et al. 2007) and may be also involved in the leaf positioning and flattening (Ohgishi et al. 2004; Harada et al. 2013). Recently ZEITLUPE and rhodopsins were also suggested to be active as photoreceptors in higher plants (Kim et al. 2007; Möglich et al. 2010; Atamna-Ismaeel et al. 2012). The signal transduction pathways directly related to these receptors are well studied (Kami et al. 2010; Casal 2013). However, the relationships between these receptors and leaf anatomy, especially with regard to long distance signaling from already expanded leaves to the newly expanding leaves (Fig. 5.10), remain little understood. Thus far, it has been suggested that hormones, ROS, sugar levels, miRNA, or expansin might be the factors responsible for such long distance signaling (Karpinski et al. 1999; Pien et al. 2001; Stessman et al. 2002; Palatnik et al. 2003).

B. Responses of Leaf Anatomy to Temperature

Compared to the acclimatory adjustments in foliar anatomy that have been documented in response to the light environment, there have been fewer reports with regard to responses to temperature. Most of the available studies report that leaves grown at lower temperatures are thicker than leaves grown in higher temperature (Boese and Huner 1990; Gorsuch et al. 2010; Dumlao et al. 2012; Stewart et al. 2016), though leaf thickness may increase when temperature is extremely high (Ben Salem-Fnayou et al. 2011, see also section IIIC). In addition, the amount of palisade tissue increased in winter-active mesophytes in response to lower temperatures (Dumlao et al. 2012). The fact that these differences parallel those between sun and shade leaves may not be coincidental because the photosystems in the chloroplast detect low temperature and high light by a comparable mechanism (Huner et al. 1998). Huner et al. (1998) proposed that both low temperature and high light cause an imbalance in the energy absorbed versus energy utilized, increasing the reduction state of plastoquinone (excitation pressure), the pH gradient across the thylakoid membrane, the production of reactive oxygen species, and depletion of phosphate. This altered chloroplastic redox balance can initiate a signal transduction pathway. Similar responses to cold temperatures and high light can be also seen at the chloroplast level. For example, Muller et al. (2009) showed that a seasonal decrease in temperature is linked to an increase in the volume of chloroplasts in evergreen species, which is similar to the previously discussed increase in the number of chloroplasts in

response to an increase in irradiance. Temperature can change the fluidity of membranes and affects protein folding, which have been suggested to act as cues for temperature-induced changes in plants (Ruelland and Zachowski 2010). Calcium is one suggested candidate as a signaling molecule involved in the transduction from perception of temperature and low water availability to plant responses to these environmental factors (Shao et al. 2008).

C. *Responses of Leaf Anatomy to Water Stress*

Plants from dry habitats often show a particular leaf anatomy called xeromorphy. The most obvious characteristic of xeromorphic leaves is a lower surface-to-volume ratio to reduce transpiration (Shields 1950), such as seen in small leaves with inwardly-rolled margins (e.g., *Erica* spp.; Metcalfe 1979). Sometimes the morphologically adaxial surface is reduced to a groove or completely eliminated, resulting in a centric leaf (Metcalfe 1979). Other typical traits are a decreased cell size and thicker cell walls, in particular the outer periclinal walls of the epidermis. Hairy leaves may sometimes increase boundary layer resistance, but ecological studies have found limited support for this notion (see section IIIF). Sclereid idioblast and lignified BSEs often occur in xeromorphic leaves, possibly playing a role in preventing tissue damage under drought (Metcalfe 1979). A meta-analysis of variation in leaf dry mass per area (LMA) showed that an increase in LMA is weakly linked to a decreased water availability in a wide range of plant species from various functional groups and habitats (Wright et al. 2004, Poorter et al. 2009). Higher mesophyll thickness in plants adapted to Mediterranean-type climates is also considered to be a water stress response (Peguero-Pina et al. 2012, 2017). This negative correlation between leaf thickness or LMA and annual precipitation can also be observed within species (Castro-Diez et al. 1997; Wang et al. 2011). The increase in LMA responding to water stress is often reported to be associated with an increase in leaf density (Groom and Lamont 1997; Bosabalidis and Kofidis 2002; Bussotti et al. 2002; Bacelar et al. 2006, 2007). However, avocado cultivars exhibited a decrease in mesophyll thickness and LMA in response to water stress (Chartzoulakis et al. 2002) and, in the case of tomato and grape cultivars, the response of mesophyll thickness and leaf density to water stress differed among accessions (Scippa et al. 2004, Galmés et al. 2013, Tomás et al. 2014). More detailed analyses of acclimation of mesophyll cell anatomy, such as changes in the thickness of cell walls and anatomy of the vascular system, are required. See the Anatomy and water transport section (section IIIB) for the contribution of the vascular system to water demand.

V. Conclusions

In the present chapter, we discussed relationships between leaf anatomy and various functions, including light absorption, CO_2 diffusion, water transport, transpiration, and mechanical strength. We also reviewed how leaf anatomy varies depending on species and environments. This suggests that leaf anatomical functions cannot all be optimized simultaneously, because (a) there are tradeoffs between the associated anatomical features that are caused by physical, chemical, and biological constraints, (b) specific anatomical traits may affect multiple functions with contrasting requirements, and (c) plants continuously experience a changing environment.

Some anatomical traits may covary. For example, thinner cell walls and greater leaf areas per unit leaf dry mass are often accompanied by a higher porosity and lower tortuosity of leaves, all of which increases mesophyll conductance. Traits that have been primarily selected for one function

could also find use for additional purposes in some species. For example, though BSEs may have evolved primarily as a structure to provide mechanical stability, they may later have been adopted as more efficient water conduits or as transparent light-guides to improve the light gradient in leaves. These complicated relationships often make it difficult to observe or analyze the function of single anatomical traits.

Elucidation of each relationship, especially covariation and trade-offs, among anatomical and physiological functions is required. Knowledge about the developmental mechanisms controlling leaf anatomy in response to the environment is still limited. Progress in these fields will be a key step to understand the factors underlying global scale correlations among leaf traits, plant strategies, and environments, as shown in the leaf economic spectrum studies. This information is also indispensable for breeding or producing plants that have certain human-desired properties. We expect the recent rapid development of bio-imaging technology to help provide some of this information and this will allow further progress in the field of leaf functional anatomy.

Glossary

abaxial Facing away from an axis (i.e., from the stem), often in reference to the lower side of a leaf.

adaxial Facing towards an axis (i.e., toward the stem), often in reference to the upper side of a leaf.

amphistomatous, amphistomatic Stomata are found on both the adaxial and abaxial sides of the leaf (often in different densities).

armed mesophyll Leaf mesophyll characterized by parenchyma cells that have protrusions (lobes) or with cell walls that have invaginations.

armed palisade Armed mesophyll characterized by lobes that are arranged perpendicular to the leaf surface, similar to regular palisade mesophyll.

bifacial leaf A leaf that develops from both adaxial and abaxial meristems.

bundle-sheath extension (BSE) Groups of cells that extend from the bundle sheath towards the upper and lower epidermis. These cells contain no chloroplasts and have cell walls that are typically thickened or lignified.

bundle sheath A tightly connected file of cells, that wholly or partially surround the vascular tissue. Bundle sheath cells are typically parenchymatous, but may sometimes become lignified (see: endodermis).

C_3 plants Plants in which the first organic acid that is produced from CO_2 by photosynthetic metabolism (3-phosphoglyceric acid) has 3 carbon atoms.

C_4 plants Plants in which the first organic acid that is produced from CO_2 by photosynthetic metabolism (oxaloacetic acid) has 4 carbon atoms.

capitate Having a distinct globular end.

centric leaf See terete leaf.

dorsiventral Along the axis connecting dorsal and ventral sides (e.g., from adaxial to the abaxial side of a leaf).

dorsiventral mesophyll The mesophyll has dissimilar dorsal (i.e., facing towards the stem) and ventral (i.e., facing away from the stem) sides.

endodermis Bundle-sheath consisting of cells with primary walls that are characterized by localized impregnation with lignin.

ensiform leaves Sword-shaped leaves; typically with a lamina that is flattened in the median plane.

epidermis One (or sometimes several) layers of tightly-connected cells that cover the surface of a plant organ. Originating from the primary meristem.

epistomatous, epistomatic Stomata are exclusively found on the adaxial side of the leaf.

fusoid cells Distinct, colorless cells located between the veins. The cells are fusiform or pyriform when viewed in transverse sections, but elongated in mediolateral and longitudinal directions and thus have more plate-like

shapes when viewed in three dimensions. There are typically large intercellular spaces between two consecutive fusoid cells.

fusiform Spindle-shaped; tapering towards each end.

heterobaric A leaf property indicating that a large fraction of the veins have bundle sheath-extensions that extend to the epidermis.

homobaric A leaf property indicating that no or only a few veins in the leaf have bundle-sheath extensions.

hyperstomatous, hyperstomatic See epistomatous.

hypostomatous, hypostomatic Stomata are exclusively found on the abaxial side of the leaf.

idioblast A single cell that is conspicuously different from the surrounding tissue.

isobilateral mesophyll See isolateral mesophyll.

isodiametric Having a nearly equal diameter in all directions.

isolateral mesophyll Mesophyll that has similar dorsal and ventral sides.

lateral Along the lateral (Y-X) plane; a plane through a body or organ parallel to the longitudinal and mediolateral axes. Divides the body in a ventral and dorsal part. Compare: paradermal.

longitudinal Proximal to distal, from base to tip.

medial Along the median (X-Z) plane; a plane trough the middle of a body or organ, parallel to the longitudinal and dorsiventral axes. Divides the body in a left and right half.

medio-lateral From side to side (or from midrib to margin).

meristem Tissue consisting out of small, thin-walled living cells with typically a large nucleus and a small vacuole. Produces new cells by cell division.

mesophyte Terrestrial plants that are not specifically adapted to a very wet or very dry environment.

mesophyll Parenchyma tissues between the epidermis and vascular tissues of a leaf. Excluding the bundle-sheath and bundle-sheath extensions. If the mesophyll cells contain chloroplasts, it is also called chlorenchyma.

mestome sheath The inner sheath of a two-layered bundle sheath, with cells that are smaller in diameter and with thicker, cell walls impregnated with lignin and possibly suberin.

ontogenetic Related to the development of an organism or part of an organism.

palisade mesophyll Leaf mesophyll characterized by parenchyma cells that are elongated in a dorsiventral direction and have no obvious lobing or cell wall invaginations.

paradermal Along a plane parallel to the leaf surface, compare: lateral, medial.

paraveinal mesophyll One or more anatomically distinct layers of mesophyll tissue that consists of laterally stretched and laterally lobed parenchyma cells. Located between the minor veins of the leaf, typically between palisade and spongy paranchyma.

parenchyma Tissue characterized by living cells with thin, non-lignified cell walls. These cells are typically isodiametric and retain their ability to divide.

parenchymatous Related to parenchyma.

peltate Shield-like; a more or less flat and circular structure, with a stalk attached to the lower surface.

periclinal In a direction parallel to the surface of an organ or tissue.

plagiotropic Having a longer axis that is oblique to the vertical.

primordium An organ or tissue in its earliest recognizable stage of development.

proximal-distal See longitudinal.

pyriform Pear-shaped.

radial Arranged like the radii of a circle.

resupinate leaves Twisting of the petiole or lamina. The result is that tissue with an adaxial developmental origin becomes located facing away from the stem.

sclereid A type of sclerenchyma cell that is not very elongated (cf. fibers) and typically has many branched pits.

sclereidal cell See sclereid.

sclerenchyma Tissue characterized by dead cells that have thick, lignified secondary walls.

sclerophyllous Related to plants with thickened and hardened foliage as a result of the devel-

opment of large amounts of sclerenchyma in the leaves.

spongy mesophyll Leaf mesophyll characterized by parenchyma cells that are not elongated in a specific direction and are not closely packed.

substomatal cavity Cavity located proximal to the stoma, which is connected to intercellular air spaces.

terete leaf Tubular or cylindrical leaf.

tortuosity A measure for the geometric complexity of a porous medium. The ratio of the effective path between two points through a pore medium relative to the straight-line distance.

tortuosity factor (τ) Square of the tortuosity.

transverse Along the transverse (Y-Z) plane; a plane through a body parallel to the mediolateral and dorsiventral axes. Divides the body in a distal (e.g., towards the leaf apex) and proximal (e.g., towards the leaf base) segment.

unifacial leaf A leaf that develops mainly from either the adaxial or abaxial meristem, leading to an absence of cells derived from one of these meristems in the greater part of the mature leaf.

uniseriate Arranged in a single file, row, or layer.

vascular tissue Plant tissue that consist of tracheary elements, sieve elements, companion cells, and associated fiber, parenchyma, and meristematic cells. Typically surrounded by a bundle sheath.

vasculature See vascular tissue.

xeromorphic With morphological features that are thought to be an adaptation to dry conditions.

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Chapter 6

Coordination Between Photosynthesis and Stomatal Behavior

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Summary

Stomata open and close in response to internal and external signals to balance CO_2 uptake for photosynthesis and water loss through transpiration. For a plant to function efficiently, this balance is essential to ensure adequate CO_2 uptake for mesophyll demands and sufficient water loss to maintain transpiration and optimal leaf temperature from evaporative cooling for maximal photosynthetic performance, while also ensuring an appropriate whole plant water status. Both stomata and mesophyll respond to external and internal cues and there is a close synchrony between stomata movements and mesophyll photosynthesis. However, the mechanism(s) that co-ordinate these two responses are unknown. Here we examine evidence for a mesophyll driven signal and discuss possible candidates for such a signal. We also provide a brief review of some of the experimental approaches adopted for exploring mesophyll-stomatal interactions. We discuss a possible role for guard cell chloroplasts and guard cell photosynthesis as a mechanism for this co-ordination. Finally, we show that stomatal responses are different on adaxial and abaxial leaf surfaces, raising further questions regarding mesophyll driven signals co-ordinating behavior. We conclude that despite numerous studies, the mesophyll signal remains to be elucidated, and that further research is needed to determine the mechanisms and signal transduction pathways that facilitate the well observed correlation between mesophyll photosynthetic rates and stomatal conductance.

I. Introduction

Stomata open and close, adjusting aperture in response to environmental and internal signals that results in a trade-off between ensure sufficient CO_2 for mesophyll photosynthesis while maintaining an appropriate plant water status. In general, stomata open in response to increasing irradiance (the degree of the response is wavelength specific as well as intensity driven; Kuiper 1964), low internal CO_2 concentration (C_i) and high vapour pressure deficits (VPD), while closure is promoted by darkness, high CO_2 and low VPD (reviewed by Assmann 1993; Willmer and Fricker 1996; Outlaw 2003; Vavasseur and Raghavendra 2005; Shimazaki et al. 2007). The typical responses outlined above are customary for stomata of C_3 and C_4 plants. However, plants that employ crassulacean acid metabolism typically opening in darkness and closing during the day (Cockburn et al. 1979) driven mostly be changes in internal CO_2 concentration. Environmental factors can directly or indirectly influence stomatal behaviour (Wong et al. 1979; Willmer and Fricker 1996). Direct effects are cues that the guard cells sense and respond to while indirect effects include those that result in modifications to assimilation rate that change [C_i] and/or other mesophyll signals that guard cells respond. One of the most important stimuli for stomatal function is light, which is made up of several components, a direct guard cell specific blue light response (see reviews by Shimazaki et al. 2007) and the so-called "red" light response. The blue light response saturates at very low fluence rates (*ca.* 10 μmol photons m^{-2} s^{-1}), is insensitive to the inhibitor of electron transport 3,4-dichlorophenyl-1,1 –dimethylurea (DCMU; Zeiger et al. 2002), and is often associated with rapid stomatal opening (Shimazaki et al. 2007) early in the morning (Zeiger et al. 2002). The red light response is mediated by photosynthesis and saturates at high fluence rates (similar to those for photosynthesis), is DCMU sensitive, and is thought to operate through mesophyll driven changes in intercellular CO_2 concentration (Tominaga et al. 2001; Olsen et al. 2002; Roelfsema et al. 2002, 2006a; Messinger et al. 2006) to which the stomata respond (Mott 1988;

Vavasseur and Raghavendra 2005; Mott et al. 2008).

Changes in stomatal aperture are the result of increasing or decreasing guard cell turgor (Heath 1949; Willmer and Fricker 1996) in response to internal and external signals. In order to do so, guard cells adjust osmotic pressure. This is achieved by loss or accumulation of ions (such as K^+) and organic solutes (e.g., malate and sucrose). However, despite decades of research into stomatal metabolism, the role of organic solutes in stomatal function is still under debate (Lawson 2009; Azoulay-Shemer et al. 2015; Horrer et al. 2016) and requires further research. This illustrates the complexity of signaling pathways and sensory mechanisms employed by guard cells that enable these complex cells to fine-tune their behavior to ensure appropriate rates of photosynthesis, water loss, and leaf cooling. Signals sensed by guard cells do not, of course, operate in isolation and therefore stomata in dynamic environments (such as those experienced in the field) receive multiple signals simultaneously, to which they respond in a hierarchical manner (Lawson and Morision 2004; Lawson et al. 2010; Lawson and Blatt 2014). Although there is now significant knowledge of many of the signaling pathways, we need a full understanding of guard cell metabolism and an appreciation of the genetics that form the basis of stomatal sensing and signaling, as well as the hierarchy of responses and how stomatal behaviour is linked to photosynthetic demands for CO_2, if we are to successfully manipulate guard cell metabolism or stomatal behavior to improve plant performance or water use efficiency (Lawson et al. 2010).

II. Anatomical Features and Physiological Responses Determine Stomatal Conductance

Maximum stomatal conductance is governed by the physical pore dimensions and the density of stomata. Assuming the maximum stomatal aperture results in an elliptical shape, maximum stomatal conductance (g_{max}) can be determined using the following equation (Parlange and Waggoner 1970; Franks and Farquhar 2007; Franks and Beerling 2009a):

$$g_{max} = \frac{\frac{Dw}{v} SD.pa_{max}}{pd + \frac{\pi}{2}\sqrt{pa_{max}/\pi}}$$

where Dw = diffusivity of water vapor in air at 25 °C (0.0000249 $m^2\ s^{-1}$) and v = molar volume of air (0.0245 $m^3\ mol^{-1}$) are both constants, SD is stomatal density (stomata m^{-2} leaf area), pa_{max} is maximum stomatal pore area (m^2) calculated as an ellipse using stomatal pore length (m) as the long axis and $\frac{1}{2}$ the stomatal pore length as the short axis; pd. is stomatal pore depth (m) considered to be equivalent to the width of an inflated, fully turgid guard cell (Franks and Beerling 2009a, b). The term:

$$\left(\frac{\pi}{2}\sqrt{\frac{pa_{max}}{\pi}}\right)$$

is the 'end correction' that takes into account the influence of diffusion shells from outside the end of stomatal pores (see Weyers and Meidner 1990). Although g_{max} provides us with an indication of the maximum achievable stomatal conductance (and has been a useful indicator in paleo-botanical studies), g_{max} is rarely realized in the field, particularly in modern day crop species (Lawson and Morison 2004; Dow et al. 2014; McElwain et al. 2016). This is due to stomatal physiological responses to their surrounding environment, and therefore, although anatomical features play an important role in stomatal conductance, these stomatal behaviour ultimately determine stomatal conductance (g_s). For example, irrespective of the number of stomata, if all are closed g_s would be minimal (depending on how tightly stomata can close). However, it is interesting to note that g_{max} and operational stomatal conductances

(those observed in the natural environment) are much closer in more "ancient species" than our modern-day crops (McElwain et al. 2016). Several studies (e.g. Brodribb et al. 2007; Boyce et al. 2009; Franks and Beerling 2009a; de Boer et al. 2016; Lawson and McElwain 2016) have shown a strong relationship between increasing stomatal density (along with supporting veins and hydraulic conductance) that facilitated the development of high photosynthetic rates in angiosperms and greater plasticity in responses to environmental factors, which have enable these species to dominate our modern-day plant kingdom. These findings provide an early example of the close relationship between stomatal conductance and photosynthetic capacity and stomatal regulation and co-ordination with photosynthetic rates, albeit over a significant period of time.

The manipulation of stomatal density is an approach that has previously been considered for increasing or decreasing g_s (Doheny-Adams et al. 2012), with greater stomatal density facilitating enhanced CO_2 diffusion and photosynthesis (Tanaka et al. 2013) while reductions in numbers increase water use efficiency (WUE; Franks et al. 2015). It should be noted that reducing stomatal numbers to save water could result in higher leaf temperatures that would be detrimental to photosynthetic processes, particularly in crops such as wheat that are sensitive to high temperature. However, stomatal numbers alone do not determine stomatal conductance. Stomatal aperture is the key determinant (Weyers and Lawson 1997; Lawson and Morison 2004) and changes in stomatal density can be compensated for by changes in aperture. For example, Büssis et al. (2006) showed that g_s was similar in wild type plants to plants with lower stomatal densities (induced by over expressing STOMATAL DENSITY AND DISTRIBUTION 1). This highlights the importance of stomatal physiological responses in determining g_s and the link with mesophyll demands for CO_2.

III. Stomatal Behavior Correlates with Mesophyll Demands for Photosynthesis

Stomatal conductance is often reported to correlate strongly with photosynthetic assimilation rates (see Wong et al. 1979), ensuring an appropriate balance between CO_2 uptake for photosynthesis and water loss through transpiration (which is essential for translocation as well as evaporative cooling). However, such observations often refer to long term or steady state measurements, as short term perturbations in the environment (e.g., irradiance) often lead to spatial and temporal non-coordination between stomatal behavior and photosynthesis (Kirschbaum et al. 1988; Tinoco-Ojanguren and Pearcy 1993; Lawson and Weyers 1999; Lawson et al. 2010; McAusland et al. 2015). This is mostly due to the fact that the response time of stomata are often an order of magnitude slower than photosynthetic responses and result in a lag in stomatal behaviour. For example, photosynthetic rates respond to changes in light within seconds of a change in intensity while changes in stomatal conductance take seconds to several 10s of minutes (Kirschbaum et al. 1988; Lawson et al. 2010) and when light intensity increases, lags and the slow nature of the stomatal response can result in low g_s that restricts CO_2 diffusion into the leaf thereby limiting photosynthesis (e.g. McAusland et al. 2016). This is illustrated in Fig. 6.1 that shows the effect of a step increase in light from 100 to 1000 μmol photons m^{-2} s^{-1} for a 1 h period before being returned to the original light intensity. Photosynthesis responds immediately to the increase in light up to a certain point, after which a slow increase in photosynthesis is observed as stomata slowly open and release the CO_2 constraint on photosynthesis. When light is returned back to 100 μmol photons m^{-2} s^{-1}, photosynthesis drops to the original value of 1.8 μmol CO_2 m^{-2} s^{-1} while stomata continue to open for 10 min or so before slowly closing and only

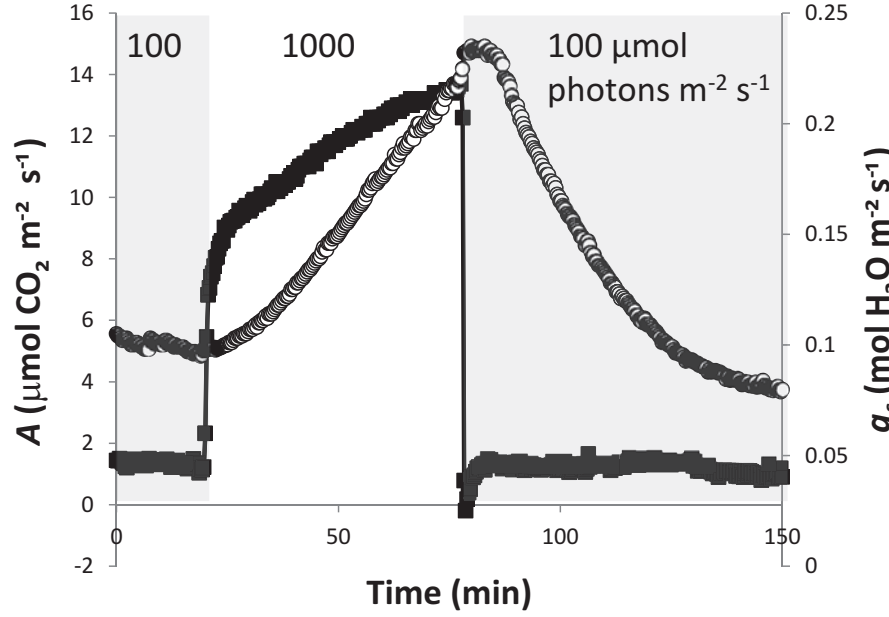

Fig. 6.1. The effect of a step change in light intensity (from 100 to 1000 µmol photons m^{-2} s^{-1} followed by a return to 100 µmol photons m^{-2} s^{-1}) on the rate of CO_2 uptake (assimilation rate = *A*, closed symbols) and stomatal conductance (*gs*, open symbols) in leaves of *Vicia faba*. Measurements were made every 2 min with cuvette CO_2 concentration maintained at 400 µmol mol^{-1}, leaf temperature at 25 °C and a vapor pressure deficit between the leaf and the surrounding atmosphere of 1 kPa. (Unpublished data of Matthews & Lawson)

reaching the original steady state conductance after a further 60 min. It has been reported that low stomatal conductance of well-watered plants can reduce photosynthetic rates by about 20% in some C_3 crop species in the field (Farquhar and Sharkey 1982; Jones 1987), an outcome that can be the result of slow stomatal responses (Lawson and Blatt 2014; McAusland et al. 2016). Several studies have also demonstrated a strong correlation between g_s and grain yield in crops, including wheat (Lu et al. 1998; Fischer et al. 1998). These studies have illustrated that both stomatal control of CO_2 uptake for photosynthesis and evaporative leaf cooling and canopy temperature can account for the observed correlations.

These observations have led to the suggestion that improvements in the co-ordination and synchrony of stomatal responses with mesophyll photosynthetic rates could improve plant WUE in plants grown in a dynamic environment (Lawson et al. 2010, 2012; Lawson and Blatt 2014). Recent studies that have modeled stomatal dynamics in response to short term fluctuations in irradiance have shown that carbon gain could be increased by 20–30% if stomata responded to changing irradiance at the same rate as photosynthesis (Lawson and Blatt 2014). Figure 6.2 shows *A* and g_s (Fig. 6.2b) responses in *Vicia faba* over a dynamic 10 h light period (Fig. 6.2a) and the effect on intrinsic water use efficiency (WUE_i). Intrinsic water use efficiency uses g_s as an estimate of water loss rather than transpiration that is traditionally used for determining water use efficiency (WUE). Generally, photosynthesis tracks the light environment; however, g_s shows a slow and steady increase for the initial 6–7 h period and then slowly decreases over the following 3 h. Stomata are not completely matched with the mesophyll demands for CO_2, resulting in dynamic WUE (Fig. 6.2c) with periods of restricted CO_2 uptake (assimilation = *A*).

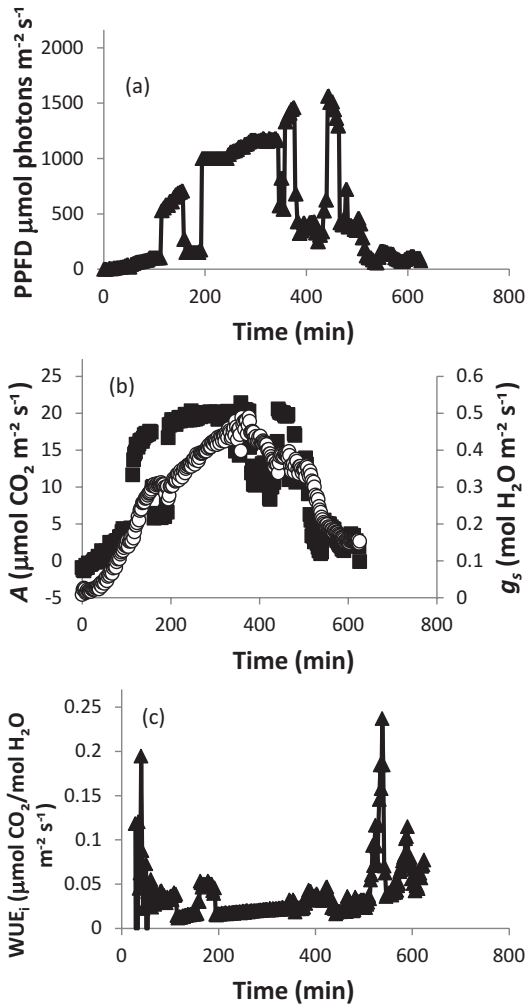

Fig. 6.2. Variation in (**a**) light intensity, (**b**) rate of CO_2 uptake (A, closed symbols) and stomatal conductance (gs, open symbols), and (**c**) intrinsic water use efficiency (WUE_i) for leaves of *Vicia faba* exposed to simulated natural light fluctuations by a Heliospectra (Heliospectra AB, Göteborg, Sweden) light. Cuvette conditions were maintained at an average leaf temperature of 25 °C and relative humidity 65%. Readings were recorded every 60s. (Unpublished data of Lawson & Matthews)

IV. Co-ordination of Stomatal Behavior and Mesophyll Photosynthesis

As g_s and A have been reported to be closely correlated (Wong et al. 1979; Farquhar and Sharkey 1982; Mansfield et al. 1990; Buckley and Mott 2013) over a range of light levels and CO_2 concentrations (Radin et al. 1988; Hetherington and Woodward 2003), it was assumed that the concentration of CO_2 inside the leaf maintains this coordination between mesophyll photosynthesis and stomatal aperture. Additionally, there are many reports in the literature that demonstrate that changes in stomatal aperture result in a constant ratio between the concentration of CO_2 inside the leaf and that of the surrounding atmosphere (C_i:C_a ratio; e.g., Mott 1988; Ball and Berry 1982). This is an attractive and logical proposal as C_i is determined by mesophyll consumption of CO_2 and the flux of CO_2 from the atmosphere into the leaf, with stomata providing the key resistance to this diffusion. Changes in light intensity would, for example, increase or decrease mesophyll consumption of CO_2, to which stomata would respond. However, several studies have suggested that stomatal responses to C_i are too small to account for the large changes in g_s that have been observed in response to light (Raschke 1975; Farquhar and Raschke 1978; Sharkey and Raschke 1981a; Farquhar and Sharkey 1982). This is in agreement with many earlier studies on epidermal peels that reported stomatal responses to light and CO_2 suggesting that C_i cannot be the only signal. More recent reports support the idea of a signal other than C_i as stomata of several different species have been shown to respond to light even when C_i is held constant (Messinger et al. 2006; Lawson et al. 2008; Wang and Song 2008). These findings confirm the work on transgenic plants (with reductions in photosynthetic capacity) showing that g_s increases in response to increasing light intensity despite the fact that rates of CO_2 uptake remained low and C_i was high (von Caemmerer et al. 2004; Baroli et al. 2008; Lawson et al. 2008). However, due to the fact that the correlation between A and g_s is broken in these plants, the work on these transgenic plants argues against a mesophyll driven signal that co-ordinates A and g_s (discussed later in the chapter) and von Caemmerer et al. (2004) suggested that

stomata respond to external CO_2 rather than internal C_i.

V. A Role for Guard Cell Chloroplasts and Photosynthesis in Co-ordinating Mesophyll Photosynthesis and Stomatal Behavior

Previous studies supported the contention that photosynthesis in the guard cells could be involved in the regulation and co-ordination of stomatal responses with mesophyll photosynthesis (e.g., Sharkey and Raschke 1981a; Messinger et al. 2006; Wang et al. 2011; Lawson et al. 2014). It is known that the chloroplasts in guard cells exhibit relatively high rates of photosynthetic electron transport (Lawson et al. 2002, 2003; Lawson 2009) and it has been speculated that this could provide the link between stomatal responses and *A*. Photosynthesis in guard cells has been shown to respond to similar stimuli as mesophyll photosynthesis (Lawson et al. 2001) and therefore one could envisage that the co-ordination between electron transport or carbon fixation in both cell types aid in the co-ordination of responses. Early studies using epidermal peels showed that red light induced stomatal opening in epidermal peels was inhibited by DCMU (Schwartz and Zeiger 1984) and, along with early patch clamp studies that illustrated plasma membrane proton pumping in response to red light, suggested that guard cell photosynthesis may provide energy for opening and/or the photosynthetic signaling product NADPH (Serrano et al. 1988; Wu and Assmann 1993). However, later studies could not confirm these findings (Roelfsema et al. 2001; Taylor and Assmann 2001).

Although it is widely accepted that all the enzymes are present for the Calvin cycle in guard cells, there is an on-going debate regarding the amount of photosynthetic carbon fixation in these cells (Lawson 2009; Daloso et al. 2015, 2016) that could provide osmotica for stomatal movement or a signal that co-ordinates mesophyll photosynthesis with stomatal aperture. A recent study by Daloso et al. (2015) has supplied further evidence for fixation of CO_2 by ribulose bisphosphate carboxylase oxygenase (as well as via phosphoenol pyruvate carboxylase) in guard cells. In the absence of any CO_2 fixation in guard cells, electron transport could provide sufficient energy for ATP driven ion exchange required for stomatal opening (Shimiazaki and Zeiger 1985). A role for guard cell photosynthesis in stomatal responses to CO_2 concentration has recently been eliminated by Azoulay-Shemer et al. (2015) who showed that stomata in transgenic *Arabidopsis thaliana* with decreased guard cell chlorophyll content still closed in response to increasing $[CO_2]$ and responded to abscisic acid (ABA). However, these studies showed that chlorophyll-less stomata had a "deflated thin-shaped phenotype" suggesting that guard cell photosynthesis is critical for establishing turgor. More recently, it was suggested that there would be a good correlation between the redox state parameter and stomatal conductance (g_s) across the entire range of red light intensities (Busch 2014). The accumulation of reducing substances might function as the possible signal inducing stomatal opening. Once again, this could provide a mechanism that enables mesophyll photosynthesis to be co-ordinated with stomatal responses. However, to date the role of the guard cell chloroplasts and photosynthesis has not be unequivocally elucidated and is an on-going avenue of research. For example, guard cells of *Paphiopedilum leeanum* have no chlorophyll, yet the stomata in this species opened in red light (Nelson and Mayo 1975) providing evident that guard cell chlorphyll is not necessary for stomatal function. Stomata in the epidermis of *Commelina communis* hardly opened in red light, indicating a minor role for photosynthesis in guard cells in stomatal responses to red light (Tominaga et al. 2001). In varie-

gated *Chlorophytum comosum*, stomata could open in response to blue light in both the green area (with active mesophyll) and the white area (which lacked mesophyll chloroplasts). Stomatal opening in response to red light was, however, only observed in the green area, not in the white area (Roeflsema et al. 2006a, b).

VI. Evidence for a Mesophyll Driven Signal: A Comparison between Stomatal Responses in Intact Leaves and in Epidermal Peels

Several studies have shown a close relationship between photosynthetic rate and stomatal conductance, under various conditions consistent with mesophyll activity control of stomatal responses. Photosynthetic metabolites could regulate stomatal responses resulting in a balance between regeneration of ribulose 1,5-bisphosphate (RuBP) by photosynthetic electron transport and RuBP carboxylation limitations (Wong et al. 1979; Grantz and Schwartz 1988; Messinger et al. 2006). The role of the mesophyll in controlling stomatal responses has been analyzed by comparing stomatal behavior in epidermal peels with those in intact leaves, with responses in epidermal peels generally being slower (Lee and Bowling 1992; Olsen et al. 2002; Mott et al. 2008) and some reports suggesting that stomata in epidermal peels respond much slower or with less magnitude to red light (Lee and Bowling 1992; Roelfsema et al. 2002). Initial experiments by Lee and Bowling (1993, 1995) showed that stomata responded to light when epidermal peels were floated on a solution with illuminated mesophyll cells or chloroplasts isolated from the leaf. However, when the epidermal peels were floated on the same buffer without mesophyll cells or chloroplasts, the stomata failed to open in response to light (Lee and Bowling 1992, 1995). Stomatal opening was also observed when the epidermal peels were floated on the supernatant of a solution from illuminated mesophyll cells (Lee and Bowling 1992). The same authors also reported that guard cell protoplasts swelled when suspended in the supernatant (Lee and Bowling 1993). Therefore, it was postulated that unknown soluble signals from mesophyll, named 'stomatin' (commonly referred to as the 'mesophyll signal'; Mott et al. 2008), controlled stomatal responses (Lee and Bowling 1992). These studies also indicated that mesophyll signals were aqueous. On the basis of these experiments, Mott et al. (2008) suggested that signals produced in the mesophyll controlled rapid and reversible stomatal responses to light and CO_2. In addition, it was indicated that the mesophyll signal was a common substance irrespective of the species. Their pioneering work illustrated the importance of the mesophyll signal in stomatal responses in a straightforward manner. However, it should be noted that in the experiment by Mott et al. (2008), the stomata in the epidermal peels could have opened widely in a hydropassive manner, as the stomata in the epidermal peels did not respond to environmental stimuli such as light and CO_2 but remained open. Therefore, it remains unclear whether stomatal responses in the epidermal peels are comparable to those in leaves.

In most of the previous studies on stomatal physiology, isolated epidermes were floated on the buffer solution, making it difficult to track behavior of the same stoma, as isolated epidermes continuously move on the solution. Moreover, when epidermal peels are floated on a buffer, almost all of the substomatal cavities are filled with the buffer, whereas the sub-stomatal cavities are filled with air in intact leaves. Therefore, it is possible that the apoplastic environment of the substomatal cavities in the isolated epidermal peels differed from that in the intact leaves. If so, stomatal behaviour would be greatly influenced by the buffer solution. To solve these problems, Fujita et al. (2013)

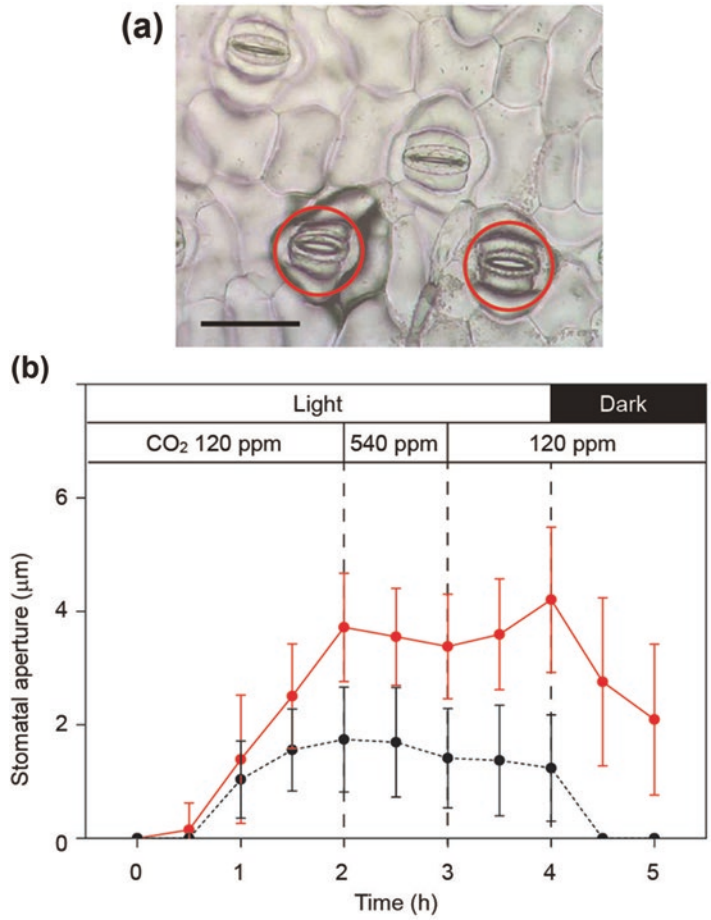

Fig. 6.3. Effects of conditions of the stomatal cavities on stomatal responses. (**a**) stomata of *Commelina communis* with air-filled sub-stomatal cavities (in red circles) and those with the buffer-filled cavities. (**b**) Responses of stomata to white light of 700 µmol photons m^{-2} s^{-1} and low versus high CO_2 concentration. Red symbols and line, stomata with air-filled cavities; black symbols and line, stomats with the buffer-filled cavities. Data represent mean values ± standard deviation of at least 10 stomata. T Fujita, unpublished data

solidified the buffer with 1% (w/v) gellangum. Because the buffer-containing gels are transparent, microscopic observations are feasible. When epidermal peels were placed on the buffer-containing gels, most substomatal cavities remained air-filled (Fig. 6.3a). Stomata with air-filled substomatal cavities were found to be more sensitive to environmental stimuli than those with buffer-filled cavities (Fig. 6.3b). It is well known that ionic concentrations of, e.g., H^+, K^+, and Cl^- in the apoplast of guard cells, subsidiary cells, and epidermal cells are different from each other (Bowling 1987; Edwards et al. 1988). When the substomatal cavities are filled with the buffer, the ionic gradient from guard cells to epidermal cells would be disturbed. Since the buffer constituents can be carefully chosen, the gel method could mimic the apoplast environment in intact leaves, and thereby stomata with the air-filled substomatal cavities, showing physiological responses similar to those in the intact leaves (Fig. 6.3b).

To analyze the effects of mesophyll on stomatal responses, Fujita et al. (2013) compared stomatal responses in leaf segments, epidermal peels, and epidermal peels trans-

planted onto the mesophyll of *Commelina communis*. In red light, stomatal opening was induced by low CO_2 (100 ppm) in the leaf segments, whereas in the epidermal peels, marked opening was not observed. In white light (that included blue wavelengths of light), stomata opened markedly in response to 100 ppm [CO_2] in both the leaf segments and epidermal peels. However, stomatal closure induced by 700 ppm [CO_2] observed in the leaf segments was largely retarded in the epidermal peels. When the epidermal peels were placed on the mesophyll, all opening and closing responses of the stomata observed in the leaf segments were restored.

VII. Characteristics of Apoplastic Mesophyll Signals: Is the Production of a Mesophyll Signal Dependent on Mesophyll Photosynthesis?

As already mentioned above, the series of studies by Lee and Bowling (1992, 1993, 1995) and by Messinger et al. (2006) suggested that mesophyll signals driven by mesophyll photosynthesis control stomatal opening. As also mentioned above, Fujita et al. (2013) showed that the mesophyll was required for rapid stomatal opening in both red and white light. Moreover, stomata in the leaf segments treated with DCMU hardly opened in red light even at 100 ppm [CO_2]. In white light, stomata in leaf segments treated with DCMU opened more slowly than those in the control leaf segments. These findings further support that stomatal opening in the leaf is dependent on photosynthesis in mesophyll cells.

During stomatal opening, a proton transporter (H^+-ATPase) in the guard cell plasma membrane actively pumps protons into the apoplastic cell wall space using energy in the phosphate bond of adenosine triphosphate (ATP; Shimazaki et al. 2007). When photosynthesis of the leaf segments was inhibited by DCMU, ATP produced by respiration of carbohydrates stored in the guard cells could be used by the H^+-ATPase (Mawson 1993). However, stomata in the leaf segments treated with DCMU did not open in red light (Fujita et al. 2013), even though carbohydrates were available. Although blue light activates H^+-ATPases in guard cell protoplasts (without mesophyll cells), red light has no such function (Taylor and Assmann 2001). Hence, it may be probable that the mesophyll signal induces stomatal opening via the activation of H^+-ATPase in the guard cells.

In addition to the regulation of H^+-ATPase, the regulation of S-type anion channels in guard cells could also be important for fast stomatal opening. In intact plants, the inhibition of S-type anion channels in guard cells occurred in red light or at low CO_2 (Roelfsema et al. 2002; Marten et al. 2008). Loss of SLAC1, a major S-type anion channel in guard cells, leads to slow stomatal closure in the dark (Negi et al. 2008; Vahisalu et al. 2008). In other words, the deactivation of S-type anion channels would be prerequisite for fast stomatal opening in the light. Mesophyll opening signals at low CO_2 in the light may deactivate S-type anion channels and thereby speed up stomatal opening.

Stomata in leaf segments treated with DCMU closed rapidly in white light at 700 ppm [CO_2] in a manner similar to that of the control (Fujita et al. 2013). What factors are responsible for the rapid stomatal closure in the DCMU-treated leaf segments? Since photophosphorylation was inhibited in DCMU-treated leaf segments, the concentration of ATP would decline. The cyclic electron transport around PS I would be also suppressed, at least, in the presence of DCMU at high concentration, although it would not be at low concentrations (Hosler and Yocum 1987). Thus, it is possible that stomatal closure was induced by a shortage of ATP in DCMU-treated segments. However, the shortage of ATP was unlikely to be the cause of the stomatal closure, because stomata in the DCMU-treated leaves continued to open

for up to several hours in white light at 100 ppm [CO_2] (Fujita et al. 2013). It was reported that S-type anion channels were activated at high CO_2 (Roelfsema et al. 2002). Mesophyll signals at 700 ppm [CO_2] may cause the activation of the S-type anion channels via some mechanisms that are independent of photosynthesis.

VIII. Mesophyll Signals Move from the Mesophyll to the Epidermis via the Apoplast

Sibbernsen and Mott (2010) found that stomatal opening declined when various liquids were injected into the intercellular spaces of leaves, leading to their suggestion that the mesophyll signal must be gaseous. In a subsequent study, stomata in the isolated epidermal peels of *Tradescantia pallida* were found to open when positive voltage and close when negative voltage was applied by an electrode positioned below the epidermal peels (Mott et al. 2014). Since the applied voltage could create some vapor phase ions, the authors suggested that the mesophyll signal was a vapor phase ion. In addition, they found that stomata in isolated epidermal peels opened in response to low pH and closed in response to high pH when suspended over solutions with different pH values. Thus, they speculated that the mesophyll signal was possibly hydronium ions in the intercellular space, not in the apoplastic solution.

To investigate whether the mesophyll signal controlling stomatal responses was aqueous, a polyethylene or cellophane film was inserted between the epidermal peels and the mesophyll segments (Fujita et al. 2013). Aqueous substances would move across the cellophane film and not across the polyethylene film. When the polyethylene film with a hole (3 × 3 mm square) was inserted between the epidermal peel and the mesophyll, the stomata did not respond to CO_2. However, when the cellophane film with a hole was inserted between the epidermal peels and the mesophyll segments, the stomata opened at 100 ppm [CO_2] and closed at 700 ppm [CO_2]. This indicated that the stomata in the cellophane film-inserted samples rapidly responded to CO_2 in a manner similar to those in leaf segments. On the basis of these findings, both the mesophyll signals inducing stomatal opening and those for closing appear to move from the mesophyll to the epidermis via the aqueous phase in the apoplast. If gaseous mesophyll signals were present, stomatal opening and closure should also be observed in the polyethylene film-inserted samples. The effects of the gaseous signals (if any) on the regulation of stomatal aperture would be less important compared to those of the aqueous mesophyll signal.

IX. Possible Mesophyll Signals

Chloroplastic ATP, NADPH and RuBP have all been proposed to be the possible candidates for the mesophyll signal (Wong et al. 1979; Farquhar and Wong 1984; Lee and Bowling 1992; Zeiger and Zhu 1998; Tominaga et al. 2001; Buckley et al. 2003). Other possibilities include malate and sugars transported from the mesophyll. Extracellular malate concentration around guard cells rose with the increase in CO_2 concentration in ambient air (Hedrich et al. 1994). The extracellular malate concentration determined the voltage-dependent properties of GCAC1, an anion-release channel in the plasma membrane of guard cells (Hedrich et al. 1994). Therefore, malate would act as a regulator of stomatal movements in response to the external CO_2 concentration (Hedrich and Marten 1993; Hedrich et al. 1994). Malate could also coordinate stomatal movements with mesophyll photosynthesis and or respiration (Lee et al. 2008; Fernie and Martinoia 2009; Araújo et al. 2011, 2013). In *Solanum lycopersicum* (tomato), the role of malate derived from mesophyll in stomatal movements was analyzed using antisense transgenic plants

with reduced expression (driven by the constitutive 35S promoter) of *SUCCINATE DEHYDROGENASE2-2* gene which encodes an iron sulphur subunit of the succinate dehydrogenase protein complex (Araújo et al. 2011). Succinate dehydrogenase catalyses the oxidation of succinate to fumarate in the tricarboxylic acid (TCA) cycle, and, therefore, the contents of TCA metabolites including malate and fumarate were reduced in the antisense plants. These antisense plants exhibited enhanced photosynthetic rates and stomatal conductance. When succinate dehydrogenase was repressed in guard cells specifically, photosynthesis and stomatal conductance were unchanged, indicating that the malate and fumarate from the mesophyll, not from the guard cells themselves, influence stomatal conductance. In *S. lycopersicum*, antisense transgenic plants with reduced expression of fumarase showed impaired photosynthesis and lower stomatal conductance (Nunes-Nesi et al. 2008). In these plants, the low amounts of fumarase resulted in decreases in the activity of the TCA cycle and the rate of dark respiration, and thereby an accumulation of malate, which induced stomatal closure (Nunes-Nesi et al. 2008). These results suggest that malate produced in the TCA cycle in the mesophyll could be important in stomatal closure.

Several sucrose and hexose transporters have been identified in the guard cells (Stadler et al. 2003; Weise et al. 2008; Bates et al. 2012). It has been shown that sucrose released from the mesophyll is accumulated in the guard cell apoplast and transported into guard cells (Lu et al. 1995, 1997; Outlaw and De Vlieghere-He 2001). It was suggested that sugars accumulated in the guard cell apoplast 'osmotically' close stomata (Ewert et al. 2000; Outlaw and De Vlieghere-He 2001; Kang et al. 2007). Besides this hypothesis, it has also been proposed that sugars released from mesophyll evoked signalling for stomatal closure. Hexokinase (HXK) is known as an enzyme catalysing the phosphorylation of hexose. It is well accepted that HXK works as a glucose sensor, coordinating glucose concentration and photosynthetic rate (Moore et al. 2003; Rolland et al. 2006; Granot et al. 2013). In *S. lycopersicum* and *A. thaliana*, stomatal opening was repressed by the overexpression of HXK in the whole plant or specifically in guard cells (Kelly et al. 2012, 2013). It was proposed that sucrose transported from mesophyll to guard cells was cleaved into glucose and fructose. HXK in the guard cell then sensed the glucose and induced stomatal closure (Kelly et al. 2013). It has also been shown that HXK evokes ABA signaling in guard cells and induces stomatal closure (Kelly et al. 2013). Various substances have been proposed as possible mesophyll signals. However, the signal or signals have yet to be unequivocally identified.

X. Adaxial and Abaxial Stomatal Responses to Light

In most amphistomatous leaves (those with stomata on both sides of the leaf), the adaxial (upper) and abaxial (lower) stomata inhabit different light environments in terms of both intensity and wavelength composition (Fig. 6.4). It should be stressed that light transmitted through the mesophyll, rather than the light reflecting from the environment, is the major light source for the abaxial stomata. Many *in vitro* and *in vivo* experimental systems such as those using epidermal peels or guard cell protoplasts have shown the adaxial and abaxial stomata have different photo-sensitivities (Darwin 1898; Dale 1961; Turner 1970; Pemadasa 1979, 1982; Sharkey and Raschke 1981b; Travis and Mansfield 1981; Goh et al. 1995, 1997, 2002). Even though the adaxial and abaxial guard cell protoplasts have similar photosynthetic capacities (Goh et al. 1997), stomatal aperture is greater in abaxial stomata than adaxial ones under the same light intensity (Goh et al. 2002).

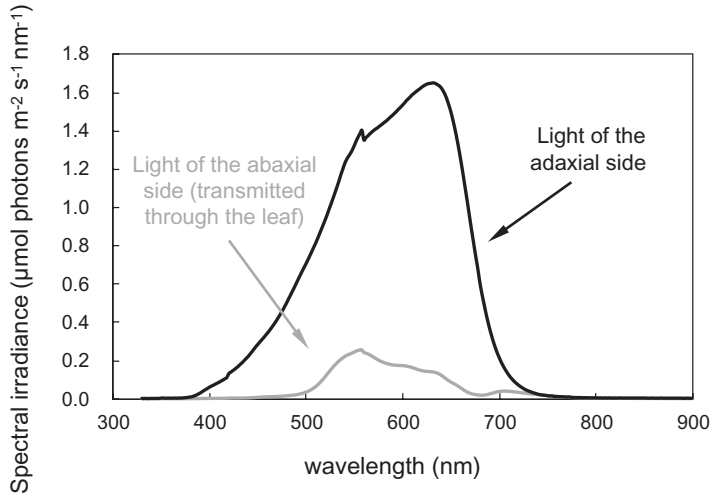

Fig. 6.4. Light conditions of both sides of the leaf in 'white' light

Wang et al. (2008) designed a gas exchange system in which a leaf was sandwiched with two half-chambers, allowing the measurement of stomatal conductances and photosynthetic CO_2 assimilation rates for two leaf surfaces of *Helianthus annuus* leaves separately and simultaneously. When directly illuminated with white light, the abaxial stomata were more sensitive than the adaxial stomata. As mentioned above, when the adaxial side is illuminated with white light, the abaxial stomata are illuminated mostly with light transmitted through the leaf. The spectrum and photon flux density of this light should be similar to those of the light transmitted through the leaf. Thus, Wang et al. (2008) compared the effects of white light, light transmitted through an optically similar leaf, and the self-transmitted light on the stomatal conductance. For example, when the responses of the abaxial stomata were examined, the leaf was either illuminated with white light from the abaxial side, light transmitted through a separate optically similar leaf from the abaxial side, or the white light from the adaxial side. The photon flux densities at the abaxial stomata were adjusted to be identical. The results showed that the self-transmitted light brought about the highest stomatal conductance followed by the leaf-transmitted light, and then white light (Wang et al. 2008). Since the photosynthetic rate was highest in the self-transmitted light, these results suggest an effect of mesophyll photosynthesis on stomatal movement (Wang et al. 2008). When investigating the relationship between photosynthetic rate and stomatal conductance, a linear relationship between the photosynthetic rate and stomatal conductance similar to that documented by Wong et al. (1979, 1985a, b, c) was obtained. Moreover, since C_i was kept constant during the experiments, the effect of mesophyll photosynthesis on stomatal movement was not mediated by changes in C_i.

The transmitted light, which contains more green light than red and blue light, induced opening in abaxial stomata, but not markedly in adaxial stomata. This suggests that abaxial stomata are probably sensitive to green light or a special mixing ratio of two (or more) monochromatic lights. To investigate this further, Wang et al. (2011) compared adaxial and abaxial stomatal responses to red, blue, and green monochromatic lights. In brief, they found that abaxial stomata were more sensitive to all three monochromatic lights than adaxial stomata. As expected, abaxial stomata responded to green

light particularly well, whereas adaxial stomata did not (Wang et al. 2011). By using DCMU to separate the effects of photosynthesis from the stomatal light response, they confirmed that red light induced stomatal movement was completely dependent on photosynthesis, whereas blue light induced stomatal movement included a photosynthesis-independent component that could not be eliminated by DCMU. Interestingly, the green light response, which was only observed in abaxial stomata, also showed a photosynthesis-independent component. This strongly suggests the existence of a green light receptor in abaxial stomata at least in sunflower leaves.

Green light is a major constituent of sunlight and enriched within leaf tissues and in the understory of canopies. Recent studies reported that green light is involved in various physiological processes, including leaf photosynthesis (Sun et al. 1998; Terashima et al. 2009), flowering (Ohgishi et al. 2004), phototropism (Steinitz et al. 1985) and shade avoidance (Zhang et al. 2011). The behavior of stomata in response to green light has also been studied previously (Frechilla et al. 2000, Talbott et al. 2002, 2006), although the main effect was the reversal of blue light induced stomatal opening. Very little is known about green light inducing stomatal opening.

Using the above-mentioned special gas-exchange system (Wang et al. 2008, 2011), green light induced stomatal opening was examined for both sides of *Nicotiana tabacum* (tobacco) leaves. As shown in Fig. 6.5a, similar to sunflower, green light could induce tobacco stomatal opening and it was more effective in abaxial stomata than in adaxial stomata. Green light induced stomatal opening was also compared between the *A. thaliana* wild type (WT) and mutant with reduced levels of the phototropins PHOT1 and PHOT2 (Fig. 6.5b, c). It is well established that phototropins (specifically PHOT1 and PHOT2) are the blue light receptors that mediate blue light induced stomatal opening (Kinoshita et al. 2001). The phototropin double mutant (*phot1phot2*) showed significantly lower values of stomatal conductance than WT in blue light. However, stomatal conductance in the *phot1phot2* plants were comparable to WT when illuminated with green light, suggesting that phototropins are not involved in the stomatal green light responses. Further studies are required to determine a possible green light receptor(s) and the role of green light in stomatal functions.

XI. Effects of Growth Light Environment on Adaxial and Abaxial Stomatal Light Responses

Since the environments of adaxial and abaxial stomata are different in both light intensity and wavelength composition, it is highly probable that the growth light environment regulates stomatal light responses. To verify this hypothesis, Wang et al. (2011) conducted an experiment in which leaves expanded in an inverted orientation. Young expanding sunflower leaves were inverted using coarse transparent net envelopes for about 14 days until full expansion (3 to 4 times larger than the younger expanding leaves). As controls, similar young leaves were kept in the same type of envelopes in the normal orientation for the same period. When fully expanded, the morphologies of inverted- and normal leaves were examined. Although leaf thickness was not altered by the inversion treatment, the morphology of cells of the palisade and spongy tissues changed significantly. In inverted leaves, palisade tissue cells were shortened and the intercellular spaces in the spongy tissue were more crowded by the cells, indicating that the direction of light illumination or the intra-leaf light largely affected mesophyll structure. These morphological changes are known to be accompanied by changes in the dorsiventrality of photosynthesis (Terashima and Hikosaka 1995). Stomatal density on the

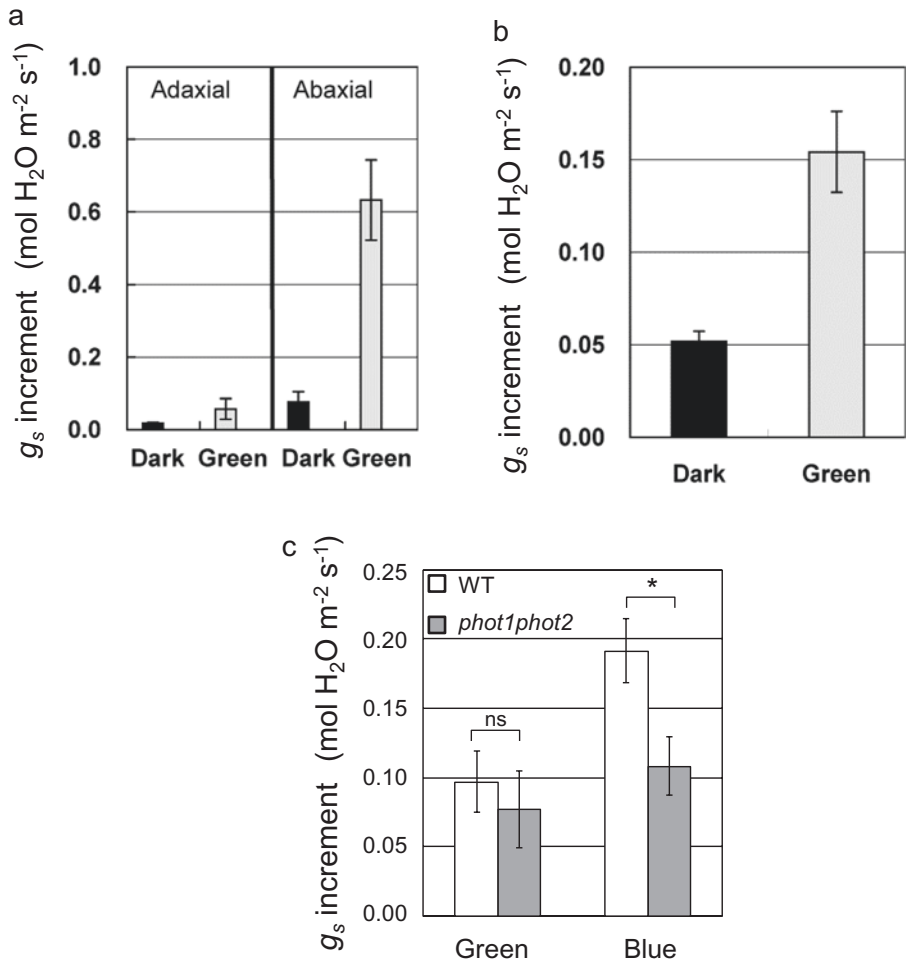

Fig. 6.5. Green light response of stomata. (**a**) Adaxial and abaxial stomatal conductance (*gs*) of a tobacco leaf in the dark and in green light. Each side of the leaf was irradiated directly by green light emitting diodes (250 μmol photons m^{-2} s^{-1}) for 60 min. *Ci* was constant at 300 μL L^{-1}. (**b**) Stomatal conductance of *Arabidopsis thaliana* (Col-0) leaves in the dark and in green light. Abaxial side of the leaf was directly irradiated with green light (250 μmol photons m^{-2} s^{-1}) for 50 min. (**c**) Increment of stomatal conductance from the dark level to green light or blue light. Both sides of the leaf were simultaneously irradiated by green light (90 μmol photons m^{-2} s^{-1} on each side) or blue light (100 μmol photons m^{-2} s^{-1} on each side) for 50 min. WT = wild type (Columbia-0) and *phot1phot2* = mutant deficient in phototropins PHOT1 and PHOT2. Data represent means ± standard deviation (leaf number≧3). (* = $P < 0.001$, ns = not significantly different, Student's *t*-test)

adaxial side of the inverted leaves was lower, although stomatal index was unaltered, indicating that stomatal differentiation was completed before the treatment commenced. Stomatal responses were not simply inverted. Neither the increase in green light sensitivity in adaxial stomata nor the decrease in abaxial stomata was observed. Wang et al. (2011) reported a decreased light sensitivity in adaxial stomata by inversion, whereas the changes of abaxial stomata were complicated (Wang et al. 2011). The decline in adaxial stomatal conductance in the inverted leaves might be attributed to the marked decrease in photosynthetic rate of the adaxial part of the mesophyll (Wang et al. 2011), once again indicating the importance of photosynthesis in stomatal movement in the

intact leaf. On the other hand, in the abaxial side, although the photosynthetic rates in inverted leaves were similar to those of control leaves, the photosynthesis-dependent component of stomatal movement (assessed as a component inhibited by DCMU) increased significantly (Wang et al. 2011). It appears that, when the leaf surface received the full light spectrum, the photosynthesis-dependent component of light driven stomatal responses became more important, whereas the photosynthesis-independent component of stomatal movements was more important when the leaf surface was subjected to transmitted light. However, the increment of photosynthesis-independent component (assessed as a component NOT inhibited by DCMU) on the adaxial side of inverted leaf was not observed, indicating that the differences between adaxial and abaxial stomata are somehow determined inherently at the genetic level. Further detailed comparative studies of guard cells in different surfaces of the leaf are awaited. A series of comparative studies of adaxial and abaxial stomata by Wang et al. (2008, 2011) revealed the importance of mesophyll photosynthesis in stomatal behavior. It is especially noteworthy that the photosynthesis-dependent component could be plastically changed by modifying growth light environment. Moreover, the sensitivity may differ between adaxial and abaxial stomata.

XII. Conclusions

The balance between stomatal behavior and mesophyll photosynthetic rate is critical for optimal water use efficiency in plants. With future prediction of decreasing water availability and increasing temperature (IPCC 2013) knowledge of these processes and interactions is critical if we are to identify and utilize stomatal and mesophyll targets to improve crop productivity for sustainable food and fuel production for the increasing global population. Here we have reported on a number of studies that provide evidence for a mesophyll driven signal. However, to date there is no conclusive evidence of what the signal is or how it functions, and the mesophyll signal therefore remains to be elucidated. Further research is needed to determine the mechanisms and signal transduction pathways that facilitate the well observed correlation between mesophyll photosynthetic rates and stomatal conductance.

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Chapter 7

CO_2 Diffusion Inside Photosynthetic Organs

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Summary

In the present chapter, we review the current state-of-the-art of knowledge on mesophyll (internal) CO_2 diffusion conductance of photosynthetic tissues (for simplification, g_m). We show that, despite concerns regarding the methodological approaches currently used for its estimation, a large and consistent body of evidence has accumulated showing that g_m is finite and significantly limiting for photosynthesis, as well as being highly variable among photosynthetic organisms and in response to environmental changes. Part of this variation results from different anatomies of the photosynthetic tissues, with a particularly strong influence of chloroplast distribution and cell wall thickness. Besides these, it appears that a biochemical modulation of g_m also occurs, likely involving aquaporins and, possibly, carbonic anhydrases and other metabolic components.

Further efforts are needed in the near future to improve CO_2 diffusion models, both for the estimation of g_m and for the precise physiological understanding of the CO_2 assimilation process in different plants, as well as to increase our knowledge of the mechanistic base for g_m and its regulation.

I. Introduction

In most plants, CO_2 is fixed into a 3-carbon (C_3) acid by the Rubisco enzyme in the chloroplast stroma. This process has been termed C_3 photosynthesis, and described as a combined diffusional and biochemical process, so that the velocity of photosynthesis depends on the capacity of CO_2 to diffuse from the atmosphere to the carboxylation sites inside chloroplasts as well as on the capacity of Rubisco to fix carbon (Gaastra

1959; Farquhar et al. 1980). Early identification of diffusional limitations to photosynthesis included stomatal diffusion on one hand, and mesophyll diffusion on the other (Gaastra 1959). Concomitantly with the spread of gas exchange analysis as the most useful tool for *in vivo* assessment of photosynthesis, models and assumptions were developed that – although allowing for the quantitative separation of stomatal diffusion from 'other' limitations to photosynthesis – do not permit an easy separation of mesophyll diffusion conductance from other 'conductances' related to the biochemical capacity of photosynthesis (Sestak et al. 1971). As a consequence, CO_2 diffusion inside photosynthetic tissues was either accounted for in an integrative term (often referred to as 'internal conductance') that included diffusional and biochemical components, or – as in the early definition of the most commonly used leaf photosynthesis model (Farquhar et al. 1980) – neglected. Neglecting potential limitations imposed by CO_2 diffusion inside the photosynthetic tissues (i.e., assuming infinite mesophyll conductance) implies the assumption that the sub-stomatal CO_2 concentration (C_i) equals the concentration in the chloroplast stroma (C_c).

Let's consider the CO_2 diffusion pathway from the atmosphere surrounding a photosynthetic tissue to the carboxylation sites inside chloroplast stroma. After diffusing through the boundary layer, the first barrier along such a pathway in typical leaves would be stomata, as cuticular conductance to CO_2 is extremely small, even far below that for water vapor, mainly because CO_2 then has to diffuse all the way across the epidermal cell (Boyer et al. 1997). In contrast, in photosynthetic tissues lacking stomata the first barrier would be a cuticle or – if the cuticle is minimal or absent – a cell wall, possibly covered with a thin film of water. In stomata-containing tissues, upon passing through the stomatal cavity, CO_2 would have to diffuse through a variable volume of intercellular air space, and then face diffusion through a cell wall. It thus follows that cell walls are the first CO_2 diffusion barriers that all photosynthetic tissues have in common. Subsequent barriers are also common to all C_3 photosynthetic tissues of eukaryotic cells: plasma membrane, cytosol, chloroplast membranes and stroma (see Fig. 7.1; Evans 2009). This series of barriers consist of alternating aqueous and lipid phases that may significantly restrict CO_2 diffusion, as was already mentioned by Gaastra (1959): 'Indeed, the process may not be as simple as the dissolution of CO_2 in the wet cell walls, followed by its diffusion towards the chloroplasts'. Therefore, it is unlikely that mesophyll diffusion resistance is negligible, and indeed different processes – some perhaps biochemical in nature – may affect the diffusion of CO_2.

With the development of additional techniques for studying photosynthesis in vivo, especially on-line carbon isotope discrimination and pulse amplitude modulation (PAM) chlorophyll fluorescence, new methods were developed allowing for estimates of the CO_2 diffusion conductance inside photosynthetic tissues (for simplicity we use the abbreviation g_m, for *mesophyll* conductance, throughout the chapter, although we acknowledge that some species, like mosses, lack a true mesophyll, and in species without stomata estimates of g_m represent a composite of cuticular, epidermal, and internal conductances). These methods allow for the quantitative separation of the contributions of stomatal and mesophyll diffusional limitations from biochemical limitations associated with the capacity of the photo- and biochemical components of photosynthesis (Evans et al. 1986; Harley et al. 1992). These techniques, which are outlined in the next section, have allowed the accumulation of a very significant amount of knowledge on how the internal diffusion of CO_2 in photosynthetic tissues is constrained and how much it contributes to limit photosynthesis rates in vivo. A search for ('mesophyll conductance' OR 'internal conductance') AND

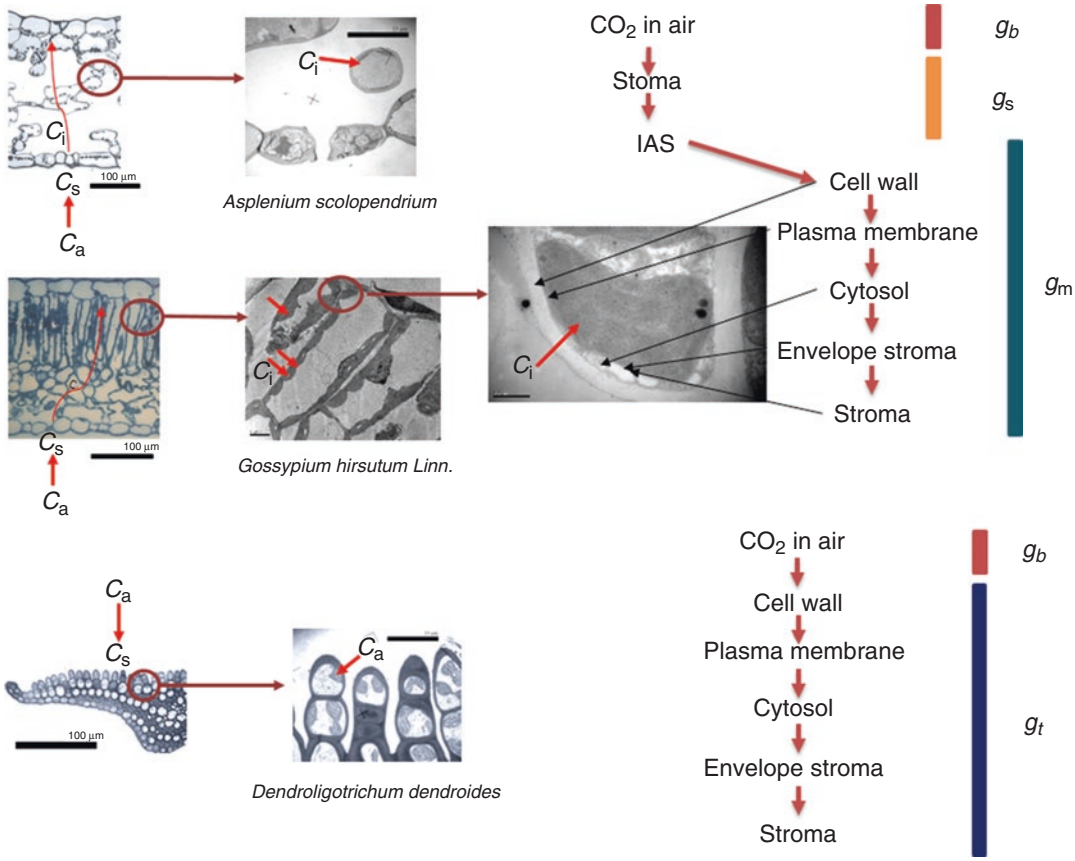

Fig. 7.1. CO_2 pathway from atmosphere to the sites of carboxylation in photosynthetic organs with stomata (upper graph, including boundary layer, stomatal and mesophyll CO_2 conductances, g_b, g_s and g_m, respectively) and photosynthetic tissues lacking stomata (lower graph, boundary layer and total CO_2 conductances, g_b and g_t, respectively). *ias* intercellular air space

'photosynthesis' in the Web of Science (Fig. 7.2) reveals the increasing importance of g_m in studies on photosynthesis, both in terms of the number of papers and citations received. In fact, it can be said that the current 'standard' analysis of photosynthetic limitations consists in quantifying stomatal conductance (g_s), g_m, and the maximum velocities of carboxylation ($V_{c,max}$) and electron transport (J_{max}), the latter based on the response of net photosynthesis (A_n) to C_c. Previously common analyses, based on the response of A_n to C_i – i.e., 'sensu' Farquhar et al. (1980) – is now considered obsolete because assuming g_m is infinite results in very significant underestimation of $V_{c,max}$ and J_{max} (Sun et al. 2014).

In this chapter, we review current knowledge on the regulation of CO_2 diffusion inside photosynthetic tissues, briefly describe the currently used methods for its estimation and their associated problems, then focus on a detailed description of the CO_2 diffusion pathway, and review the variability of g_m among different species and plant groups, its potential structural and biochemical determinants, and analyze how g_m responds to changes in environmental variables.

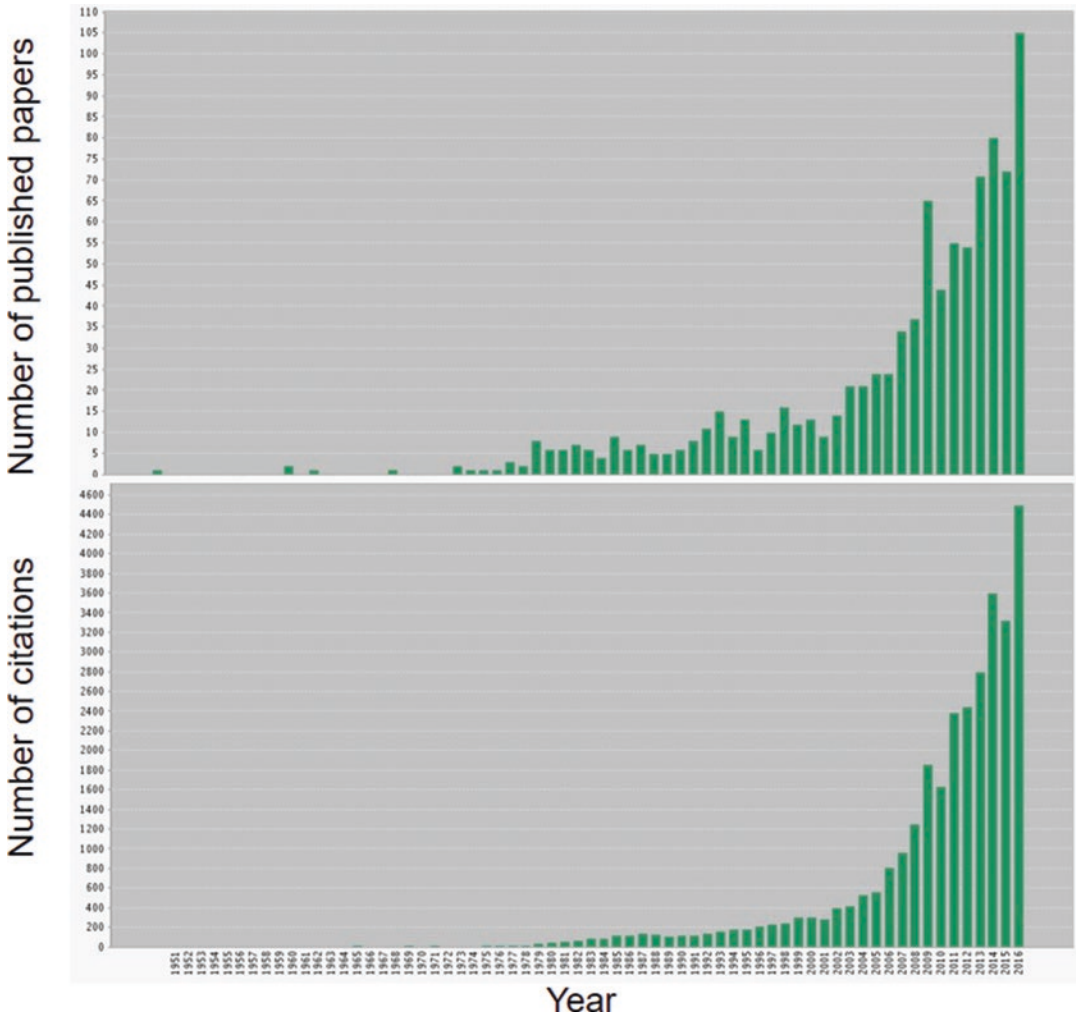

Fig. 7.2. Number of articles published on mesophyll conductance and citations received found by searching the Web of Science – Thompson Reuters in January 2017

II. How to Estimate Internal CO_2 Diffusion Conductance?

Since g_m is an expression of the ease with which CO_2 diffuses from just inside the stomatal pore to the sites of carboxylation (Fig. 7.1), its determination requires the estimation of these concentrations and the flux of CO_2 between them. Hence, g_m is typically operationally defined as:

$$g_m = \frac{A_n}{C_i - C_c} \tag{7.1}$$

A_n can be measured by gas-exchange equipment, but C_i is rarely directly measured (Boyer and Kawamitsu 2011; Tominaga and Kawamitsu 2015) and is instead typically estimated from the conductance of water vapor diffusing out of the leaf (Moss and Rawlins 1963; von Caemmerer and Farquhar 1981). It should be noticed that estimating C_i is simple when most transpiration occurs through stomata, but not when it occurs through cuticles (Tominaga and Kawamitsu 2015). Thus, the estimation of C_i cannot be done easily in photosynthetic organisms or tissues lacking stomata (e.g., bryophyte

gametophytes). Although for simplicity we will use the term g_m throughout this chapter for all organisms and tissues, in these latter cases what is estimated is the total conductance of the whole photosynthetic tissue (i.e., mesophyll conductance, but also surface conductance through water layers on the tissue; Meyer et al. 2008). No methods are currently available that allow direct measurement of C_c. Instead, various approaches have been developed to estimate C_c, and thus g_m, based on theoretical considerations that are described below. As a result, estimates of g_m depend not only on the experimental methodology, but also on the underlying assumptions of theoretical models.

All methods for estimating g_m rely on measurements of gas exchange, and minimizing errors or biases with gas-exchange measurements is thus a prerequisite for obtaining reliable estimates for g_m. Enclosing a large leaf surface in a gas-exchange cuvette increases the differences in the CO_2 and water concentrations of the air entering and leaving the chamber, which improves the accuracy of the measurement (Pons et al. 2009). However, care should be taken to prevent gradients in light intensity and temperature over the leaf surface in such larger chambers (Pons et al. 2009), and the chamber air should be well mixed and at positive pressure compared to the external air (so that any leaks are advective and non-discriminatory). Leakage can be minimized by enclosing the leaf chamber in another container that is supplied with exhaust air from the gas-exchange system and by using large cuvettes with high flow rates (Long and Bernacchi 2003; Flexas et al. 2007a; Rodeghiero et al. 2007). Even with these precautions, very often leaks cannot be completely avoided. Gas-exchange measurements can be corrected after quantification of leakage by measuring gas fluxes on inert leaves (Flexas et al. 2007a; Rodeghiero et al. 2007). Leaf temperature is a key parameter used to determine stomatal conductance and estimate C_i, but reliable measurement of the leaf temperature remains a problem in most gas-exchange setups (Morrow and Slatyer 1971; Mott and Peak 2011).

Compared to biochemical factors, diffusion limitations have a different effect on the curvature of the response of A_n to CO_2 or O_2 (Ethier and Livingston 2004). Thus, assuming g_m is constant over a range of different C_i or O_2 concentrations, it is possible to estimate g_m from gas-exchange measurements alone (Ethier and Livingston 2004; Sharkey et al. 2007; Dubois et al. 2008; Bunce 2009). However, it is more common to estimate g_m by combining gas-exchange measurements with simultaneous measurements of carbon isotope discrimination or chlorophyll fluorescence. This also allows for examining possible changes in g_m in response to a change in CO_2 or O_2 concentration. Methods to estimate g_m have been extensively reviewed in the past (Warren 2006; Pons et al. 2009). Here, we will limit ourselves to discuss only some recent new developments in the widely used isotopic and fluorescence-based methods, highlighting the problems, precautions, and uncertainties that should be considered when using these methods. Despite their shortcomings, these methods can be and have been used to provide realistic estimates of g_m in many different plant species and under a variety of conditions, which are the basis for this review.

A. The Isotopic Methods

The isotope discrimination methods are based on the slower diffusion of a heavier carbon isotopologue ($^{13}CO_2$) compared to a lighter isotopologue ($^{12}CO_2$) through photosynthetic tissue. Because $^{13}CO_2$ is discriminated against during biochemical reactions and diffusion, air leaving a gas-exchange cuvette is normally enriched in $^{13}CO_2$ compared to the air entering the cuvette. The isotopic composition of this air can be determined using isotope ratio mass spectrometry, or more recently also using spectroscopic (optical) isotope measurement

technologies, allowing for the measurement of the observed amount of discrimination against $^{13}CO_2$ by the tissue enclosed in a gas-exchange cuvette (Δ_o). The discrimination against $^{13}CO_2$ (Δ) can also be predicted by calculating and combining the effect of several discrimination events along the reaction-diffusion pathway (Farquhar et al. 1982, 1989; Evans et al. 1986; Evans and von Caemmerer 2013). The fractionations at every step along the pathway depend on the medium and physico-chemical processes involved, and are conveniently described using several fractionation factors. Thus, a_b is a fractionation factor associated with diffusion through the air in the boundary layers (2.9‰) and a is the fractionation due to diffusion through the air in the stomatal pore and between photosynthetic tissues (4.4‰). Often, a_b and a are combined into a single factor $\bar{a}$, weighted by the relative resistances of boundary layer and stomata (Tazoe et al. 2011; Evans and von Caemmerer 2013). a_i is a combined fractionation factor for dissolution of CO_2 and diffusion through the liquid phase (1.8‰), b is the net fractionation factor associated with carboxylation, and is dependent on the fractionation by both Rubisco (b_3 = 29‰; Roeske and O'Leary 1984) and phosphoenolpyruvate carboxylases (PEPC; b_4 = 5.7 ‰). b is computed as $b = (1 - \gamma)b_3 + \gamma b_4$ (Farquhar and Richards 1984), where γ is the molar proportion of carbon fixed by PEPC, thought to be between 5 to 10% of that of Rubisco in C_3 plants (e.g., Williams and Kennedy 1978 – that is, typically b is assumed to be around 28‰), e is the fractionation due to mitochondrial respiration in the light (e is often assumed to be negligible if plants are grown and measured under the same atmospheric conditions; Wingate et al. 2007), and f is the fractionation factor for photorespiration. Theoretical and experimental analyses (Gillon and Griffiths 1997; Lanigan et al. 2008; Igamberdiev et al. 2004; Tcherkez 2006) have estimated f to be around 11‰, but recently Evans and von Caemmerer (2013) reported a somewhat higher value (16.2‰).

Farquhar and Cernusak (2012) explained that the isotopic method did not take into account the ternary correction that is commonly used to correct gas-exchange parameters (Jarman 1974; von Caemmerer and Farquhar 1981). This correction is necessary to account for the effect of water molecules diffusing out of the stomata colliding (and thus slowing down) with CO_2 molecules diffusing into the leaf. Therefore, Farquhar and Cernusak (2012) derived a correction factor, $t = (1 + \bar{a})E/(2g_{ac})$, that should be used to correct the fractionation. This correction factor depends on the transpiration rate (E) and the total conductance to CO_2 through stomata and boundary layers (g_{ac}). Fractionation factors for the diffusion pathway through stomata and boundary layers ($\bar{a}$) are corrected with a factor $1/(1 - t)$ (thus $\bar{a}^t = \bar{a}/(1 - t)$), whereas fractionation factors in the liquid phase are corrected with $(1 + t)/(1 - t)$ (e.g., $b^t = b(1 + t)/(1 - t)$). As a result of this correction, g_m values will decrease, sometimes significantly, compared to older data where this correction has not been applied. The effect is greater at larger leaf-to-air vapor pressure differences, at high CO_2 concentrations, and for plants with low photosynthetic capacities (Farquhar and Cernusak 2012). Taking into account these ternary corrections, the expected discrimination against $^{13}CO_2$ is calculated as the sum of the fractionation factors weighted by the relative partial pressures of CO_2 along the pathway (Farquhar et al. 1982, 1989; Evans et al. 1986):

$$\Delta = \frac{\bar{a}^t\left(C_a - C_i\right) + a_i^t\left(C_i - C_c\right) + b^t C_c - \dfrac{e^t R_d\left(C_c - \Gamma^*\right)}{A_n + R_d} - f^t\Gamma^*}{C_a} \tag{7.2}$$

where R_d is the respiration in the light and Γ* is the CO_2 compensation point of photosynthesis in the absence of CO_2 release other than from photorespiration.

The discrimination that is expected if g_m is infinite (i.e., $C_i = C_c$), and in the absence of any (photo)respiratory fractionation ($e = 0$, $f = 0$), is:

$$\Delta_i = \overline{a}^t + \left(b^t - \overline{a}^t\right)\frac{C_i}{C_a} \tag{7.3}$$

Since $C_c = C_i - A_n/g_m$ (Eq. 7.1), the difference between this expected (Δ_i) and observed amount of discrimination (Δ_o) equals:

$$\Delta_i - \Delta_o = \left(b^t - a_i^t - \frac{e^t R_d}{A_n + R_d}\right)\frac{A_n}{g_m C_a} + \frac{e^t R_d\left(C_i - \Gamma^*\right)}{\left(A_n + R_d\right)C_a} + \frac{f^t \Gamma^*}{C_a} \tag{7.4}$$

Assuming that $e^t R_d/(A_n + R_d)$ is small, this allows g_m to be estimated from the slope of the relation between $\Delta_i - \Delta_o$ and A_n/C_a. This "slope method" was originally employed by Evans et al. (1986) and it allows estimation of g_m without making assumptions on the magnitude of the respiratory and photorespiratory fractionation. It requires obtaining a range of A_n/C_a values by varying, for example, CO_2 or O_2 concentrations, and thus relies on the assumption that g_m is not affected by these factors. Light may also be used to obtain variation in A_n/C_a, but with the precaution of using light intensities above a species-dependent PPFD threshold, ~250 μmol quanta m^{-2} s^{-1}, below which deepest cell layers in the mesophyll receive insufficient light energy and contribute little to whole-leaf g_m (Théroux-Rancourt and Gilbert 2017). In cases where $e^t R_d / (A_n + R_d)$ is not negligible, the non-linearity of Eq. 7.4 can be taken into account by an iterative fitting procedure (Lanigan et al. 2008; Tazoe et al. 2009).

Alternatively, when using the "single-point method" (Lloyd et al. 1992; Tazoe et al. 2011; Cano et al. 2014), Eq. 7.2 is rewritten as:

$$g_m = \frac{\left(b^t - a_i^t - \dfrac{e^t R_d}{A_n + R_d}\right)A_n}{\overline{a}^t C_a + \left(b^t - \overline{a}^t\right)C_i - \Delta_o C_a - \dfrac{e^t R_d\left(C_i - \Gamma^*\right)}{A_n + R_d} - f^t \Gamma^*} \tag{7.5}$$

allowing for direct estimates of g_m by taking a single measurement of Δ_o simultaneously with gas exchange. This method is less time-consuming compared to the slope method, and since it does not require obtaining a range of A_n/C_a values, it allows examining the response of g_m to changes in light, CO_2, or O_2. In the past, the fractionation effects by respiration and photorespiration have sometimes been ignored, further simplifying Eq. 7.5; however, in the presence of O_2, respiratory and photorespiratory fractionation is not negligible and has to be taken into account (Gillon and Griffiths 1997; Douthe et al. 2011).

Unfortunately, many uncertainties still remain about the exact magnitude of the fractionation factors. The fractionation associated with respiration is small, but is thought to be somewhat variable depending on the

respiratory substrate (Ghashghaie et al. 2003; Tcherkez et al. 2003, 2004). In addition, carbon substrates used for respiration have a slow turn-over and are therefore likely to have been fixed before the start of the experiments. If there are differences in the isotopic composition between the air used during gas-exchange measurements and the air used to grow the plants, e becomes non-negligible and has be taken into account (Wingate et al. 2007). Photorespiratory carbon has a faster turnover, but there is a considerable variation in estimates for f (Gillon and Griffiths 1997; Ghashghaie et al. 2003; Igamberdiev et al. 2004; Lanigan et al. 2008; Evans and von Caemmerer 2013). Most of these estimates and theoretical considerations suggest a fractionation factor $f = 11‰$ (Tcherkez 2006), which is commonly used in many recent studies. However, an experimental analysis by Evans and von Caemmerer (2013) resulted in an estimate as high as 16.2‰. The latter study showed that the estimate for f depends strongly on the value used for the fractionation by CO_2 carboxylation (b), which may partially explain the observed variation in this factor. The common approach used to estimate f relies on the assumption that g_m is constant over different O_2 concentrations. This assumption may not be valid if the resistance between mitochondria and chloroplasts (r_{ch}) is large relative to the resistance of the cell wall (Tholen et al. 2012a; Evans and von Caemmerer 2013; Gu and Sun 2014; see also Sect. 3). In such cases, more (photo)respiratory CO_2 diffuses outward to the atmosphere and has a greater effect on the observed isotope discrimination. In this case, the effect of e^t and f^t in Eqs. 7.4 and 7.5 becomes enlarged with a factor $(1 + r_{ch}(A_n + R_d))/(C_i - \Gamma^*)$; Evans and von Caemmerer 2013; Gu and Sun 2014).

The fractionation associated with carboxylation is also not well-known, but has a large effect on the g_m estimate in both isotopic methods (Pons et al. 2009; Gu and Sun 2014). Although b for Rubisco in higher plants is thought to be about 29–30‰ (Roeske and O'Leary 1984; Brugnoli and Farquhar 2000), phosphoenolpyruvate carboxylases (PEPC) also contribute to this factor. It is commonly assumed that about 5–10% of the CO_2 is fixed by PEPC, lowering b to about 28‰, but this could also make b somewhat variable and dependent on, for example, the CO_2 concentration or light intensity (O'Leary 1982; Farquhar and Richards 1984; Melzer and O'Leary 1987; Brugnoli et al. 1988; Douthe et al. 2012).

In addition to these theoretical uncertainties, a good estimate of g_m using the isotopic methods require accurate estimates of the isotopic composition of the air flowing through a gas-exchange cuvette. Continuous flow and dual-inlet isotope ratio mass spectrometry have been most frequently used, with the latter technique allowing for standard deviations on the isotopic composition of a gas ($\delta^{13}C$-CO_2) below 0.03‰ (Pons et al. 2009). Great progress has been made during the last several years in spectroscopic (optical) isotope measurement technologies that allow online measurements of carbon isotopes. The high sensitivity and specificity of tunable-diode laser absorption spectroscopy (TDLAS) make it very suitable for g_m measurements (Tazoe et al. 2011; Warren et al. 2011; Douthe et al. 2012; Griffis 2013). These instruments allow for a reference gas standard error of $\delta^{13}C$-CO_2 below 0.05‰ with averaging times within a minute (Tazoe et al. 2011; Douthe et al. 2012). Alternative techniques such as Cavity Ringdown Spectroscopy (CRDS) and Off-Axis Integrated Cavity Output Spectroscopy (OA-ICOS) have only rarely been used for CO_2 isotope measurements and their performance remains poorly quantified (Griffis 2013). For OA-ICOS, standard errors in reference gas $\delta^{13}C$-CO_2 were better than 0.2‰ only when averaging for up to 10 minutes (Tholen et al. 2012a). Although this allows for accurate estimates of g_m if large CO_2 drawdowns in the gas-exchange cuvette can be created, it requires stable gas-exchange parameters over a longer time period. It

should be noted that the $\delta^{13}C$-CO_2 values obtained by spectroscopic methods are often concentration-dependent and may also respond strongly on the amount of O_2 and water present, requiring constant calibration during a measurement cycle (Tazoe et al. 2011; Tholen et al. 2012a).

B. The Chlorophyll Fluorescence Method

Chlorophyll fluorescence has been used as an alternative approach to estimate the concentration of CO_2 in the chloroplast stroma. The most used method is the variable J method (Di Marco et al. 1990; Harley et al. 1992) based on the measurement of chlorophyll fluorescence under photorespiratory conditions. Following Genty et al. (1989), this allows for the calculation of the whole-chain electron transport rate:

$$J_F = \alpha\beta \mathrm{PPFD} \Phi_{PSII} \tag{7.6}$$

where α is leaf absorptance, β is the fraction of photons absorbed by photosystem II (PS II) often assumed to be 0.5 (Eichelmann and Laisk 2000), PPFD is the photosynthetically active photon flux density incident on the leaf, and $\Phi_{PS\,II}$ is the photochemical yield of photosystem II, typically measured using a commercially available PAM fluorometer.

The electron transport rate is also related to gas-exchange parameters and can be described as (Farquhar et al. 1980; Harley et al. 1992; Yin et al. 2009):

$$J_C = \frac{\left(A_n + R_d\right)\left(p_1 C_c + p_2 \Gamma^*\right)}{\lambda\left(C_c - \Gamma^*\right)} \tag{7.7}$$

The values p_1 and p_2 depend on the stoichiometry of the electron transport chain, provided the rate of photosynthesis is limited by RuBP regeneration. RuBP regeneration may be limited either by insufficient NADPH production (which implies that the linear electron transport is enough to meet the requirements for PCR and PCO cycle activity) or ATP production. The latter case takes into account a variable number of protons required for synthesizing an ATP and other processes that increase the ratio of protons moved across the thylakoid membrane, e.g., Q-cycle, pseudocycling electron transport, or Mehler reduction, and λ is the cyclic-pseudocyclic electron flow coefficient (von Caemmerer 2000; Yin et al. 2009). For C_3 photosynthesis, it is commonly assumed that under many conditions the supply of NADPH is limiting electron transport, and in this case $p_1 = 4$, $p_2 = 8$ and $\lambda = 1$. Hence, the partial pressure of CO_2 in the chloroplast equals:

$$C_c = \frac{\Gamma^*\left(J_C + 8\left(A_n + R_d\right)\right)}{J_C - 4\left(A_n + R_d\right)} \tag{7.8}$$

which subsequently allows for calculation of g_m combined with Equation 7.1:

$$g_m = \frac{A_n}{C_i - \dfrac{\Gamma^*\left(J_C + 8\left(A_n + R_d\right)\right)}{J_C - 4\left(A_n + R_d\right)}} \tag{7.9}$$

It is noteworthy that Eq. 7.9 can only be used if J-4(A_n + R_d) is not zero, that is, under photorespiratory conditions. An additional complication is that electron transport rates derived from fluorescence measurements (J_F) are not necessarily equal to the electron transport rates estimated from gas-exchange measurements (J_C). Even if $\alpha\beta$ from equation 7.6 is known, alternative electron sinks such as nitrate reduction or the Mehler reaction (Haupt-Herting and Fock 2002; Rachmilevitch et al. 2004) may cause a discrepancy between J_F and J_C. To account for such discrepancies, the relationship between J_F and J_C can be analyzed by measuring both under non-photorespiratory conditions (1 or 2% O_2, where $J_C \cong 4(A_n + R_d)$) and over a range of CO_2 or light conditions. It is common to derive a calibration factor by plotting the gas-exchange derived quantum yield,

$\Phi_{CO2} = 4(A_n+R_d)/PPFD$, against $\Phi_{PS\ II}$; the slope of this relation equals the factor ($\alpha\beta$) from Eq. 7.6, if there is no significant contribution of alternative electron sinks (Valentini et al. 1995; Flexas et al. 2007b; Hassiotou et al. 2009; Cano et al. 2014). To give more equal weighting between measurements at different light intensities, it is, however, recommended to derive a correction factor directly from the relation between J_C and J_F (Gilbert et al. 2011). Significant offsets or non-linearities in this relationship could indicate the presence of alternative electron sinks or variation in other model parameters such as β or R_d, and it is preferable that such effects are accounted for by non-linear curve fitting (Warren and Dreyer 2006; Gilbert et al. 2011). Although the variable J method has been widely applied to study the effect of varying light and CO_2 on g_m (Flexas et al. 2007b; Hassiotou et al. 2009; Vrabl et al. 2009), analyses have shown that the method is very sensitive to errors in parameter estimates when photorespiration rates are low (Harley et al. 1992; Pons et al. 2009; Gilbert et al. 2011; Gu and Sun 2014). This has led to suggestions of restricting the use of the method within a CO_2 range between 10 and 30 Pa, to avoid low photorespiration rates at high CO_2, and to minimize the sensitivity of g_m to R_d and Γ^* at low CO_2, or at moderate light intensities where A_n is limited by RuBP regeneration. In addition, the calibration between J_F and J_C is best performed under conditions that match as close as possible the experimental conditions under which g_m is estimated using Eq. 7.9 (Harley et al. 1992; Gilbert et al. 2011).

A number of methodological issues may affect the variable J method. For example, the electron flow through PS II per unit quantum flux, $\Phi_{PS\,II}$, is given by the increase in fluorescence of an illuminated leaf relative to the maximal fluorescence (Genty et al. 1989):

$$\Phi_{PS\,II} = \frac{F_m' - F_s'}{F_m'} \tag{7.10}$$

where F_s' is the steady state fluorescence in the light and F_m' is the maximal fluorescence in the light during a short saturating pulse of light. However, due to rapid turnover of electron acceptors, it may be difficult to measure the true maximum F_m', especially with fluorometers that have a relatively low flash intensity, and the commonly estimated F_m' approaches the true maximum F_m' asymptotically (Markgraf and Berry 1990; Loriaux et al. 2013). This leads to an underestimation of $\Phi_{PS\ II}$ that has potentially large effects on the g_m estimate (Loriaux et al. 2013). Such bias can be avoided by extrapolating the true maximum F_m' from multiple measurements with different flash intensities, or using a multiphase flash approach that rapidly varies the irradiance over the course of a single flash (Loriaux et al. 2013). An additional problem relates to the possibility that photosystem I fluorescence contributes more to F_s' than to F_m', resulting in further underestimations of $\Phi_{PS\ II}$ (Genty et al. 1990; Agati et al. 2000; Franck et al. 2002). These underestimations cannot explain the typically observed decrease in g_m with rising CO_2 concentrations (Flexas et al. 2007b; Hassiotou et al. 2009; Vrabl et al. 2009), as such an effect would require either an overestimation of $\Phi_{PS\ II}$ or biased estimates for R_d or Γ^* (Gilbert et al. 2011; Gu and Sun 2014). An overestimation of $\Phi_{PS\ II}$ may, for example, result from a mismatch between chloroplasts sampled by typical fluorometers and those contributing to gas-exchange parameters (Evans 2009; Pons et al. 2009; Théroux-Rancourt and Gilbert 2017). To measure fluorescence, many fluorometers use modulated red or blue light, which is typically absorbed by chloroplasts close to the leaf surface. Since these chloroplasts generally have a higher electron transport capacity, measurements using modulated red or blue light are likely to overestimate whole-leaf $\Phi_{PS\ II}$ (Evans 2009). On the other hand, the chloroplasts near the illuminated surface tend to be light-saturated at lower incident PPFD than those in the bottom (Terashima et al. 2009,

2016). These experimental issues stress the need for careful calibration between J_F and J_C at relatively high light intensities, as mentioned above.

Both the isotopic and fluorescence method rely on estimates of the non-photorespiratory CO_2 release in the light (R_d) and on the CO_2 light compensation point of photosynthesis (Γ^*) in the absence of R_d. Harley et al. (1992) and Gu and Sun (2014) have shown that variation in these parameters has a large effect on the g_m estimate. Γ^* is related to the kinetic properties of Rubisco and while its value assumed to be relatively conserved over a range of C_3 species (Walker and Ort 2015), recent evidences suggest it is not and may cause large biases on the estimation of g_m (Walker et al. 2013; Perdomo et al. 2016). Both parameters are usually estimated from the common intersection point of several A_n-C_i curves measured at different light intensities (the Laisk-method; Laisk 1977; Brooks and Farquhar 1985). von Caemmerer et al. (1994) pointed out that the points where the curves intersect (C_i^*) refers to an intercellular, not chloroplastic, CO_2 concentration. They showed that:

$$C_i^* = \Gamma^* - \frac{R_d}{g_m} \tag{7.11}$$

It is recommended to account for this when calculating g_m using Eq. 7.4, 7.5, or 7.9 by substituting Γ^* with $C_i^* + R_d/g_m$ in these equations and solve again for g_m.

Estimating Γ^* and R_d from gas-exchange measurements involves measuring low fluxes at low CO_2 concentrations, which lowers signal-to-noise ratios and increases problems related to leakage (Pons et al. 2009). Such measurements are therefore best made in large leaf chambers with appropriate precautions against leakage (Flexas et al. 2007a; Rodeghiero et al. 2007). In case the resistance between the sites of (photo)respiration and CO_2 fixation is significant (see Sect. 3), the A_n-C_i curves no longer intersect at exactly the same point and this may result in biased estimates for Γ^* and R_d (Tholen et al. 2012a).

Walker and Ort (2015) suggested various improvements to the Laisk-method to prevent typical problems such as, for example, Rubisco deactivation at the required non-saturating light and low C_i conditions. In addition, they developed a more robust method to average different intersection points for the often observed case where the different A_n-C_i curves do not intersect at exactly the same point. Nevertheless, a modelling study by Gu and Sun (2014) suggested that the Laisk-method may inevitably result in significant bias in the estimation of R_d and Γ^*, highlighting the need for alternative methods to estimate Γ^* and R_d. Galmés et al. (2006) suggested that in vitro Γ* derived from the relative specificity of Rubisco are recommended over in vivo estimation of C_i^* by the Laisk method. Several alternative methods to estimate R_d have already been developed (Kok 1948; Laisk 1977; Pinelli and Loreto 2003; Yin et al. 2009; Busch et al. 2013; Martins et al. 2013), and show good agreement with the Laisk method, contributing to the currently accepted view that non-photorespiratory CO_2 release in the light (R_d) is lower than CO_2 emission in the dark (R_n), which is also supported by metabolic analyses that indicate that light inhibits respiratory metabolism and down-regulates CO_2 emitting processes, such as the pentose phosphate pathway (Tcherkez and Ribas-Carbó 2012; Tcherkez et al. 2012; Tcherkez 2013).

III. The CO_2 Pathway

A. *Sources and Sinks of CO_2 Inside Photosynthetic Organs*

Net photosynthesis (A_n) is the balance between uptake of CO_2 from the atmosphere into the leaf and the release of CO_2 to the surrounding atmosphere, often expressed on a leaf area basis. As mentioned before, we restrict our analysis to C_3 photosynthetic metabolism and exclude organisms with

active CO_2 concentrating mechanisms (CCMs), where the CO_2 partial pressure inside photosynthetic cells is higher than in the environment outside the organism (Raven and Beardall 2016). Before reviewing the models that have been used to describe the CO_2 pathway, we will first describe the main sinks for carbon within a leaf or other photosynthetic organ, the sources of CO_2 that potentially may be fixed, and the pathway of CO_2 from sources to sinks.

1. *Sinks of CO_2 Inside Photosynthetic Organs*

The enzyme that carries out most of the carboxylation is ribulose-1,5-bisphosphate carboxylase-oxygenase (Rubisco). It is located inside the chloroplast stroma and uses CO_2 as its inorganic carbon substrate (Weissbach et al. 1956; O'Leary 1982). In addition, a small fraction of the total amount of CO_2 fixed may be contributed by other carboxylases, such as PEPC, which uses bicarbonate as its inorganic carbon substrate and generates four-carbon (C_4) acids. In C_3 plants, carboxylation by PEPC is thought to contribute 5–10% of the total amount of carbon fixed at ambient CO_2 concentrations (O'Leary 1982; Melzer and O'Leary 1987). PEPC also catalyzes the initial CO_2 uptake in C_4 and CAM plants, and similar to two fold levels of PEPC over Rubisco are found in C_4 plants (Latzko and Kelly 1983; Williams and Kennedy 1978); however, in C_3 plants, the ratio between Rubisco and PEPC is typically about 15:1 (Melzer and O'Leary 1987). PEPC activity seems mainly confined to the cytosol (Chollet et al. 1996), but chloroplastic isoforms exist and may also account for a part of the total fixation rate (Masumoto et al. 2010).

2. *Sources of CO_2 Inside Photosynthetic Organs*

The main source of CO_2 for photosynthesis is the atmosphere. From there, CO_2 passes through a boundary layer close the photosynthetic organs. In most leaves, CO_2 enters mainly through the stomata because the conductance of CO_2 through the epidermis is minimal as a result of the presence of a cuticle and the low diffusivity of CO_2 in the cytosol inside the epidermal cells (Boyer et al. 1997; Boyer 2015). However, there are other sources of CO_2 in addition to the atmosphere. In photosynthetic cells, CO_2 is released by photorespiratory and non-photorespiratory CO_2 release in the light, processes that occur simultaneously to carboxylation. In the presence of oxygen, oxygenation of RuBP by Rubisco produces phosphoglycolate whose recycling during photorespiration eventually results in CO_2-release by glycine decarboxylation in the mitochondria (Ogren 1984; Tcherkez 2015). In addition, non-photorespiratory CO_2 release in the light takes place in chloroplasts (chloroplastic pyruvate dehydrogenase, NADP-dependent isocitrate dehydrogenase, NADP-dependent malic enzyme, and pentose phosphates), mitochondria (mitochondrial pyruvate dehydrogenase, TCAP, NAD-dependent malic enzyme), and in the cytosol (NADP-dependent isocitrate dehydrogenase, pentose phosphates pathway) (Tcherkez et al. 2012; Tcherkez 2013). Other living cells of photosynthetic organs with no functional chloroplasts also release respiratory CO_2, for example in the epidermis, including living trichomes, and in non-photosynthetic living cells inside and surrounding the vascular tissue (Devi et al. 1995; Long et al. 2015). Furthermore, respired CO_2 generated in distant heterotrophic tissues may travel upwards dissolved, or in the form of organic acids present in the xylem sap, and can be re-fixed by chloroplasts inside cells bordering the vascular system in the stem, branches, petioles, and leaves (Levy et al. 1999; Hibberd and Quick 2002; McGuire et al. 2009; Bloemen et al. 2013). In species with branches covered by thick bark, CO_2 in the xylem may travel long distances, even reaching leaves, while in species with green twigs, most of this extra source of CO_2 might be used for carboxylation in the twigs themselves (Pfanz et al. 2002; Rosell et al.

2015). Under certain circumstances, carbon may accumulate in photosynthetic cells, and can be released later on. For example, CO_2 may accumulate inside the vacuole during dark periods or in shaded leaves (at irradiances lower than the photocompensation point). This CO_2 may contribute to photosynthesis when the irradiance increases (Prof J. Berry, personal communication).

3. *CO_2 Diffusion Inside Photosynthetic Organs*

The atmosphere surrounding the photosynthetic tissues is considered well-mixed, but close to the leaf surface a boundary layer develops. In this boundary layer, small gradients of CO_2 exist and the conductance of this boundary has to be taken into account when estimating g_s. CO_2 diffuses from higher to lower concentrations (i.e., following the gradient in CO_2), from the boundary layer into the internal air space through the stomatal pores (see Fig. 7.1 for photosynthetic organs lacking stomata). Within the leaf, at the interface between air and living tissue, a thin water film covers the cell walls. Photosynthesis removes CO_2 from the stromal solution and hence contributes to create a strong diffusional gradient, so that CO_2 within the intercellular air space of the leaf enters into the apoplastic solution and passes through the cell wall, plasma membrane, cytosol, the double membranes of chloroplasts, and is finally fixed by Rubisco in the chloroplast stroma (Fig. 7.3). Hence, CO_2 diffusion into photosynthetic organs comprises movement through a gas phase (intercellular air spaces) and a liquid phase (after dissolving in the liquid matrix of the cell wall). Transport of CO_2 through liquid media may be facilitated by the conversion of CO_2 to bicarbonate by carbonic anhydrases, which is especially important in the basic media of the chloroplastic stroma (Poincelot 1979; Cowan 1986; Badger and Price 1994; but see Price et al. 1994). Diffusion through plasma and chloroplast membranes may be facilitated by aquaporins that are able to conduct H_2O and CO_2 (Hanba et al. 2004; Flexas et al. 2006b; Evans et al. 2009; Heinen et al. 2009; Kaldenhoff 2012; Mori et al. 2014). The structural consideration of several of these diffusion barriers are discussed in detail in Sect. 5 and facilitation mechanisms are described in Sect. 6.

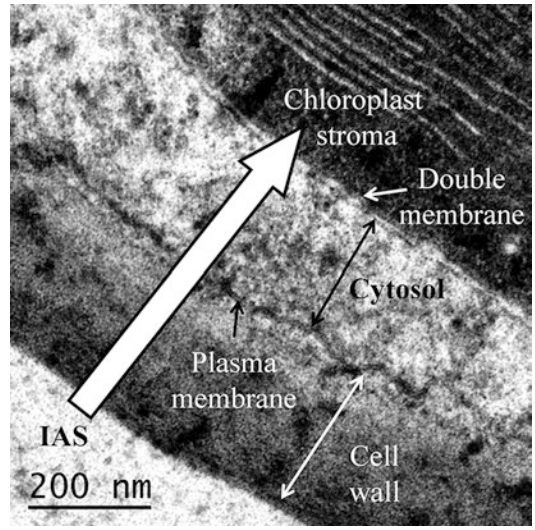

Fig. 7.3. The CO_2 pathway (large white arrow outlined in black) in the liquid phase between intercellular air spaces (ias) and chloroplast stroma. CO_2 dissolves in the water-filled pores of the cell walls and passes through the plasma membrane, some portion of the cytosol, the double membrane of the chloroplasts and is ultimately fixed by Rubisco in the chloroplast stroma. Image from a spongy mesophyll cell of a European beech (*Fagus sylvatica* L.) leaf growing in full sunlight. Transmission electron micrograph by F Javier Cano

Leaves and other photosynthetic organs are complex three-dimensional structures with many CO_2 sources and sinks that generate gradients in the partial pressure of CO_2. PEPC is uniformly distributed within the cytosol, but Rubisco is concentrated into chloroplasts. Therefore, each chloroplast should be treated as a unique sink of CO_2. Chloroplasts are able to move within the cell to balance the energy input and redox state (Kasahara et al. 2002; Oikawa et al. 2003), and this may have consequences for the diffusion of CO_2 (Tholen et al. 2008). The three

dimensional structure of the leaf can also be influenced by the irradiance flux during development (Terashima et al. 2006; Tosens et al. 2012a; Wuyts et al. 2012). For example, more chloroplasts are localized in the palisade than in the spongy tissue (Vogelmann et al. 1996). These conditions make it extremely difficult to model CO_2 diffusion in detail.

B. Models for CO_2 Diffusion

Nearly all current models of photosynthesis couple (directly or indirectly) an anatomy-related CO_2 diffusion model with biochemical processes driving carbon fixation, thereby linking a CO_2-demand and supply function (Farquhar et al. 1980, 2001; Laisk and Oja 1998; Zhu et al. 2013). For simplicity, these models contain multiple assumptions, many of which are related to the nature of the CO_2 diffusion path within a leaf, and these assumptions may reduce the applicability of these models under certain conditions. Simplicity in modeling CO_2 diffusion is also widely used in many models of stomatal conductance, including those commonly used in gas exchange measurements (von Caemmerer and Farquhar 1981). Fick's first law describes how the diffusive flux depends on a concentration gradient and only applies to steady state systems, where concentrations stay constant (Parkhurst 1994). A commonly used simplification is to apply the one-dimensional form of Fick's first law to describe diffusion within photosynthetic organs, analogous to Ohm's law of electrical resistance. This allows diffusion to be described using a simple resistance (or conductance) parameter (Eq. 7.1). More complex reaction-diffusion models are instead based on Fick's second law and allow for a more appropriate description of diffusion in the presence of several sources and sinks that affect the concentration (Parkhurst 1994). Reaction-diffusion models can also be one-dimensional (Cowan 1986), but allow for a convenient description of biochemical reactions and diffusion in multiple dimensions (Parkhurst 1994).

The introduction of a resistance model to describe the diffusion of CO_2 in plant physiology came as early as 1900 (Brown and Escombe 1900), but our understanding of the generalized pathway of CO_2 movement from the atmosphere, and the processes involved in uptake of CO_2 by a leaf, was significantly defined by the pioneering work of Gaastra (1959). Gaastra (1959) used simple modeling based on the analogy with electrical circuits and the similarities between Ohm's law and Fick's first law of diffusion to define the diffusion through a series of resistances including the boundary layer, stomata, and mesophyll. Today, most leaf photosynthetic models use resistances (or their reciprocal: conductances) to describe one dimensional CO_2 movement (Ball et al. 1987; Leuning 1995; Evans et al. 2009; Tosens et al. 2012b). One dimensional models offer simplicity and are useful for understanding gas exchange between the atmosphere and chloroplasts. At the cost of additional complexity, for example in terms of further variables to characterize mesophyll structure and geometry, some diffusion models offer a more comprehensive mathematical approach to represent three dimensional anatomy (Parkhurst 1994; Vesala et al. 1996; Aalto and Juurola 2002; Tholen and Zhu 2011; Ho et al. 2016). Nevertheless, these approaches may substantially differ in the predicted photosynthetic rates. For example, using a one dimensional resistance-related model (von Caemmerer and Farquhar 1981), stomata are represented by assuming that the epidermis is a homogeneous porous barrier. This would result in a linear decrease in C_i in the intercellular airspaces at increasing distances from the epidermis. Because a one-dimensional resistance model does not allow for lateral CO_2 gradients, i.e., CO_2 gradients parallel to the leaf surface, resulting from the spatially distinct stomata in the epidermis, the overall C_i concentration is overestimated. This effect becomes important in

thick and densely compacted photosynthetic tissue or under conditions where the CO_2 gradient between the substomatal region and the atmosphere is high, for example when the rate photosynthesis is high, or stomatal conductance is low (Parkhurst and Givnish 1986; Parkhurst 1994).

The three-dimensional nature of a photosynthetic organ, which includes the location of chloroplasts and mitochondria within each mesophyll cell, characterizes CO_2 diffusion. Gradients in light environment within the leaf may drive gradients in CO_2 concentration along the mesophyll profile, especially under non-saturating light conditions (Evans 2009; Ho et al. 2016; Théroux-Rancourt and Gilbert. 2017). In addition, factors other than CO_2 that regulate enzyme activities and CO_2 diffusivity (e.g., temperature and pH) may change along the mesophyll profile together with the light environment (Tholen and Zhu 2011; Buckley et al. 2017). Furthermore, chloroplast movement (Gorton et al. 1999; Wada et al. 2003; Tholen et al. 2008) and chloroplasts with different photosynthetic capacity according to the local light environment during leaf development (Evans and Vogelmann 2003; Brodersen et al. 2008; Terashima et al. 2016) may complicate the quantification of the CO_2 gradient at each chloroplast position. On the other hand, applying optimization theory (e.g., nitrogen) to each layer inside the mesophyll allows for proportionality between environment (e.g., light) and photosynthetic capacity at each layer, which facilitates modelling exercises, but also allows direct scaling of the equations for photosynthesis from the chloroplast to the whole mesophyll (Terashima and Saeki 1985; Farquhar 1989). The complexity of such a three dimensional structure suggests the use of reaction-diffusion models as the best alternative to describe the CO_2 partial pressure at every point along the CO_2 diffusion pathway (Parkhurst and Mott 1990; Parkhurst 1994). In such a complex structure, mesophyll conductance can be calculated from the average CO_2 concentrations in the intercellular space and in the chloroplast stroma (Eq. 7.1), but because of the complexity of the underlying system, the resulting resistance does not only depend on anatomy and diffusivity of cellular components, but also on factors affecting assimilation and (photo)respiration rates (Parkhurst 1994).

It remains challenging to modify biochemical models of leaf photosynthesis with an adequate three dimensional description of CO_2 diffusion (Farquhar et al. 2001). The newest model of leaf photosynthesis combines three dimensional imaging techniques with a biochemical model of photosynthesis (Ho et al. 2016). This allows for a spatial representation of the mesophyll and makes it possible to implement a three dimensional reaction-diffusion model linked with a description of the light attenuation within the leaf. Although the progress in modelling CO_2 diffusion within the leaf is remarkable, the image technique used (synchrotron radiation X-ray laminography) was insufficient to identify organelles such as chloroplasts, which were incorporated into the model based on a uniform-distributed pattern facing intercellular air spaces. Moreover, the diffusion model in the liquid phase relied on diffusivity and permeability of cellular components that have to be defined a priori (Ho et al. 2016). Since in vivo measurements of these parameters are unavailable, such models cannot be used to directly pinpoint to what extent specific factors limit the photosynthetic rate. Instead, they may provide a framework that can help to design new hypotheses and that can improve our understanding of the underlying processes.

C. The CO_2 Fluxes Inside Photosynthesizing Cells

Contrary to the situation in the substomatal cavity, where the source of CO_2 derives mainly from the atmosphere surrounding the leaf, inside mesophyll cells there is a significant source of CO_2 other than that coming

from intercellular air spaces in the form of (photo)respiratory CO_2 release. The (photo) respiratory release takes place in several cellular compartments, but at different locations from those involved in fixation of CO_2 by Rubisco (see Sect. 3.1. Sources and Sinks of CO_2 Inside Photosynthetic Organs).

Spatial separation between carboxylation, taking place inside the chloroplasts, and the (photo)respiratory CO_2 release from mitochondria was considered in some of the earlier models for C_3 photosynthesis (Moss 1966; Lake 1967). For example, Lake (1967), considered the CO_2 released from non-photosynthesizing cells and the photorespiratory CO_2 release inside photosynthesizing cells in a delta-scheme of a resistance model. More recently and based on a three dimensional reaction-diffusion model, Tholen and Zhu (2011) predicted that this separation of CO_2 release and fixation may have consequences for g_m. Later on, Tholen et al. (2012a) modified the commonly used resistance in series approach of mesophyll resistance (see Evans et al. 2009 and Sect. 5. Structural Determinants of Mesophyll Conductance) to take this spatial separation into account. They suggested that the flux of CO_2 that sustains the carboxylation rate may face two resistances in series between the intercellular airspace and the sites of CO_2 fixation: the resistance of cell wall and plasma membrane (grouped in r_{wp}) and the resistances of the double membranes of the chloroplasts and the stroma resistance (grouped as r_{ch}). Photorespiratory CO_2 , i.e., combination of CO_2 fluxes emitted in the cytosol of photosynthesizing cells and grouped as photorespiration (F) and non-photorespiratory CO_2 release in the light (R_d), would only have to face r_{ch} (see Fig. 7.4). The following equations can be used to describe the partial pressure of CO_2 at the site of fixation:

$$C_C = C_i - \left(A_n + F + R_d\right)\left(r_{wp} + r_{ch}\right) + \left(F + R_d\right) r_{wp} \tag{7.12}$$

$$C_C = C_i - A_n\left(r_{wp} + r_{ch}\right) - \left(F + R_d\right) r_{ch} \tag{7.13}$$

The problem of internal resistances for the fluxes of CO_2 from the mitochondria and from outside the cell had been addressed previously by Price et al. (2011) and reviewed by von Caemmerer (2013) resulting in the same equation 7.13 for C_c.

From Eqs. 7.1 and 7.13 it follows that a non-negligible r_{ch} would result in g_m declining with increased photorespiration rates. Although this has been experimentally observed, the magnitude of this effect is rather small and may be unsuitable for obtaining reliable estimates of r_{ch} and r_{wp} (Tholen et al. 2012a). Moreover, a response of g_m to high O_2 or low CO_2 may be explained by other factors, such as incorrect model assumptions (Sect. 2; von Caemmerer 2013; Gu and Sun 2014).

It must be stressed that the model described by Eq. 7.13 is still a simplification and the resistance between mitochondria and intercellular airspaces is not necessarily equal to the resistance experienced by atmospheric CO_2 diffusing across the cell wall (here both termed r_{wp}). Moreover, Eq. 7.12 assumes that the cytosol has no resistance and the flux of CO_2 from the mitochondria completely mixes with that from the intercellular airspace before carboxylation takes place (as if the mitochondria were in the cytosolic space between chloroplasts and cell wall). However, examination of the relative position of chloroplasts and mitochondria in the mesophyll cells (Van Gestel et al. 2002; Oikawa et al. 2003; Wada and Suetsugu 2004; Sage and Sage 2009; Hatakeyama and

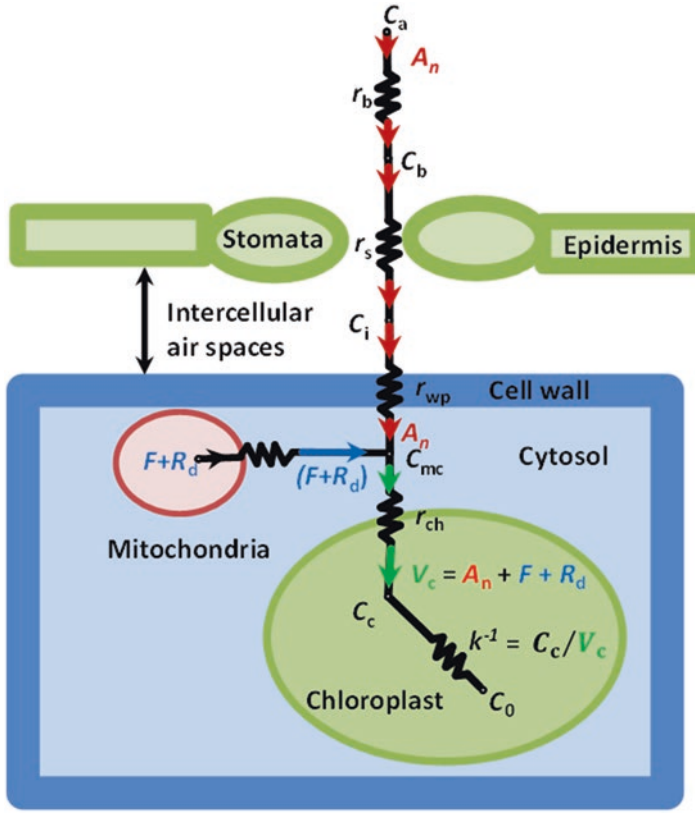

Fig. 7.4. Schematic representation of the source- sink pathways for CO_2 to the sites of carboxylation (chloroplast stroma) proposed by Tholen et al. (2012a). Red arrows represent the net CO_2 influx (A_n) from the atmosphere and the blue ones the (photo)respiratory release ($F + R_d$) assigned to mitochondria. In the cytosol of a mesophyll cell (C_{mc}) both CO_2 fluxes merge into the carboxylation rate (V_c, green arrow). Once CO_2 is fixed by Rubisco the partial pressure of CO_2 becomes null (C_0). The biochemical resistance associated with the carboxylation rate is represented by k^{-1}, and is defined as the inverse of the carboxylation efficiency ($k = V_c/C_c$). The resistance of cell wall and plasma membrane are grouped in r_{wp} and the resistances of the double membranes of the chloroplasts and the stroma resistance are grouped as r_{ch}. r_b and r_s represent the resistance of the boundary layer and stomata to the diffusion of CO_2 from the atmosphere (C_a) to outside the stomata (C_b) and from there to the intercellular air spaces (C_i), through the stomata. Resistance of the intercellular air space to CO_2 diffusion is omitted in this model

Ueno 2017) shows that the mitochondria are often located more towards the center of the cell and a large surface area of the cell is occupied by chloroplasts. This suggests that photorespiratory CO_2 may often diffuse through the chloroplasts or chloroplast stromules to reach the atmosphere, much reducing the effect of $F + R_d$ in Eqs. 7.12 and 7.13.

One solution to deal with the problem of organelle position is treating r_{ch} as an 'effective' resistance that incorporates part of the structure. Mitochondria being located behind a chloroplast will thus reduce the effective r_{ch} (even if the stromatal, cytosolic, and membrane resistances stay constant). In the extreme case where the chloroplasts occupy all the surface of mesophyll cells facing intercellular air spaces, r_{ch} would approach zero and Eq. 7.13 will be equivalent to the classical equation 7.1 (von Caemmerer 2013). However, the concept of 'effective' resistance remains problematic because it conflates the permeabilities with structural parameters such as chloroplast arrangement.

Yin and Struik (2017) recently proposed to generalize the model by adding an additional parameter to account for the proportion of $F + R_d$ that would affect g_m. It remains unclear at this time if such more complex models can be experimentally verified. If the goal is to describe subcellular diffusion accurately and in detail, it seems that using reaction diffusion models are a better option (Rand and Cooke 1980; Parkhurst 1994; Tholen and Zhu 2011). For all models, the biggest uncertainties remain regarding the biophysical diffusion properties of the different components of the diffusion pathway and the role of biochemical facilitation of diffusion (see Evans et al. 2009 and Sects. 5 and 6 of the present chapter for more details).

IV. Mesophyll Conductance to CO_2 in Different Plant Groups and Its Co-Regulation with Leaf Hydraulics

A. *Mesophyll Conductance to CO_2 in Different Plant Groups*

Thus far, g_m has been estimated for more than 200 species of land plants (i.e., embryophytes). More than 80% of the estimated values are for angiosperms and gymnosperms (i.e., spermatophytes; Rho et al. 2012; Flexas et al. 2013a; Muir et al. 2014; DaMatta et al. 2016; Veromann-Jürgenson et al. 2017), plus some recent values for ferns (15%; Volkova et al. 2009; Carriquí et al. 2015; Tosens et al. 2015), while very few data are available for mosses (Williams and Flanagan 1998) and for liverworts and hornworts (Meyer et al. 2008). Still, by comparing the existing datasets for these different phylogenetic groups, interesting information can be extracted (Fig. 7.5). There are differences in the average g_m between the taxonomic groups. These differences in g_m – as well as those for stomatal conductance, g_s – seem to follow an evolutionary trend. Highest values for g_m are found in spermatophytes, especially for grasses and herbs, contrasting with the low values for conifers. Lower average values than in the conifers were found in ferns and, although with very few data available, the lowest values were observed for non-vascular lineages, i.e., mosses, liverworts, and hornworts.

Comparing the average rate of g_m for the different functional groups inside angiosperms, some significant differences can also be found. The lowest values are for evergreen shrubs and trees, similar to those for gymnosperms. The next highest groups are the semi-deciduous and deciduous shrubs and trees, with values of g_m hovering around 0.2 mol CO_2 m^{-2} s^{-1}, and finally herbs and grasses have the highest estimated values of g_m. Therefore, these results suggest that part of the observed variation corresponds to the adaptation to different habitats or life forms, in addition to possible phylogenetic trends. For instance, only within a single genus, *Solanum*, the values of g_m estimated at light saturation and ambient temperature for different species ranged between 0.15 and 0.25 mol CO_2 m^{-2} s^{-1} (Muir et al. 2014). Even larger ranges have been found for *Quercus* (0.03–0.09 mol CO_2 m^{-2} s^{-1}; Peguero-Pina et al. 2017) and *Limonium* species (0.14 and 0.35 mol CO_2 m^{-2} s^{-1}; Galmes et al. 2017), or even within a single species, *Vitis vinifera* (0.14 and 0.22 mol CO_2 m^{-2} s^{-1}; Tomas et al. 2014).

Estimates of g_m for ferns are two-fold lower than the average value for conifers and seven-fold lower than values for grasses. This is coupled with the fact that ferns have a very different stomatal functioning, showing predominantly passive hydraulic stomatal control and strong hydraulic limitation of photosynthesis (Brodribb and McAdam 2011; Zhang et al. 2014). Moreover, Tosens et al. (2015) showed that these low values are at least in part caused by characteristic frond anatomical traits, especially because of notably large cell wall thickness and low chloroplast distribution towards intercellular air spaces (See Sect. 5). Even within ferns and

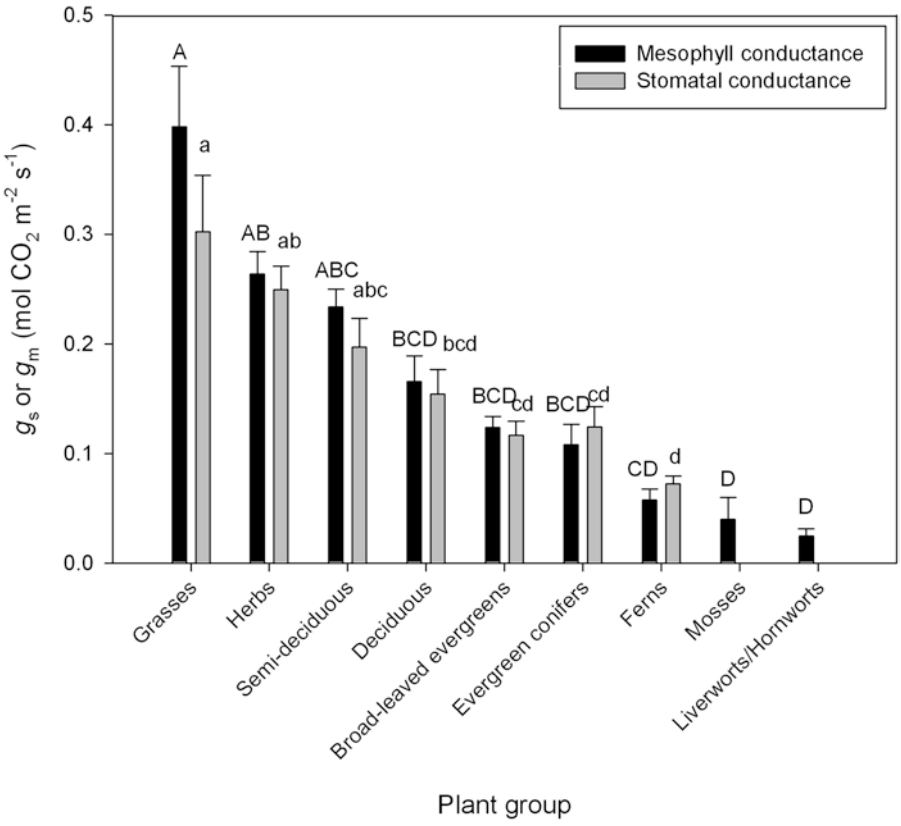

Fig. 7.5. Taxonomic distribution of stomatal (g_s) and mesophyll (g_m) conductances. Values are averages ± standard error for each group of plants. (Data from liverworts/hornworts is from Meyer et al. (2008), data for mosses is from Williams and Flanagan (1998), data for ferns is from Tosens et al. (2015), and data for all other groups from Rho et al. (2012), Flexas et al. (2013a, b), Carriquí et al. (2015), Muir et al. (2014), and DaMatta et al. (2016). Only data at light saturation and ambient temperature were considered. Differences between means are indicated in capital letters for g_m and in lower case letters for g_s (Tukey Test, $p < 0.05$, n = 9, 38, 2, 33, 83, 13, 33, 2, and 6 for grasses, herbs, semi-deciduous, deciduous, broad-leaved evergreen, evergreen conifers, ferns, and mosses and liverworts/hornworts species, respectively)

fern allies, significant variability is observed, with horsetails showing the largest and lycophytes the lowest values (Tosens et al. 2015). Variability is also marked by fern lifestyle, as it has been proposed that some reophytic ferns could absorb CO_2 directly from the leaf surfaces, as their epidermal chloroplasts face outside instead of the intercellular spaces (Kato and Imaichi 1992; Murakami 1995).

Regarding bryophytes, estimates of g_m or g_t (as they do not have stomata, see Sect. 1) are much lower compared to those for vascular plants, especially in comparison with values for grasses, which are 10 to 16-fold higher. The gametophytes of bryophytes lack stomata and show a low degree of cuticularization (Proctor 2010), having leaf-like organs that range from a simple substrate-attached thallus of a few cell-layers thick to a one cell layer thick leaf-like structure (Hanson et al. 2014). The low number of estimations for bryophytes is in part associated with the difficulty of measurement, especially under optimal photosynthetic conditions.

In summary, a large variability in g_m is found among organisms, but the phylogenetic trend obtained so far needs to be reinforced by compiling data of still under-represented groups. These data should

be acquired under the same developmental and environmental conditions to elucidate phylogenetic implications from g_m. This knowledge will help understand the evolution of g_m and the mechanisms involved in its regulation.

B. Co-Regulation of Mesophyll Conductance with Leaf Hydraulics

CO_2 pathways inside the leaf are shared, at least partially and often in the opposite direction, with water. Leaves exchange both water vapor and CO_2 with the atmosphere through stomata and both substances diffuse through the gas-phase in the sub-stomatal and intercellular cavities. Once liquid water leaves the leaf xylem, it moves via apoplastic, symplastic, and transcellular pathways (partially mediated by aquaporins, which facilitates diffusion of water but also of CO_2; See Sect. 6) to the evaporation sites, thereby sharing in part diffusion pathways with the CO_2 in the mesophyll (Sack et al. 2004). In this sense, some correlation between g_m, g_s, and leaf hydraulic conductance (K_{leaf}) may be expected.

1. Leaf Hydraulic Conductance (K_{leaf})

g_m and K_{leaf} quantify the effectiveness of the diffusion or mobilization of different substances, in partially distinct leaf tissues, by different physical processes, and are estimated with completely different measurement techniques. Until recently, both characteristics were not studied together. Despite this, both traits contribute strongly to the determination of maximum rates of photosynthesis and its dynamics under varying environmental conditions, and they partly share an anatomical basis. This translates to some extent in a correlation between g_m and K_{leaf}. Flexas et al. (2013a) first showed a correlation between both parameters under steady-state conditions across a diverse range of species, which was recently reinforced by Xiong et al. (2015a) by comparing *Oryza* genotypes (Fig. 7.6). Flexas et al. (2013a) also demonstrated that both g_m and K_{leaf} are dynamic traits at a huge range of time scales (Sack and Holbrook 2006; Flexas et al. 2008) and show similar responses to several different environmental variables,

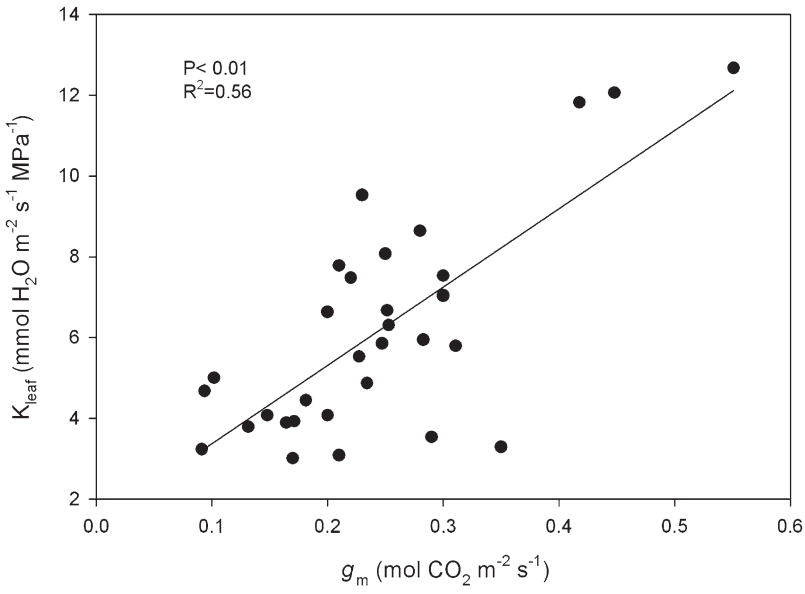

Fig. 7.6. Correlation of mesophyll conductance (g_m) with leaf hydraulic conductance (K_{leaf}) across *Oryza* genotypes. (Data from Xiong et al. 2015a)

such as to a decline in water status or to an increase in light, VPD, or temperature. This supports the notion that g_m and $K_{leaf.}$ are mechanistically coordinated. Nevertheless, mechanisms that would explain such co-regulation between both conductances are not fully understood (Flexas et al. 2012; Griffiths and Helliker 2013). K_{leaf} consists of the xylem hydraulic conductance and the outside-xylem hydraulic conductance. Since CO_2 shares only the part outside of the xylem with water, it has been speculated that the latter part of K_{leaf} would correlate better with g_m (Flexas et al. 2013a).

2. *Stomatal Conductance (g_s)*

From a theoretical perspective, a co-regulation between g_s and g_m may be expected, simply because high g_s and g_m are both needed in order to obtain maximized photosynthesis. Another reason to expect a correlation between both traits is the close relationship often found between K_{leaf} and g_s (Sack and Holbrook 2006). Indeed, g_s and g_m tend to be positively related across diverse species (Flexas et al. 2013a) and show parallel responses to drought stress, irradiance, CO_2, and temperature (Buckley and Warren 2014). Nevertheless, g_s and g_m also respond independently of one other under some conditions. For instance, during the midday depression of photosynthesis, when an initial decrease of g_s was accompanied by an increase of g_m (Pons and Welschen 2003).

The inter-dependency of g_s and g_m has been debated from a theoretical water-use efficiency perspective (i.e., WUE = A_n/g_s), as some uncoupling between both traits (at constant photosynthetic activity) would be advantageous. Water-use efficiency can be increased by increasing the g_m to g_s ratio under steady-state conditions. This has been observed under several conditions and in different species. In most cases, an increased g_m/g_s ratio resulted in a decreased net photosynthesis, such as in tree populations grown under a dry climate compared with their relatives from a temperate climate (Duan et al. 2009; Soolanayakanahally et al. 2009), in tomato plants subjected to drought-stress (Galmés et al. 2011), and in grasses which show a progressive increase in g_m along the leaf, from the base to the leaf tip (Kodama et al. 2011). However, few studies found concomitant increases of the g_m/g_s ratio and A_n, as, for example, in some conifer species where g_m/g_s was higher as height declined and A_n increased (Montpied et al. 2009; Woodruff et al. 2009; Boegelein et al. 2012).

V. Structural Determinants of Mesophyll Conductance

As stated in previous sections, mesophyll conductance (g_m) is defined as the efficiency of CO_2 diffusion from intercellular spaces across the cell wall, plasma membrane, cytosol, chloroplast envelope, and stroma to Rubisco (Evans et al. 2009; Terashima et al. 2011; Tholen and Zhu 2011). Evidence suggests that each of these components has a certain diffusion resistance to CO_2, and their contributions to CO_2 diffusion resistance vary with species (see Sect. 4; Tosens et al. 2012b, 2015; Giuliani et al. 2013; Tomas et al. 2013) and environment (see Sect. 7; Kogami et al. 2001; Oguchi et al. 2003; Galmés et al. 2013; Xiong et al. 2015b, 2016). Therefore, redesigning the leaf anatomy has a large potential to improve photosynthetic efficiency (Tholen et al. 2012b; Ort et al. 2015). In this section, we summarized our current understanding of how components in the mesophyll influence g_m.

Intercellular airspaces are the first layer of resistance to CO_2 diffusion inside many photosynthetic tissues. Resistance to diffusion through intercellular airspace has been investigated by comparing gas exchange under normal air with that in helox (air where helium replaces nitrogen to increase diffusivity; 21% O_2, 79% He) in several studies (Parkhurst and Mott 1990) and showed that the changes of photosynthesis in helox

depends on the species under investigation; for example, the enhancement of photosynthesis was greater in hypostomatous leaves. The CO_2 diffusion conductance in the intercellular air space consists of dorsiventral and lateral conductance, and both conductances are influenced by the effective porosity of the mesophyll, leaf thickness, and the density and arrangement of the stomata (i.e., hypostomatous, epistomatous, amphistomatous). Several studies have investigated lateral diffusion and claimed that it may be of the same order of magnitude as the dorsiventral diffusion in homobaric leaves (Morison et al. 2005; Lawson and Morison 2006; Pieruschka et al. 2006). In heterobaric leaves, lateral diffusion may be strongly limited due to the insulating effect of the tightly-packed bundle-sheath extensions along the leaf vasculature. In addition to leaf thickness and mesophyll porosity, the dorsiventral conductance is also affected by the temperature gradient within the leaf that is strongly affected by environmental conditions and leaf anatomy (Rockwell et al. 2014; Buckley 2015; Buckley et al. 2017). Although it is likely that gaseous diffusion plays an important role in g_m variation among species and genotypes (see Chap. 5) and in response to different environmental conditions, it is frequently ignored because the diffusion of CO_2 in gaseous phase is 10^4 times faster than that in liquid phase (Evans et al. 2009; Tholen et al. 2012b).

After diffusion through intercellular airspaces, CO_2 dissolves in the apoplastic water at cell wall surface. Mesophyll cells are surrounded by a polysaccharide-rich primary wall that, together with the middle lamella, accounts for most of the apoplast in growing tissues. Hence, the resistance to CO_2 diffusion across the cell wall is related to cell wall porosity, tortuosity, and thickness. The role of the cell wall porosity and tortuosity on CO_2 diffusion is not well documented. In contrast, an effect of cell wall thickness on g_m has been widely acknowledged. A negative relationship between g_m and cell wall thickness (T_{cw}) was observed across species or genotypes of a given species (Evans et al. 2009; Terashima et al. 2011; Tomas et al. 2013). Due to the low diffusion rate of CO_2 in the liquid phase, the most effective pathway via cell wall and cytosol is the shortest pathway, i.e., that across the chloroplast surface exposed to the intercellular airspace (Terashima et al. 2011). Many studies have reported that g_m positively correlates with chloroplast surface exposed to the intercellular airspace (S_c) across species, genotypes of the same species, and different treatments (Tholen et al. 2008; Galmés et al. 2011; Terashima et al. 2011; Tomas et al. 2013; Carriquí et al. 2015; Tosens et al. 2015; Xiong et al. 2015b, 2016). Hence, the relation between T_{cw} and CO_2 diffusion conductance per unit chloroplast surface exposed to the intercellular airspace (g_m/S_c) can be plotted to evaluate the limitation of cell wall thickness on g_m (Evans et al. 2009; Terashima et al. 2011; Tomas et al. 2013). It has been observed in some studies that T_{cw} and S_c can be affected by environmental changes, for instance drought and light (Tholen et al. 2008; Tosens et al. 2012a; Galmés et al. 2013), and this impacts g_m. Also, the supply of nutrients can also affect chloroplast development and T_{cw}; chloroplast size can be dramatically enhanced by N supplement in rice (Xiong et al. 2015b, c; Fig 7.7).

Since g_m is affected by leaf anatomical traits, models have been developed that relied on anatomical and physical parameters. In several one dimensional diffusion models using the resistance (or conductance) concept, the diffusivity of each individual component along the diffusion path was combined to estimate g_m (Tosens et al. 2012b; Tomas et al. 2013; Peguero-Pina et al. 2015). First, g_m is divided in a gas-phase conductance between the sub-stomatal cavities and the outer surface of cell walls (g_{ias}), and a liquid-phase conductance between the outer surface of the cell walls and the site of carboxylation in the chloroplast stroma (g_{liq}):

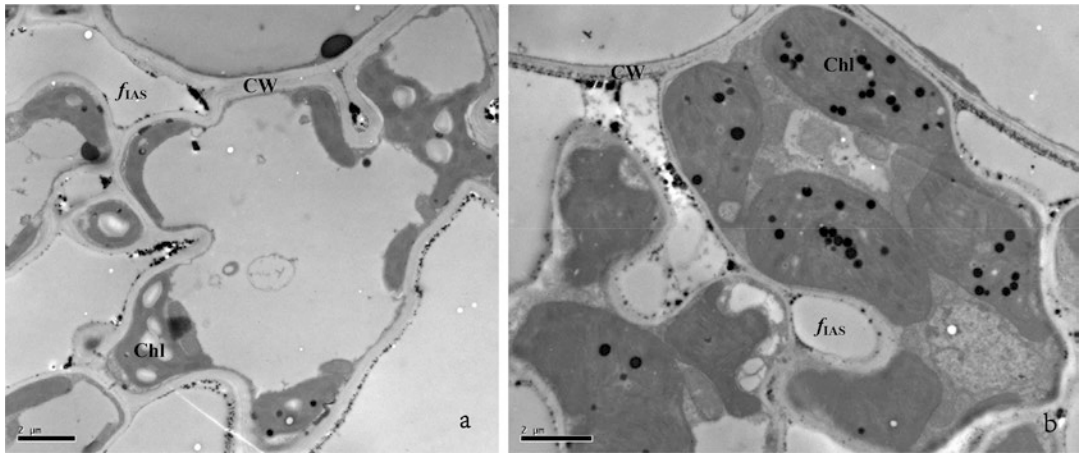

Fig. 7.7. Effects of N supplement on chloroplast size and cell wall thickness of mesophyll cells in rice (cv. Wuyungeng 7). (**a**) Low N supplement and (**b**) high N supplement. Plants were grown in 13 L pots outdoors in Wuhan, China, between May and July of 2013. Leaf samples were collected 55 days after transplanting. The minimum, maximum, and daily mean temperatures during the growth period were 21.1, 34.7, and 28.4°C, respectively

$$g_m = \frac{1}{\frac{1}{g_{ias}} + \frac{RT_k}{H \cdot g_{liq}}} \tag{7.14}$$

where R is the gas constant, T_k is the absolute temperature, and H is the Henry constant.

The g_{ias} is calculated based on the fraction of mesophyll volume occupied by intercellular air space (f_{ias}) and the diffusion path length in the gas phase (L_{ias}), which is generally assumed to be half of the mesophyll thickness for hypostomatous leaves:

$$g_{ias} = \frac{D_a \cdot f_{ias}}{L_{ias} \cdot \varsigma} \tag{7.15}$$

where D_a ($m^2\ s^{-1}$) is the diffusion coefficient for CO_2 in the gas phase (1.51×10^{-5} at 25 °C), and ς is the diffusion path tortuosity ($m\ m^{-1}$). In theory, the ς can be obtained from leaf sections with the help of a microscope, but this estimation is imprecise due to the great variation of cell shapes. Hence, an estimated value of ς is often used in practice. Another debatable aspect relates to the determination of L_{ias}. Because the internal endpoint of the diffusive pathways represented by g_s (and therefore the "assumed location" of C_i) is variable with environmental conditions and anatomy, including not only the stomata pore but also and additional and unknown section of the mesophyll, L_{ias} calculated in this way may be somewhat biased (Buckley et al. 2017). In practice, rather than estimating L_{ias} the leaf thickness is often used as a proxy.

The g_{liq} is calculated as the sum of serial diffusion resistances (r):

$$g_{liq} = \frac{1}{\sum \frac{1}{g_i}} \cdot S_c = \frac{1}{\sum r_i} \cdot S_c \tag{7.16}$$

where g_i ($1/r_i$) is the conductance of a given component of the diffusion pathway (i.e., through cell wall, plasmalemma, cytosol, chloroplast envelope, or chloroplast stroma). Because g_{liq} is also limited by surface area available for diffusion, the conductance scales with S_c (Tosens et al. 2012b; Tomas et al. 2013). The conductance of a given component of the diffusion pathway can be calculated as:

$$g_i = \frac{D_w \cdot p_i \cdot \gamma_i}{\Delta L_i} \quad (7.17)$$

where D_w is the aqueous phase diffusion coefficient for CO_2, p_i is the effective porosity, L_i is the diffusion path length that is usually represented by the thickness of a component, and γ_i is a dimensionless factor accounting for a decrease of diffusion conductance in the cytosol and in the stoma compared with free diffusion in water. Due to technical limitations, estimates for p_i are often used, but this introduces a large amount of uncertainty in the model predictions. The advantage of this type of model is that it can link g_m directly to individual leaf anatomical properties (Peguero-Pina et al. 2015) and allow the identification of factors that are most limiting for diffusion. A disadvantage, as described earlier, is that such a model does not account well for the effect of multiple CO_2 sinks and sources in the leaf. In addition, even without having to describe the leaf anatomy in detail, many parameters in these models rely on values that cannot yet be accurately measured.

Traditionally, it was thought that lipophilic gases, including CO_2, could pass easily through the lipid bilayers of for example plasma and chloroplast membranes, because of their high lipid phase solubility (Meyer 1899; Overton 1901). CO_2 indeed shows very high solubilities in solvents containing carbon–oxygen bonds (such as the ester bonds in oils or in a phospholipid bilayer) and this results in higher solubility of CO_2 compared to gases like N_2 or O_2. However, at the same time CO_2 is almost as soluble in water as in phospholipids (the phospholipid-water partition coefficient is therefore close to 1; Perisanu 2001; Endeward et al. 2014). The high CO_2 permeability of lipid bilayers is thus associated not only with the solubility of the gas molecule in the membrane, but also dependent on the diffusion resistance (i.e. diffusivity) of the gas molecule within the membrane (Finkelstein 1976). The measured CO_2 transport rate through biological membranes is 10–1000 times lower than that obtained for pure lipid bilayers (Yang et al. 2000; Evans et al. 2009; Otto et al. 2010; Kaldenhoff 2012; Uehlein et al. 2012; Muir et al. 2014). A biological membrane contains many proteins; for instance the chloroplast thylakoid membranes consists out of more than 70% protein and less than 30% lipid (Tremmel et al. 2003; Engelman 2005). Taking into account protein shape, membrane-intrinsic and membrane attached-proteins, the membrane surface area available for easy diffusion must be reduced significantly, but such membranes also contain other molecules that reduce the diffusivity of CO_2, like for example sterols (Itel et al. 2012). These properties of biological membranes explain why plant membranes may not be very permeable to CO_2 and in this case aquaporins (AQPs), a family of pore-forming integral membrane proteins that function as diffusion facilitators for small molecules, could be mandatory for an efficient CO_2 diffusion. In addition, there is abundant functional evidence showing that some AQPs enhance CO_2 diffusion into mesophyll and stomatal guard cells (see Sect. 6 and Groszmann et al. 2017).

In addition to diffusion through cell wall and biological membranes, CO_2 also diffuses through the cytosol and chloroplast stroma (Evans et al. 2009). Although the cytosol layer between plasma membrane and chloroplast envelope membrane is normally very thin, the pathway through the cytosol may be somewhat longer when the amount of chloroplast surface area exposed to intercellular airspaces is small (Tomas et al. 2013). Diffusion through the cytosol and chloroplast may be mediated by carbonic anhydrases (see Sect. 6). In addition to all these cellular components, the thylakoid membranes, starch granules, and mitochondria may all impact CO_2 diffusion.

Understanding the contribution of each structural component in the mesophyll to g_m is important for identifying factors that could

be altered to achieve enhanced rates of photosynthesis. However, it is not yet possible to estimate the resistances of these components directly. Several studies have evaluated these resistances based on model simulations (Evans et al. 2009; Terashima et al. 2011; Tholen and Zhu 2011; Tomas et al. 2013; Berghuijs et al. 2015; Veromann-Jürgenson et al. 2017). Results from these studies indicate that cell wall thickness and chloroplast surface exposed to the intercellular air space are probably the two structural traits that have the greatest impact on restricting CO_2 diffusion inside leaves. However, these simulations are based on a large number of assumptions rather than direct experimental estimations. Moreover, the effects of cell wall porosity and composition, and carbonic anhydrases in cytosol and stroma, are still unclear (see next section). In summary, the fundamental structural factors that affect g_m remain poorly understood.

VI. Biochemical Determinants of Mesophyll Conductance

Because mesophyll conductance to CO_2 (g_m) has been shown to considerably and rapidly vary in response to environmental changes (see Sect. 7; Centritto et al. 2003; Uehlein et al. 2003; Flexas et al. 2008; Miyazawa et al. 2008), it has been suggested that, in addition to the effect of morphoanatomical leaf traits discussed in the previous section, biochemical determinants such as enzymatic or protein-facilitated diffusion may also exert some influence over g_m (Bernacchi et al. 2002). There are two main proposed facilitation mechanisms that could improve the diffusivities of CO_2 in lipid and aqueous phases (Terashima et al. 2011; Tomas et al. 2013): membrane-bound aquaporins (Uehlein et al. 2003, 2008; Hanba et al. 2004; Flexas et al. 2006b; Miyazawa et al. 2008; Heckwolf et al. 2011; Kawase et al. 2013; Yaneff et al. 2015) and cytosol and stromal forms of carbonic anhydrases (Price et al. 1994; Williams et al. 1996; Gillon and Yakir 2000; Perez-Martin et al. 2014). A biochemical control of g_m would not only help explain rapid changes of g_m in response to environmental changes, but also the discrepancies often found between in vivo estimates of g_m and estimates based on physical models and the anatomical properties of the leaves (g_{mA}; see Sect. 5 and Tomas et al. 2013 for details on its estimation). For instance, when leaf metabolism is altered in response to stressful environmental conditions, such as drought in grapevines (Tomas et al. 2014) or photoinhibition in *Ligustrum vulgare* (Fini et al. 2016), differences between g_m measured with the variable-*J* method and g_{mA} estimated by anatomical parameters become very large, likely reflecting increasing contributions of biochemical or photochemical constraints. In addition, rearrangement of anatomy can further limit g_m in leaves (Tomas et al. 2014; Fini et al. 2016). Currently used resistance models for g_m do not consider the effects of aquaporins that serve as channels for CO_2 (COO-porins, see below) on membrane permeability for g_{mA} estimation (but see Evans et al. 2009 for model extensions that could take this effect into account). This may partially explain some of the observed discrepancies between g_m and g_{mA} (Tosens et al. 2012b; Tomas et al. 2014; Fini et al. 2016).

A. *The Role of Aquaporins (COO-porins)*

Experimental evidence indicates that specific aquaporins facilitate CO_2 diffusion and are involved in the control of mesophyll CO_2 conductance (g_m) in leaves (Uehlein et al. 2003; Otto et al. 2010; Abascal et al. 2014; Yaneff et al. 2015). Uniquely among plant aquaporins, PIPs also transport CO_2 (Groszmann et al. 2017), being sometimes referred to as COO-porins (Terashima et al. 2006). In particular, there is solid evidence from heterologous and homologous systems that some PIP1 and PIP2 aquaporins are functional CO_2 channels, which may allow leaves to sustain high rates of photosynthesis

(Perez-Martin et al. 2014; Sade et al. 2014; Yaneff et al. 2015; Groszmann et al. 2017).

Based on experiments with mercury-induced blocking of aquaporins, Terashima and Ono (2002) were the first to suggest a role for aquaporins in the regulation of g_m. Afterwards, Uehlein et al. (2003) showed in vitro that the tobacco PIP1 aquaporin could transport CO_2 when expressed in the heterologous *Xenopus* oocyte system. Additional experiments with mercury (Miyazawa et al. 2008) and with plants expressing altered levels of several aquaporins (Hanba et al. 2004; Flexas et al. 2006a; Uehlein et al. 2008; Heckwolf et al. 2011; Kawase et al. 2013; Sade et al. 2014) have provided strong and consistent evidence for a relationship between aquaporins and g_m. Nevertheless, in all these studies modification of aquaporin expression has resulted not only in modifications of g_m, but also in altered stomatal conductance, and Bi et al. (2015) concluded that PIP proteins not only play essential roles in whole leaf water and CO_2 flux but have important roles in the regulation of stomatal movement.

Besides other uncertainties, the mechanism by which aquaporins (AQP) alter g_m remains unclear. While one possibility is the simple role of specific COO-porins as CO_2 channels, as originally suggested (Uehlein et al. 2003), it is unclear why the very same AQP can contribute to water and/or CO_2 transport depending on its tissue-specific expression in plants (Uehlein et al. 2008; Sade et al. 2014; Verdoucq et al. 2014). Otto et al. (2010) showed that artificially-constructed aquaporin tetramers with different proportions of PIP1 and PIP2 aquaporins induced opposite changes in water and CO_2 permeability, which matches in vivo observations of g_m and water conductivity in PIP1 anti-sense and over-expressing tobacco (Flexas et al. 2012). Aquaporin heteromerization has recently been demonstrated to occur in nature and to affect the intrinsic permeability of membranes (Yaneff et al. 2014, 2015). But the relationship between g_m and AQPs can be even more complex, as illustrated by a recent finding showing an interaction between bacterial harpin Hpa1 and *Arabidopsis thaliana* PIP1;4 in the modulation of g_m and photosynthesis (Li et al. 2015). In addition, reduced mesophyll CO_2 conductance in an *A. thaliana AtPIP1;2* T-DNA insertion line resulted in an altered transcription pattern resembling that obtained under limited CO_2 concentrations rather than that obtained under water stress, supporting the hypothesis that the function of AtPIP1;2 is to facilitate CO_2 and not water diffusion. It also opens the possibility that the role of AQP is not (only) mediated by direct channelling of CO_2 through membranes (Boudichevskaia et al. 2015).

Despite the lack of consensus as to how AQPs influence g_m, their activity may explain some physiological observations related to environmentally-induced regulation of photosynthesis. As an example, Li et al. (2009) observed decreased photosynthetic nitrogen use efficiency (PNUE) in rice under high-N supply, and suggested that this may be due to a reduction in C_c, resulting in a lower Rubisco activation state and ultimately limiting photosynthesis under high-leaf-N conditions. Recently, the same group studied how AQPs mediated chloroplastic CO_2 concentration under different N-supply levels, finding that C_c and g_m decreased in the *Ospip1;1* mutant line as compared to wild-type rice. They could then confirm that under high-N supply, the decreased PNUE was indeed associated with insufficient C_c mediated by AQP effects on mesophyll conductance (Ding et al. 2016). Another promising aspect of plant aquaporin is their cellular trafficking between subcellular membranes, which determines changes in the amount and density of AQP (Uehlein et al. 2008; Maurel et al. 2009; Li et al. 2014). Even though, the signals that target aquaporins to other cell compartments are poorly known, their dynamic rearrangements seem mainly modulated by their phosphorylation status (Verdoucq et al. 2014) and other cell parameters including cytosolic

protons (H^+), calcium (Ca^{2+}) and H_2O_2 accumulation. The relocation of AQP arises as an useful mechanism to improve the understanding of the rapid responses of g_m and their acclimation to environmental conditions and hormonal stimuli (Boursiac et al. 2008; Maurel et al. 2009; Li et al. 2014; Groszmann et al. 2017). In general, phosphorylated AtPIP2;1 reaches the plasma membrane (PM) by trafficking through the Golgi apparatus prior to synthesis in the endoplasmic reticulum (ER). In this sense, kinase activity could lead to the internalization or endocytosis of AtPIP2;1, inducing the trafficking of PIPs to putative endosomal compartments supporting aquaporin storage, recycling or degradation. PIP internalization may be seen as an acclimating response to adverse conditions, such as osmotic, salt and salicylic acid stresses, and for several species like Hot pepper, Maize, *Mesembryanthemum* and *Arabidopsis* (Boursiac et al. 2005, 2008; Maurel et al. 2009; Sade et al. 2014; Li et al. 2014; Groszmann et al. 2017).

B. The Role of Carbonic Anhydrase (CAs)

The flux of carbon dioxide from atmosphere to the sites of carboxylation depends on the concentration gradient and the diffusivity of the liquid phase (Eq. 7.1). CO_2 in solution is slowly hydrated to bicarbonate, but the rate of this reaction can be drastically increased in the presence of CA. If CA activity is not limiting, this results in bicarbonate concentrations (and thus concentration gradients) that are up to 50 times higher than those of CO_2 in an alkaline environment such as the chloroplast stroma (Evans et al. 2009). Because the diffusion coefficient of bicarbonate is about half that of CO_2, the presence of CA allows for diffusion to be enhanced by about 25x at the pH values found in the chloroplast stroma (Evans et al. 2009). Since a significant part of the diffusion pathway is through the chloroplast stroma, and most of the CA activity in leaves is localized to the chloroplast stroma (Evans et al. 2009), a role for CA in facilitating diffusion in photosynthetic tissue has long been suspected (Cowan 1986).

There is some descriptive evidence for a correlation between CA expression and activity and g_m (Gillon and Yakir 2000; Perez-Martin et al. 2014). Theoretical studies predict that CA would indeed increase g_m, but would only have a minor effect on photosynthesis (<10%; Cowan 1986; Tholen and Zhu 2011). In agreement with this, reduction of CA activity in *Nicotiana tabacum* reduced mesophyll conductance by up to 30%, but no significant differences in photosynthesis were found (Price et al. 1994; Williams et al. 1996). Gillon and Yakir (2000) suggested that an effect of CA on photosynthesis may be more noticeable in species with a low g_m.

In leaves, CA isoforms are expressed in different tissues and several subcellular compartments (Fabre et al. 2007), but little is known about the physiological function of membrane-bound and cytosolic CA isoforms. Rumeau et al. (1996) estimated that up to one-third of the total CA activity is present in the cytosol. When the path through the cytosol is long, such as may occur in plants with a low S_c, some facilitation effect can be expected but the effect on photosynthesis should again be minimal (Tholen and Zhu 2011). A recent report by DiMario et al. (2016) found no significant effect of several cytosolic CA isoforms on photosynthesis in *Arabidopsis thaliana*.

C. The Potential Role of Other Biochemical Processes

Besides AQPs and CAs, other metabolic processes have been shown to be related to the regulation of g_m. In the case of transgenic tobacco expressing excess phytochrome (Sharkey et al. 1991) or *Arabidopsis* mutants unresponsive to ethylene (Tholen 2005), effects on g_m may be the result of alterations in chloroplast shape or arrangements, but in other cases the mechanisms remain even

more elusive than in the case of AQPs. Among these, several metabolic components related to cell redox regulation, including Complex I of the mitochondrial electron transport (Priault et al. 2006; Juszczuk et al. 2007; Galle et al. 2010) and Thioredoxin f (Aranjuelo et al. 2015).

Two recent reports have proposed other metabolic components for the regulation of g_m. First, Gong et al. (2015) showed that the transgenic expression in rice of the cyanobacterial *Ictb* gene, which is involved in carbon concentration mechanisms, resulted in increased g_m and photosynthesis. Second, Medeiros et al. (2015) reported that inefficient regulation of stomatal closure via repression of the organic acid transport *AtQUAC1* culminates in higher growth and photosynthetic rates through increased g_s and g_m.

In conclusion, some evidence indicates that there are biochemical determinants of g_m, but the exact nature of these and its precise mechanistic operation remains elusive.

VII. Environmental Responses of Mesophyll Conductance

Many studies have reported that g_m responds to diverse environmental factors both in the short and long terms (Flexas et al. 2008, 2012; Niinemets et al. 2009). Recently, some researchers pointed out that the responses of g_m to some environmental factors might have problems in terms of artifacts of calculation (see Sects. 2 and 3 for detailed discussion; (Tholen et al. 2012a). However, mesophyll conductance remains recognized as a major factor that explains responses of photosynthesis to environmental factors.

Under drought conditions, g_m decreased concomitantly with stomatal conductance (g_s) in many plant species in short-term experiments ranging from several minutes to hours (Flexas et al. 2006a), as well as in long-term experiments on a scale from weeks to months (Grassi and Magnani 2005; Diaz-Espejo et al. 2007; Galmés et al. 2007; Flexas et al. 2009; Galle et al. 2009; Tosens et al. 2012a; Cano et al. 2013). These responses of g_m to drought conditions were summarized in Flexas et al. (2008), and new experiments have since provided further supporting evidence (Ferrio et al. 2012; Theroux-Rancourt et al. 2014). In addition, some reports have demonstrated that the decrease in g_m recovers slowly after rewatering, further contributing to limited photosynthesis during drought cycles (Flexas et al. 2009; Galle et al. 2009; Warren et al. 2011; Cano et al. 2014). In some cases, however, g_m was not affected even under drought. For instance, g_m was not reduced in soybean until g_s was reduced by up to 90% (Bunce 2009).

The influence of reduced g_s under drought conditions creates an uncertainty in the g_m calculation, because g_m may depend on the CO_2 concentration (Flexas et al. 2007b; Tholen et al. 2012a). If g_m decreases with increasing levels of photorespiration (see Sect. 3), a relationship between g_s and g_m can be expected. To eliminate this effect, several studies have been conducted under low oxygen levels, but this implies that only the isotope discrimination-based technique can be used (see Sect. 2) and there is still a minor effect from respired CO_2 (Evans et al. 1994; Tazoe et al. 2011; Mizokami et al. 2015). In addition to this, under drought conditions, patchy stomatal closure and the contribution of cuticular conductance could induce uncertainty in the estimation of C_i, (Terashima et al. 1988; Boyer et al. 1997; Meyer and Genty 1998) which may result in an incorrect estimation of g_m. As an alternative approach, using the chlorophyll fluorescence-based technique and different ambient CO_2 concentrations, Theroux-Rancourt et al. (2014) demonstrated that g_m is reduced under drought even when compared at identical C_i with well-watered controls, hence providing support for drought-induced reductions of g_m occurring that are not a consequence of an effect of photorespiration on g_m.

Decreased g_m and g_s under drought has important implications, because the ratio g_m/g_s is positively related to water use efficiency (Flexas et al. 2013b). For instance, Theroux-Rancourt et al. (2015) have shown that poplar clones with delayed response of g_m to drought and/or faster recovery after rewatering present larger WUE. Manipulation of the ratio g_m/g_s has been proposed as a key to simultaneously improve photosynthesis and WUE in drought-prone agriculture (Flexas et al. 2015). Consequently, understanding the mechanisms of drought-regulation of g_m would be crucial, but they are still largely unknown. Flexas et al. (2006a) demonstrated that the application of abscisic acid (ABA) from roots in soybean and tobacco resulted in lower C_c and g_m. In contrast, Vrabl et al. (2009) showed that *Helianthus annuus* treated with ABA for three days exhibited no decrease in g_m but had reduced g_s. However, these studies may have had complications in terms of stomatal closure in response to ABA. Recently, Mizokami et al. (2015) examined the possible effects of ABA on decreasing g_m under drought conditions by using tobacco ABA-deficient mutants. Based on indirect evidence using inhibitors (Miyazawa et al. 2008) or gene expression assessment (Perez-Martin et al. 2014), a role for aquaporins and, perhaps, carbonic anhydrases in the regulation of g_m under drought has been proposed (see Sect. 6).

Light responses of g_m differ between conditions of short-term and long-term treatments. As for short-term responses, an apparent decrease in g_m has been shown when measured under reduced light intensity in tobacco (Flexas et al. 2007b) and rice (Xiong et al. 2015b). On the other hand, Tazoe et al. (2009) showed that g_m was independent of variations in light intensity. They carefully considered the effects of light intensity changes and took into account the effects of fractionation associated with photorespiration and day-respiration in calculating g_m. Nevertheless, using the same techniques and considerations, Douthe et al. (2011, 2012) found significant light-induced short term changes of g_m in *Eucalyptus* seedlings. Recently, Gu and Sun (2014) suggested some methodological artifacts that may be responsible for the dependence of g_m on light intensity. Theroux-Rancourt et al. (2017) re-evaluated the response of g_m to light intensity considering a light gradient within a leaf. Altogether, the responses of g_m to short-term light intensity changes require some re-evaluation.

Some reports have also shown an effect of light quality on g_m. Rapid response of g_m to light quality was shown in Tholen et al. (2008) and ascribed to an effect of blue light on chloroplast movement. In their work, these light-induced chloroplast arrangements were shown to affect the S_c in *Arabidopsis thaliana*. Differences in g_m in response to different light qualities were also reported by Loreto et al. (2009).

As for the long-term, the response of g_m to light intensity is explained mainly by well-known differences in leaf morphological traits between sun and shade leaves. Usually, leaves that developed under higher light intensity (sun leaves) exhibited higher g_m than leaves that developed under lower light (Piel et al. 2002; Warren et al. 2007; Niinemets et al. 2009; Cano et al. 2011, 2013). When the morphological traits between sun and shade leaves were compared, sun leaves tended to have thicker leaves and S_c was greater in sun than in shade leaves in several species, thereby enhancing diffusion (Hanba et al. 2002; Oguchi et al. 2005; Tosens et al. 2012b).

In some species, g_m responds strongly to leaf temperature (Bernacchi et al. 2002; Warren and Dreyer 2006; Yamori et al. 2006; Scafaro et al. 2011; Evans and von Caemmerer 2013; von Caemmerer and Evans 2015; Xiong et al. 2015b). In spinach, the optimum temperature for g_m is dependent on the growth temperature, and was 25°C and 20°C for plants grown at day/night air temperature of 30/25 °C and 15/10 °C, respectively (Yamori et al. 2006). According

to Bernacchi et al. (2002), g_m increases exponentially following an increase in temperature from 10°C to 35°C in tobacco, but decreases thereafter. The Q_{10} of g_m was approximately 2.2, which is comparable to the 1.8–2.0 found by Yamori et al. (2006). Because the Q_{10} of CO_2 diffusion in water is about 1.25, larger Q_{10} values described for g_m suggest that this is regulated by a protein-facilitated process (see Sect. 6). On the other hand, in *Eperua grandiflora*, g_m decreased slightly with increasing temperature from 28 °C to 38 °C (Pons and Welschen 2003). The reasons for these different responses of g_m to temperature are unclear. To address this, Evans and von Caemmerer (2013) and von Caemmerer and Evans (2015) used the carbon isotope method, taking into account the new ternary correction (see Sect. 2) to investigate the temperature dependency of g_m in nine different plant species. A variety of responses were observed; for example, g_m varied about three-fold from 15 °C to 40 °C in tobacco, while almost no changes occurred in *Lophostemon confertus* and *Triticum aestivum* over the same range. Therefore, the responses of g_m to temperature seem to be species-dependent.

A decrease in g_m with an increase in CO_2 was reported for many species using different measuring techniques (Flexas et al. 2007a, b; Vrabl et al. 2009; Douthe et al. 2011; Tazoe et al. 2011; Xiong et al. 2015a, b). However, in wheat, g_m hardly decreased in response to an increase in CO_2, but g_s decreased (Tazoe et al. 2009). Some of the observed rapid responses could be methodological artefacts (Gu and Sun 2014). Nevertheless, it seems likely that g_m in wheat is much less responsive to environmental cues such as CO_2 and temperature compared to other species (Tazoe et al. 2009; von Caemmerer and Evans 2015). By using a three-dimensional reaction model, Tholen and Zhu (2011) suggested that leakage of HCO_3 through the chloroplast envelopes may be a possible explanation for the decrease in g_m at high CO_2.

There are few reports of plants grown in elevated CO_2 for longer periods and there appear to be no general trends for the effect of this treatment on g_m (Singsaas et al. 2004; Bernacchi et al. 2005; Crous et al. 2013; Singh et al. 2013; Kitao et al. 2015). For example, according to Singsaas et al. (2004), cucumber grown at a CO_2 concentration of 750 μmol mol^{-1} showed slightly lower g_m compared to plants grown at 500 μmol mol^{-1}; however, sweetgum grown at 560 μmol mol^{-1} had a larger g_m than that grown at 360 μmol mol^{-1}. g_m was unaffected by growth at different CO_2 concentrations in most of the examined species (Singsaas et al. 2004; Crous et al. 2013). Kitao et al. (2015) demonstrated that *Betula* plants grown at elevated CO_2 and low nitrogen supply showed decreased g_m following an increase in leaf starch content. Many other environmental changes have been reported to alter g_m and examples of these are listed in Table 7.1.

VIII. Conclusions

Since the popularization of methods for the estimation of mesophyll conductance CO_2 (g_m) using IRGA-based gas exchange measurements coupled with either chlorophyll fluorescence or carbon isotope discrimination, it has been revealed that resistance to CO_2 diffusion exerts a significant limitation on photosynthesis, in higher plants this limitation being of similar magnitude to that exerted by stomata. In parallel to the increased use of these methods, concerns have also been raised regarding both potential errors during their application and misestimations associated with the simplicity of their underlying models. Consequently, a significant research effort is being made to develop and improve new models for a better understanding of g_m. Despite these shortcomings, current estimates of g_m indicate clearly that it differs largely between species and g_m seems to follow an evolutionary trend with the highest values measured in mono-

Table 7.1. Summary of g_m responses to environmental changes

Environmental changes	Responses of g_m	References	Methods
<Drought stress>			
Minutes to hours (excised leaves)	Decreased g_m	Flexas et al. (2006a)	CFlu[a]
Days to months	Decreased g_m	Flexas et al. (2006a, 2009)	CFlu
		Galmes et al. (2007)	CFlu
		Miyazawa et al. (2008)	CFlu
		Galle et al. (2009)	CFlu
		Ferrio et al. (2012)	CFlu
		Tosens et al. (2012a)	CFlu
		Brilli et al. (2013)	CFlu
		Perez-Martin et al. (2014)	CFlu
		Mizokami et al. (2015)	CFlu
		Theroux Rancourt et al. (2015)	CFlu
		Warren et al. (2011)	CIso
		Cano et al. (2014)	Cflu, Ciso, Cfit
	Decreased g_m (only g_s fell below 0.15 mol m^{-2} s^{-1})	Silva et al. (2015)	CFlu
	Unaffected g_m (up to g_s reduced to 90%)	Theroux-Rancourt et al. (2014)	CFlu
		Bunce (2009)	CFlu
Months to years	Decreased g_m	Grassi and Magnani (2005)	CFlu
		Diaz-Espejo et al. (2007)	Cfit
		Cano et al. (2013)	Cfit
		Aranda et al. (2012)	CFlu,Cfit
<ABA application>			
Minutes to hours	Decreased g_m	Flexas et al. (2006a)	CFlu
		Mizokami et al. (2015)	CIso
		Sorrentino et al. (2016)	CFlu
	Unaffected g_m	Flexas et al. (2013a)	CFlu
Days	Unaffected g_m	Vrabl et al. (2009)	Cflu, CIso
<Re-watering>			
Hours to days	Increased g_m (recover)	Flexas et al. (2009)	CFlu
		Galle et al. (2009)	CFlu
		Warren et al. (2011)	CIso
		Cano et al. (2014)	Cflu, Ciso, Cfit
		Perez-Martin et al. (2014)	CFlu
		Theroux Rancourt et al. (2015)	CFlu
	Increased and uneffected g_m (species dependent)	Silva et al. (2015)	Cfit
		Galmes et al. (2007)	CFlu
<Light intensity>			
Minutes to hours	Increased g_m with increase in light intensity	Flexas et al. (2007b)	Cflu, Ciso, Cfit
	Unaffected g_m	Tazoe et al. (2009)	Ciso
	Increased g_m with increasing light intensity only in plants cultivated with high nitrogen supply	Xiong et al. (2015b)	Cflu
	Increased g_m with increase in light intensity	Douthe et al. (2011)	Ciso
		Douthe et al. (2012)	Ciso

(continued)

Table 7.1. (continued)

Environmental changes	Responses of g_m	References	Methods
Days to months	Sun leaves have higher g_m than shade leaves	Piel et al. (2002)	Cflu
		Hanba et al. (2002)	Ciso
		Laisk et al. (2005)	Cflu
		Warren et al. (2007)	Cflu
		Cano et al. (2011)	Cflu
		Tosens et al. (2012a)	Cflu
		Lloyd et al. (1992)	Ciso
		Syvertsen et al. (1995)	Ciso
		Niinemets et al. (2006)	Cflu
		Cano et al. (2013)	Cfit
		Montpied et al. (2009)	Cfit
		Singsaas et al. (2004)	Cflu
<Light quality>			
Minutes to hours	Decreased g_m in response to high blue light intensity (chloroplast avoidance response)	Tholen et al. (2008)	Ciso, Cfit
		Loreto et al. (2009)	Cflu
		Pallozzi et al. (2013)	Cflu
<Fluctuating light>	Decreased g_m (60mins treatment)	Huang et al. (2015)	Cflu
<Temperature>			
Minutes to hours	Decreased g_m (from 28 °C to 38 °C)	Pons and Welschen (2003)	Cflu
	Increased g_m (from 10 °C to 35 °C)	Bernacchi et al. (2002)	Cflu
	Decreased g_m (over 37.5 °C)	Bernacchi et al. (2002)	Cflu
	Increased g_m (from 10 °C to 20 °C)	Yamori et al. (2006)	Ciso
	Increased g_m (from 10 °C to 20 °C)	Warren and Dreyer (2006)	Cflu
	Increased g_m (from 20 °C to 40 °C)	Scafaro et al. (2011)	Ciso
	Increased g_m (from 15 °C to 40 °C)	Evans and von Caemmerer (2013)	Ciso
	Increased g_m (from 15 °C to 30 °C : N. tabacum)	Walker et al. (2013)	Cflu
	Increased g_m (from 15 °C to 40 °C: N. tabacum, G. hirsutum, G. max and E. pauciflora)	von Caemmerer and Evans (2015)	Ciso
	Unaffected g_m (from 15 °C to 35 °C: A. thaliana)	Walker et al. (2013)	Cflu
	Unaffected g_m (from 15 °C to 40 °C: L. confertus and T. aestivum)	von Caemmerer and Evans (2015)	Ciso
	Largely increased g_m (from 20 °C to 40 °C, rice cultivated with high nitrogen supply)	Xiong et al. (2015b)	Cflu
	Slightly increased g_m (from 20 °C to 40 °C, rice cultivated with low nitrogen supply)	Xiong et al. (2015b)	Cflu
Days to months	Different responses between plants cultivated at 30 °C and 15 °C measurement temperature at over 20 °C	Yamori et al. (2006)	Ciso
<CO_2 concentration>			
Minutes to hours	Decreased g_m with increase in C_i	Flexas et al. (2007b)	Cflu, Ciso, Cfit
		Tazoe et al. (2011)	Ciso
		Douthe et al. (2011)	Ciso
		Xiong et al. (2015b)	Cflu
	Unaffected g_m	Tazoe et al. (2009)	Ciso

(continued)

Table 7.1. (continued)

Environmental changes	Responses of g_m	References	Methods
Months to years	Slightly decreased g_m in high CO_2 cultivated Cucumber	Singsaas et al. (2004)	Cflu
	Slightly increased g_m in high CO_2 cultivated Sweetgum (Sun leaf)	Singsaas et al. (2004)	Cflu
	Unaffected Sweetgum)	Singsaas et al. (2004)	Cflu
	Unaffected g_m	Bernacchi et al. (2005)	Cflu
	Decreased g_m in high CO_2 and low nitrogen cultivated plants	Kitao et al. (2015)	Cflu, Cfit
	Unaffected g_m	Crous et al. (2013)	Ciso
	Increased g_m in high CO_2 grown plants	Singh et al. (2013)	Cfit
	Decreased g_m in high CO_2 grown plants	Chen et al. (2014)	Cfit
<O_2 concentration>	Increased g_m at 2% O_2	Tazoe et al. (2011)	Ciso
	Increased g_m at 1% O_2	Douthe et al. (2012)	Ciso
	Increased g_m in response to low O_2	Tholen et al. (2012b)	Ciso
	Unaffected g_m	Evans and von Caemmerer (2013)	Ciso
<UV-B>	Decreased g_m	Martínez-Lüscher et al. (2015)	Cflu
<Salinity>	Decreased g_m	Sade et al. (2014)	Cflu
<K supply>	Increased g_m (months)	Battie-laclau et al. (2014)	Cfit
<Copper supply>	Decreased g_m (days)	Bazihizina et al. (2015)	Cflu

[a]*Ciso* Carbon isotope method, *Cflu* Chlorophyll fluorescence method, *Cfit* Curve fitting method

cotyledonous grasses. g_m is also revealed as a highly plastic trait, responding significantly to changes in, for example, light during leaf development and availability of nutrients. This variability in g_m is strongly related to differences in anatomical traits, most notably cell wall thickness and chloroplast surface facing intercellular air spaces. Moreover, g_m responds to changes in environmental parameters from minutes to hours, suggesting the involvement of biochemical regulation likely related to aquaporins and/or carbonic anhydrases. Its co-regulation with the extra-xylem component of leaf hydraulic conductivity is currently under debate; if true, CO_2 and water may share the same pathway from mesophyll cell walls to the stomata pores, but in the opposing sense. We suggest that significant efforts are necessary to: (1) develop improved models and (2) understand the physical and biochemical mechanisms underlying the regulation of this important leaf trait for photosynthesis.

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Chapter 8

Molecular Mechanisms Affecting Cell Wall Properties and Leaf Architecture

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Summary

Leaf architecture is determined by cell shape, size, and density. As plant cells are enclosed by a rigid cell wall, changes to leaf architecture have to occur through downstream genetic systems that induce alterations in (1) cell wall composition, (2) synthesis, assembly, and orientation of cytoskeletal elements and/or (3) the degree of cross-linkage between wall components in response to upstream developmental and environmental cues. This chapter reviews how leaf architecture is influenced by molecular mechanisms that modulate the above wall modification processes. Upstream signaling systems such as salicylic (SA), jasmonic (JA), and gibberellic (GA) acid have significant effects on leaf architecture. GA promotes and JA and SA suppress growth. Leaf architectural changes are brought about by these upstream systems in concert or in an interactive manner, and the associated downstream molecular systems that are involved in executing changes to cell wall properties will be discussed. Evidence will be provided to show that xyloglucan endotransglucosylase/hydrolase and pectin methyltransferase/pectin methylesterase/pectin methylesterase inhibitor systems are key downstream execution points of leaf architectural changes common to different upstream molecular systems. Optimization of leaf architecture maximizes light interception,

gas exchange properties, and photosynthesis. In addition, plant growth has been shown to be more sensitive to leaf area than to area-based photosynthesis rate. Therefore, understanding genes and molecular mechanisms that affect cell wall properties and leaf architecture has broader implications in terms of crop improvement, and candidate genes that can be manipulated to optimize leaf architecture in order to maximize net carbon assimilation and plant growth will be proposed.

I. Introduction

A. Leaf Growth and Architecture

In general, a leaf is composed of upper and lower epidermes and layers of mesophyll cells usually organized into palisade and spongy tissue (Graham et al. 2006; Lambers et al. 2008). Traversing through the leaf mesophyll is a network of vasculature composed of two groups of specialized cells: xylem and phloem (Graham et al. 2006). Leaf growth occurs in three phases: (1) leaf initiation through leaf primordia formation in the apical meristem, (2) establishment of polar axes of the leaf (leaf-length, leaf-width, and leaf-depth directions), and (3) leaf expansion (Sinha 1999; Bowman et al. 2002; Kim and Cho 2006). Leaf architecture is determined by a large number of characteristics such as the size, shape, symmetry, venation, organization, and petiole characteristics (Ellis et al. 2009) that define leaf morphology as well as anatomical features such as cell types and their size, shape, density, and the size and distribution of intercellular air spaces. Leaf morphology and leaf cell anatomy can have large influences on photosynthetic rate per unit area and, even more, on whole-plant photosynthetic rate. In this chapter we will focus on genes and associated molecular mechanisms that affect leaf size/area, shape, and epidermal cell and mesophyll characteristics, with special reference to how these affect photosynthesis.

As plant cells are encircled by a rigid cell wall, cell wall biosynthesis and modification is required in order for the proper execution of all three growth phases and to establish specific leaf architecture (Sinha 1999; Buchanan et al. 2000; Kim et al. 2002; Baskin 2005; Cosgrove 2005; Caffall and Mohnen 2009; Guerriero et al. 2014; Ochoa-Villarreal et al. 2012; Tenhaken 2015). These modifications include: (1) alteration in cell wall composition, (2) alterations in the synthesis, assembly, and orientation of cytoskeletal elements such as microtubules and actin filaments, and/or (3) alterations in the degree of cross-linking within and between cell wall components. For example, initiation of leaf primordia has been shown to depend on cell wall composition while establishment of the polar axes of the leaf requires synthesis and proper arrangement of the cortical cytoskeleton (Sinha 1999; Buchanan et al. 2000; Bowman et al. 2002; Kim et al. 2002; Baskin 2005; Cosgrove 2005; Kim and Cho 2006; Caffall and Mohnen 2009; Ochoa-Villarreal et al. 2012; Guerriero et al. 2014; Tenhaken 2015). The extent to which cells can expand depends on both turgor pressure and the physical properties of the cell wall (Kim et al. 2002; Baskin 2005; Guerriero et al. 2014). During growth of a cell, the cell wall has to be sufficiently ductile to submit to the internal force of turgor and to allow expansion (Kim et al. 2002; Baskin 2005; Guerriero et al. 2014). It also has to synthesize new cell wall material to effectively encapsulate and reinforce the growing cell surface (Buchanan et al. 2000; Graham et al. 2006). Therefore, constant synthesis and modification of cell wall material, arrangement of cytoskeletons and the formation, disruption, and reformation of cross-linkages need to take place and these processes are under strict genetic regulation. Changes in cell wall architecture can

occur in response to both external and internal signals. Developmental cues such as altered rates of cell division in leaf primordia have been shown to affect the extent to which cells can expand in a process known as "compensated cell enlargement" (Fujikura et al. 2007). In general, upstream signals perceived from developmental and environmental cues will need to affect downstream targets that are directly involved in modulating cell wall properties to direct changes in leaf architecture.

A large number of genes that code for enzymes and transcription factors involved in directly modulating cell wall properties have been characterized through genetic manipulations. This chapter will summarize how altered expression of some of these genes affects leaf architecture. Some of the key genes and molecular mechanisms specifically involved in modulating the three cell wall modification processes mentioned above will also be discussed.

B. Alterations in Leaf Growth and Architecture Mediated by CAMTA/SA, PHYB/GA/PIF, and JAZ/JA Upstream Molecular Signaling Pathways

Three key upstream molecular systems namely the salicylic (SA), jasmonic (JA), and gibberellic (GA) acid signalling pathways and their influence on cell wall properties and leaf architecture will also be discussed in this chapter. We will look closely at the roles of *CALMODULIN BINDING TRANSCRIPTION ACTIVATOR* (*CAMTA), PHYTOCHROME-B* (*PHYB)*, and *JASMONATE ZIM*-domain *(JAZ)* repressor proteins and their associated mechanisms in regulating leaf architecture; these genes are associated with SA, GA, and JA signalling pathways, respectively. *CAMTA*, *PHYB*, and *JAZ* genes were specifically selected owing to the significant alterations in leaf growth observed in the corresponding mutant plants (Figs. 8.1, 8.2 and 8.3). Some evidence for altered leaf growth upon altering expression of *PHYB*, *JAZ*, and *CAMTA* genes has been presented (Reed et al. 1993; Tsukaya et al. 2002; Foo et al. 2006; Finlayson et al. 2007; Doherty et al. 2009; Karve et al. 2012; Yang et al. 2012; Kim et al. 2013; Campos et al. 2016). Here we will look at how mesophyll architecture is altered in these mutant lines, and will discuss in the following sections the downstream molecular systems involved in altering cell wall properties in response to the above upstream systems.

Recently, *CAMTA1, 2*, and *3* genes were shown to suppress genes of the isochorismate synthase (*ICS1*) pathway of SA biosynthesis under warm temperature (Fig. 8.1) (Kim et al. 2013). SA biosynthesis is upregulated in the *camta2/3* while the *sid2–1* mutant line contains a loss-of function allele of *ICS1* and is incapable of producing SA (Kim et al. 2013). Leaf growth in terms of both projected and total leaf area was significantly reduced in the *camta2/3* double mutant compared to wild-type, while it was partially rescued in *camta2/3sid2–1* (Fig. 8.2a) (unpublished data by Y-S.K., S.M.W., T.D.S., and M.F.T.). Downregulation of *CAMTA* expression caused marked changes in the mesophyll architecture that included the production of thin leaves carrying a large number of small, densely packed mesophyll cells and a reduction of intercellular air spaces (Fig. 8.2b–c) (unpublished data by Y-S.K., S.M.W., T.D.S., and M.F.T.). These data show that changes in leaf architecture in *camta2/3* occurs in an SA dependent manner.

Under shade or a lower red to far red (FR) light ratio, PHYB is converted to its inactive form (Pr) which promotes the degradation of DELLA proteins (negative regulators of PIF) (Fig. 8.1) (Kozuka et al. 2005; Jaillais and Chory 2010; Colebrook et al. 2014; Mazzella et al. 2014; Havko et al. 2016). The degradation of DELLA proteins relieves inhibition of PIF transcription factors leading to growth (Kozuka et al. 2005; Jaillais and Chory 2010; Colebrook et al. 2014; Mazzella et al. 2014; Campos et al. 2016; Havko et al. 2016). Under unshaded light or higher red to FR light, PHYB remains in its active form (Pfr)

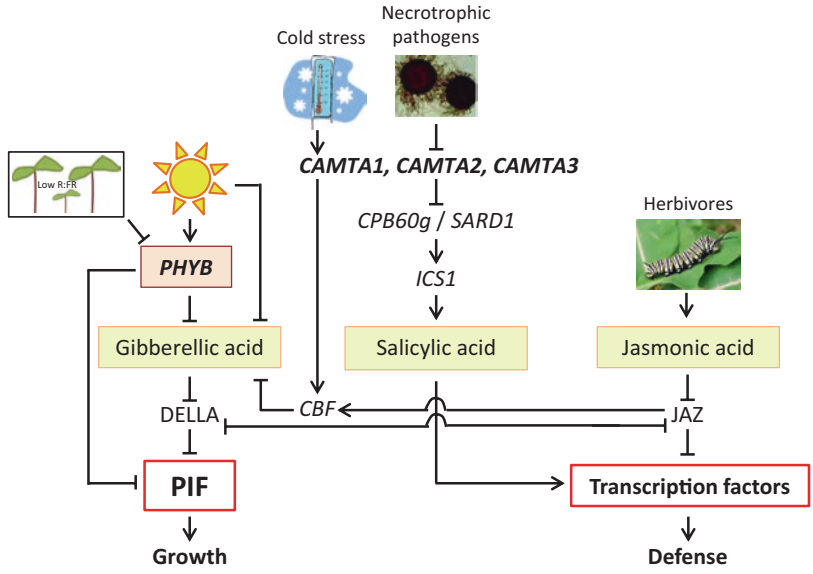

Fig. 8.1. **Modulation of growth by *CAMTA*/SA, *PHYB*/GA/PIF, and JAZ/JA upstream molecular signalling pathways and their interactions.** A schematic diagram is presented summarizing the interactions between SA, GA and JA signalling pathways which result in growth-defense trade-offs in plants. GA promotes shoot cell elongation and growth (Jaillais and Chory 2010; Chapman et al. 2012; Karve et al. 2012; Gommers et al. 2013; Leduc et al. 2014; Mazzella et al. 2014; Behringer and Schwechheimer 2015; Chaiwanon et al. 2016). Under light, a decrease in GA occurs as a result of both a reduction in the transcription of genes involved in GA biosynthesis, and an increase in gibberellin-2-oxidase which increases GA catabolism (Folta et al. 2003; Hisamatsu et al. 2005; Foo et al. 2006; Achard et al. 2007; Weller et al. 2009; Pierik et al. 2011; Hirose et al. 2012; Colebrook et al. 2014; Mazzella et al. 2014). Genes involved in GA biosynthesis may be downregulated by PHYB (Hisamatsu et al. 2005; Pierik et al. 2011; Hirose et al. 2012; Colebrook et al. 2014). Under shade or a lower red to far red (low R:FR) light ratio, PHYB is converted to its inactive form (Pr) which promotes the degradation of DELLA proteins (negative regulators of phytochrome-interacting factors, PIF). *CAMTA* mediated SA signalling under warm temperature induces SA-mediated defense responses (Kim et al. 2013). Activation of the CRT/DRE *binding factor* (*CBF*) pathway in low temperature by *CAMTA* genes, improves freezing tolerance in plants (Doherty et al. 2009; Kim et al. 2013) (Fig. 8.1). Rapid cold induction of *CBF* genes triggers the transcription of a large number of transcription factors that induce transcription of genes involved in freezing tolerance (Lee and Thomashow 2012). Herbivory-triggered jasmonic acid (JA) synthesis leads to the degradation of JAZ proteins, relieving the inhibition of several transcription factors, including group IIIe bHLHs (e.g., MYC2), and enhancing defence related processes (Hou et al. 2010; Havko et al. 2016; Campos et al. 2016). Antagonistic interactions between JAZ and DELLA proteins play a part in regulating the growth-defense trade-off mediated by GA and JA (Hou et al. 2010; Yang et al. 2012; Havko et al. 2016; Campos et al. 2016). Both CAMTA and JAZ can influence GA through CBF proteins. (Lee and Thomashow 2012) (Colour figure online)

Fig. 8.2. (continued) at rubisco calculated using the $\delta^{13}C_{VPDB}$ values (right panel), are presented for *A. thaliana* Col-0 wild-type, and *sid2–1*, *camta2/3*, and *camta2/3sid2–1* mutant lines (unpublished data by Y-S.K., S.M.W., T.D.S., M.F.T.). The $\delta^{13}C_{VPDB}$ value is a measure of discrimination against $^{13}CO_2$ by a leaf. A smaller negative $\delta^{13}C_{VPDB}$ value indicates lower discrimination against $^{13}CO_2$ and lower CO_2 partial pressure at rubisco. Plants were grown hydroponically in 1/2-strength Hoagland's solution under a light intensity of 120 μmol m^{-2} s^{-1}, an 8-h photoperiod, day- and night-time temperatures of 22 °C and 20 °C, respectively, and 60% relative humidity. In (**a**), rosettes were photographed 41 days after seeding. In (**b**), leaf thickness is denoted by red double arrows. In (b-d), data are from 44-day old leaves. In (**c**) and (**d**) n = 3–4 plants per line. In (**c**) values represent the mean ± SE. In (**d**) box plots display the full range of variation and the line that divides the box in half marks the median. The mean is denoted by the small box in the middle of each box plot. The upper and lower whiskers represent scores outside the middle 50%. Statistical differences at α = 0.05 are marked with lower case letters (Colour figure online)

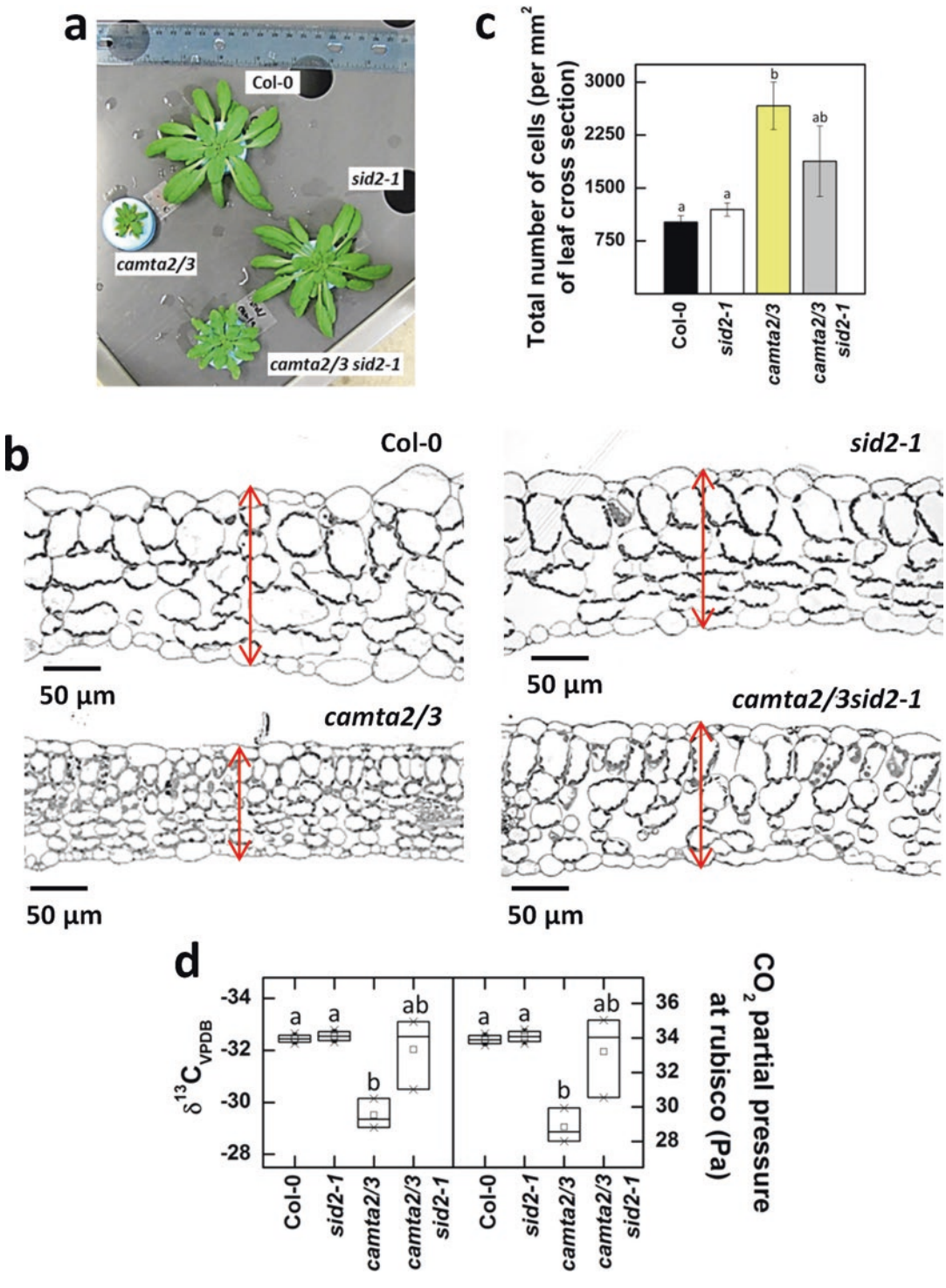

Fig. 8.2. **The effect of altered *CAMTA2* and *CAMTA3* gene expression on leaf architecture and CO_2 diffusion through the leaf mesophyll.** (**a**) Photographs comparing rosettes sizes, (**b**) representative micrographs of leaf cross sections, (**c**) total number of cells, and (**d**) the $\delta^{13}C_{VPDB}$ value calculated as the ratio of ^{13}C to ^{12}C isotopes in leaf tissue relative to a Vienna-Pee-Dee Belemnite standard (VPDB) (left panel) and the CO_2 partial pressure

which leads to suppression of PIF mediated growth promotion (Jaillais and Chory 2010; Karve et al. 2012; Colebrook et al. 2014; Mazzella et al. 2014; Havko et al. 2016). Herbivory-triggered JA synthesis leads to the degradation of JAZ proteins, relieving the inhibition of several transcription factors, including group IIIe bHLHs (e.g., MYC2), and enhancing defence related processes (Fig. 8.1) (Hou et al. 2010; Campos et al. 2016; Havko et al. 2016). Antagonistic interactions between JAZ and DELLA proteins play a part in regulating the growth-defense trade-off mediated by GA and JA (Hou et al. 2010; Yang et al. 2012; Campos et al. 2016; Havko et al. 2016). Leaf growth in the *phyB* mutant line has been examined (Tsukaya et al. 2002; Kozuka et al. 2005; Jaillais and Chory 2010; Colebrook et al. 2014; Mazzella et al. 2014; Campos et al. 2016; Havko et al. 2016). However, new data from *jaz*Q and *jaz*Q*phyB* mutant lines reveal leaf area to be smaller in *jaz*Q (Fig. 8.3a) (Campos et al. 2016). In contrast, a significant increase in both petiole length and projected and total leaf area, as well as flattened leaves were seen in *phyB*; these leaf characteristics were also evident in *jaz*Q*phyB* (Fig. 8.3a). Examination of the leaf cross sections revealed wider and shorter palisade tissue cells in the transverse sections, a reduced number of cell layers, a slight reduction in intercellular air spaces, and thinner leaves in both *phyB* and *jaz*Q*phyB*; such changes were not observed in *jaz*Q (Fig. 8.3b) (Campos et al. 2016). In summary, the above studies provide evidence that *CAMTA*/SA, *JAZ*/JA, and *PHYB*/GA/PIF effects on leaf growth are accompanied by significant effects on mesophyll architecture as well as a role of underlying downstream molecular systems that modulate cell wall properties.

Use of a variety of different techniques such as microscopy, leaf gas exchange measurements, ^{13}C discrimination analyses, and growth modeling enables greater understanding of the impact of leaf architecture on photosynthesis and plant growth. In addition, combination of physiological measurements gathered from the above techniques with gene expression data from RNA sequencing (RNA-seq) can help unravel molecular mechanisms affecting cell wall properties and leaf architecture. This chapter discusses downstream genetic mechanisms through which upstream molecular systems execute their effects on leaf architecture. In addition, key common downstream genes and molecular mechanisms that alter cell wall properties and consequently leaf architecture in response to SA, GA, and JA upstream signaling systems and the resulting effects on photosynthesis and overall plant growth are also reviewed. Candidate genes that may help to optimize leaf architecture in order to maximize net C assimilation and plant growth will also be presented.

II. Regulation of Cell Wall Composition

The development of the cell wall includes the formation of a middle lamella and the primary wall during initial growth, which in some cells is followed by formation of a secondary wall for further strength (Buchanan et al. 2000; Caffall and Mohnen 2009). The major constituents of the plant cell wall are cellulose (30%), hemicelluloses (30%), and pectins (35%) (Buchanan et al. 2000; Cosgrove 2005; Caffall and Mohnen 2009; Ochoa-Villarreal et al. 2012; Tenhaken 2015). A large gene superfamily, *CELLULOSE SYNTHASE (CESA)/ CELLULOSE SYNTHASE LIKE* (*CSL*), includes genes that share significant sequence similarity. These genes code for enzymes catalyzing cellulose and hemicellulose synthesis, respectively (Cosgrove 2005; Burton et al. 2006; Suzuki et al. 2006; Held et al. 2008; Doblin et al. 2009; Dwivany et al. 2009; Yoshikawa et al. 2013).

Cellulose is synthesized by isoforms of the *CESA* family of cellulose synthase enzymes. Based on studies on *A. thaliana,*

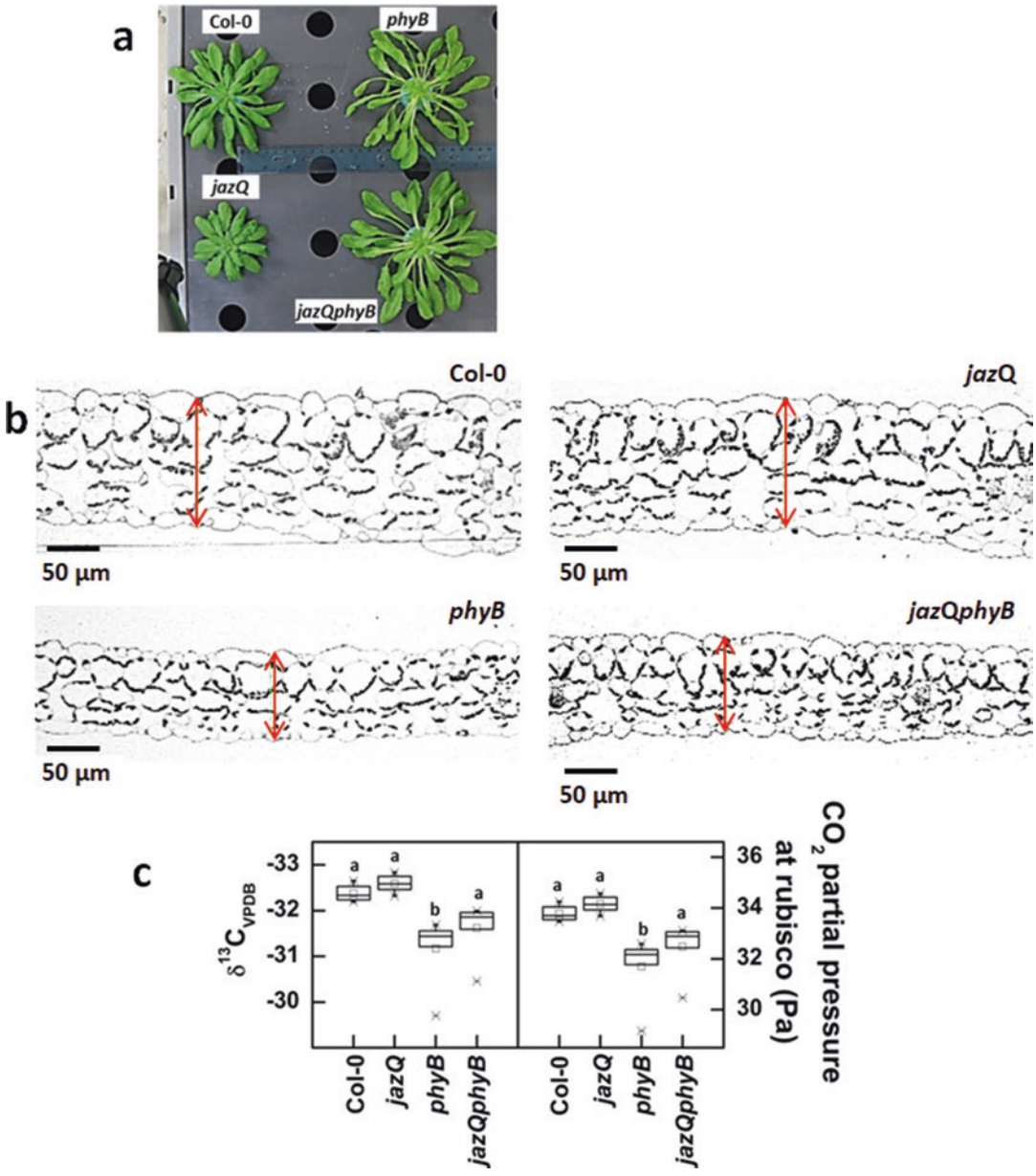

Fig. 8.3. **The effect of altered *PHYB* and *JAZ* gene expression on leaf architecture and CO_2 diffusion through the leaf mesophyll.** (**a**) Photographs comparing rosettes sizes, (**b**) representative micrographs of leaf cross sections, and (**c**) the $\delta^{13}C_{VPDB}$ value calculated as the ratio of ^{13}C to ^{12}C isotopes in leaf tissue relative to a Vienna-Pee-Dee Belemnite standard (VPDB) (left panel), and the CO_2 partial pressure at rubisco calculated using the $\delta^{13}C_{VPDB}$ values (right panel), are presented for *A. thaliana* Col-0 wild-type and *phyb*, *jazQ*, and *jazQ-phyB* mutant lines. $\delta^{13}C_{VPDB}$ is described in the Fig. 8.2 legend. Plants were grown hydroponically in 1/2-strength Hoagland's solution under a light intensity of 120 µmol m^{-2} s^{-1}, 8-h photoperiod, day- and night-time temperatures of 22 °C and 20 °C, respectively, and 60% relative humidity. In (**a**), rosettes were photographed 55 days after seeding. In (**b**), leaf thickness is denoted by red double arrows. In (**b**–**c**), data are from 22-day old leaves. In (**c**) box plots display the full range of variation and the line that divides the box in half marks the median. The mean is denoted by the small box in the middle of each box plot. The upper and lower whiskers represent scores outside the middle 50%; n = 6 plants per line. Statistical differences at $\alpha = 0.05$ are marked with lower case letters. (**a**–**b** – Campos et al. 2016; **c** – unpublished data by M.L.C., Y.Y., I.T.M., S.M.W., T.D.S., and G.A.H.) (Colour figure online)

Nicotiana benthamiana, Gossypium hirsutum, Hordeum vulgare, Oryza sativa, Sorghum bicolor, and *Zea mays*, 8–12 *CESA* genes have been found to exist in plants (Pear et al. 1996; Burton et al. 2000, 2004; Robert et al. 2004; Tan et al. 2015). These cellulose synthase proteins interact to form a hexameric complex (Burton et al. 2004; Robert et al. 2004; Cosgrove 2005). The constituent *CESA* in the cellulose synthase complex differs based on whether the complex is associated with the primary or secondary cell wall (Burton et al. 2004; Robert et al. 2004; Cosgrove 2005). *CESA3* and *CESA5* in *Z. mays* and *CESA4* in *A. thaliana* have been shown to be highly expressed in leaf blades (Holland et al. 2000; Burton et al. 2004).

A major portion of hemicellulose is made of xyloglucans followed by xylans, mannans, and other types of polymers such as mixed linkage glucan. The type and abundance of hemicellulose varies depending on the plant species. For example, dicot cell walls contain xyloglucans, xylans, mannans, and glucomannans while β -(1,3;1,4)-glucans are only found in Poales and other monocot groups. Arabinoxylans are the most prominent hemicelluose in graminae. *CSLA – CSLJ* genes are responsible for hemicellulose synthesis as follows: *CSLA* – β-mannan and glucomannan synthases, *CSLC* – β-glucan synthases, and *CSLF* and *CSLH* – mixed linkage glucan synthases (Cosgrove 2005; Burton et al. 2006; Suzuki et al. 2006; Held et al. 2008; Doblin et al. 2009; Dwivany et al. 2009; Chou et al. 2012; Yoshikawa et al. 2013). Recent studies show that xyloglucan synthesis is catalyzed by a multiprotein complex of *CSLC4* and xylosyltransferases (*XXT*) (Chou et al. 2012).

Pectin is a complex heteropolysaccharide rich in galacturonic acid. It is the most abundant group of polymers in the primary cell wall. While pectin comprises 35% of primary cell wall in diocts and non-graminaceous monocots, it is about 2–10% in grasses (Ochoa-Villarreal et al. 2012). Pectin has various structural types, primarily homogalacturonan, rhamnogalacturonan I, and rhamnogalacturonan II (Buchanan et al. 2000; Caffall and Mohnen 2009; Ochoa-Villarreal et al. 2012; Xiao and Anderson 2013; Tenhaken 2015). Pectin is also highly substituted by side-chain modifications, such as methylesterification of the carboxyl groups of the galacturonic acid (Mouille et al. 2007; Caffall and Mohnen 2009; Wolf et al. 2009; Ochoa-Villarreal et al. 2012; Xiao and Anderson 2013; Kim et al. 2015; Tenhaken 2015). Homogalacturonan is synthesized in the Golgi apparatus and secreted as a highly methylesterified polymer. Methylesterification is catalysed by pectin methyltransferases (PMT) in the Golgi lumen. Pectin methylesterases (PMEs) present in the cell wall then de-methylesterify homogalacturonan. The interplay of PME-inhibitors (PMEI) and PMEs defines the levels of methylesterification in the cell wall, which is critical for cell expansion and overall plant growth and development. This review addresses the molecular mechanisms that affect leaf architecture by regulating cellulose, hemicellulose, and pectin synthesis.

A. Alterations in Cellulose Synthase Gene Expression

Naturally occurring small interfering RNA (siRNA) in developing leaves can suppress *CESA* expression (Held et al. 2008). In *H. vulgare*, a significant negative correlation was found between the expression levels of *CESA* in primary cell walls and antisense siRNA for *CESA* and leaf length, whereas a significant positive relationship was seen between leaf length and antisense siRNA expression levels for *CESA* (Held et al. 2008). The decrease in *CESA* transcript levels corresponded with a decrease in the rate of cellulose synthesis. Virus-induced gene silencing (VIGS) specifically targeting *CESA1* resulted in the suppression of *CESA* as well as *CSLA*, *CSLF*, and *GLYCOSYL TRANSFERASE8* (*GT8*) genes, which are glycosyl transferases. Consequently, an over-

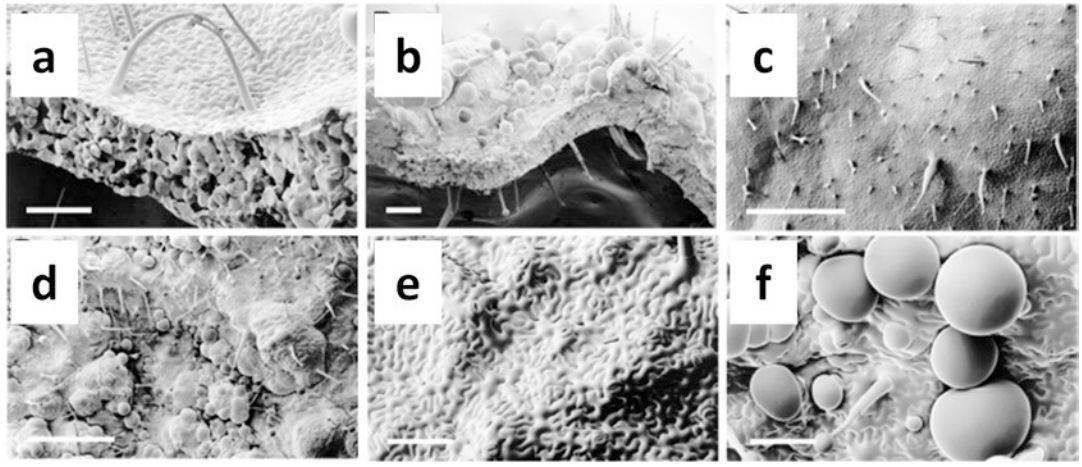

Fig. 8.4. **Effect of altered cellulose synthase gene expression on leaf architecture.** *CESA* was suppressed in *N. benthamiana (Nt)* via virus-induced gene silencing via a potato virus X vector (PVX) containing a putative *CESA* cDNA (PVX–*NtCESA-1b*) (Burton et al. 2000) (**a–f**). Scanning electron micrographs of (**a**) the adaxial side of PVX control leaf with a smooth epidermal surface, and trichomes, and mesophyll with adequate air spaces, (**b**) abaxial surface of PVX–*NtCESA-1b* with surface distortions, thinner leaf mesophyll with significantly reduced air spaces, (**c**) and (**d**) abaxial surface views of PVX control and PVX–*NtCESA-1b* leaves, respectively, (**e**) and (**f**) higher magnification views of PVX control and PVX–*NtCESA-1b* leaves, respectively, are shown. Bars in (**a**) and (**b**) = 200 μm; bars in (**c**) and (**d**) = 1 mm; bars in (**e**) and (**f**) = 100 μm. (Reproduced from Burton et al. 2000)

all reduction in cellulose synthesis and incorporation of mixed linkage glucans were observed in developing leaves. siRNA for *CESA* and *CSL* genes have also been found in *A. thaliana* and *O. sativa* (Held et al. 2008). These data indicate that antisense siRNA can regulate *CESA*/*CSL* and *GT8* gene expression and alter both cellulose and hemicellulose biosynthesis during early stages of leaf growth. It is also thought that difficulty in overexpressing *CESA1* may be because of the effects of siRNA (Held et al. 2008). *Arabidopsis thaliana* mutant lines that are defective in *CESA1* expression showed reduced cellulose content accompanied by a significant decrease in leaf and cotyledon areas (Arioli et al. 1998; Williamson et al. 2001; Beeckman et al. 2002). However, leaf shape was not affected indicating that altered cellulose content does not affect direction of expansion (Williamson et al. 2001; Beeckman et al. 2002) although arrangement of cellulose microfibrils would.

Recent studies indicate that effects on cell expansion brought about by changes in cellulose content are likely due in part to altered methylation status of pectin and that the synthesis of cellulose is tightly coupled with the synthesis of pectin and the degree of pectin methylesterification and vice versa. For example, VIGS of *CESA1* and *CESA2* in *N. benthamiana* resulted in a significant reduction in *CESA1* and a decrease in cellulose that was compensated for by an increase in pectin (Burton et al. 2000). Interestingly, the degree of pectin methylesterification also showed a marked decrease with a subsequent increase in Ca^{2+} mediated cross linkages that helped strengthen the cell wall weakened by the lack of cellulose. Plants with suppressed *CESA1* showed a significant reduction in leaf area and alterations in mesophyll architecture (Fig. 8.4a–f), which were similar to that seen in mutants with altered PMT gene expression (see Section IVB). The study by Burton et al. (2000) indicates that expression of *CESA* can regulate expression of *PMEI* and/or *PME*; demethylesterification requires enhanced activity of *PME* and decreased expression or activity of its inhibitor, *PMEI*.

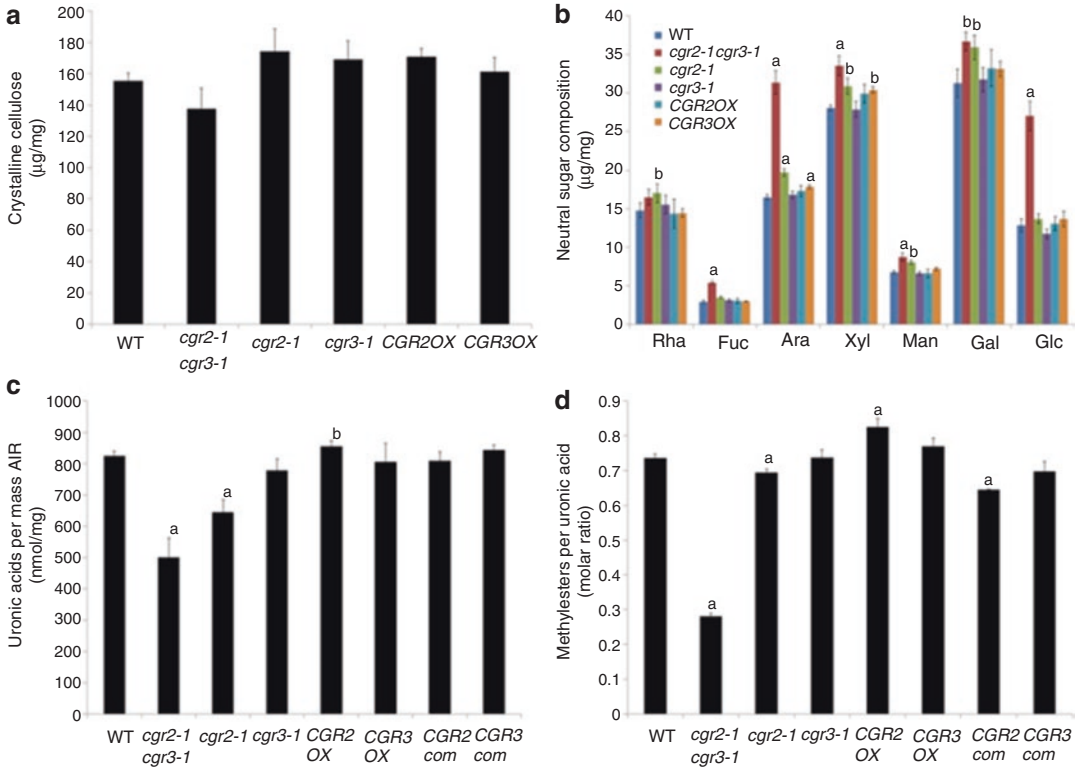

Fig. 8.5. **The effect of altered expression of pectin methyltransferase (*CGR*) on cell wall composition.** (**a**) Quantification of crystalline cellulose, (**b**) neutral sugar, (**c**) uronic acids, and (**d**) the degree of methylesterification, from the alcohol insoluble residue (AIR) of leaf tissue of *A. thaliana* transgenic lines showing suppressed (*cgr2*–1 *cgr3*–1, *cgr2*–1, *cgr3*–1), and enhanced (*CGR2*OX, *CGR3*OX) *CGR2* or *CGR3* gene expression. In (**b**), AIR from leaf tissue was analyzed for quantification of neutral sugars using alditol acetate derivatives. In (**c**), uronic acids from AIR were measured using a colorimetric method (Filisetti-Cozzi and Carpita 1991). D-galacturonic acid was used as a standard to calculate concentration. In (**d**), release of methanol from methyl esters in AIR was measured after saponification (Wood and Siddiqui, 1971). Methanol was used as a standard to calculate concentration. Values are means + SD (n = 3 for each genotype). Values indicated by letters are statistically significantly different from the wild type (a, $P < 0.01$,and b, $P < 0.05$) by Student's t test. (Reproduced from Kim et al. 2015)

Held et al. (2008) did not measure *PME* or *PMEI* transcripts. Recent studies provide compelling evidence that altered expression of PMTs, which catalyze methylesterification of pectin in the Golgi, can cause changes in cellulose content (Kim et al. 2015; Weraduwage et al. 2016). Interestingly, overexpression of a PMT *COTTON GOLGI-RELATED 2* (*CGR2*) led to an enhancement in pectin, methylated pectin as well as the crystalline cellulose content; conversely, suppression PMTs (*CGR2* and *CGR3*) led to a decrease in these components (Fig. 8.5) (Kim et al. 2015; Weraduwage et al. 2016). The above data show that not only does *CESA* regulate *PME* and *PMEI* expression, but also that the expression of PMTs can control the degree of pectin methylesterification and cellulose synthesis.

In summary, we see that *CESA* and PMT/*PME*/*PMEI* molecular systems work in coordination to support cell wall synthesis and modification during cell expansion and have significant effects on leaf architecture.

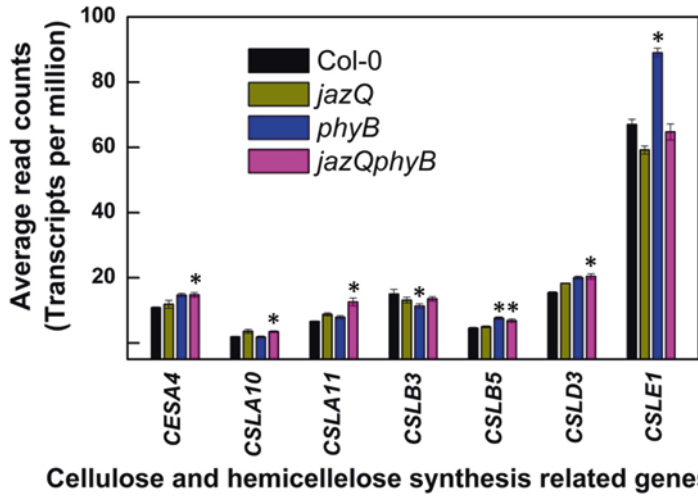

Fig. 8.6. **PIF mediated effects on genes associated with cellulose and hemicellulose synthesis.** Expression levels of *Cellulose synthase* (*CESA*) and *Cellulose synthase like* (*CSL*, involved in hemicellulose synthesis) genes are presented for *A. thaliana* Col-0 wild-type, and *jazQ*, *phyB*, and *jazQphyB* mutant lines as determined by leaf messenger RNA sequencing (Campos et al. 2016). Values represent the mean ± SE and n = 3 plants per line. Statistically different expression levels in comparisons to Col-0 found according to the DESeq algorithm ($P < 0.1$, using a Benjamini-Hochberg adjusted for multiple testing) are marked with asterisks

High PMT expression supports cell expansion and growth, enhanced *CESA* expression, and cellulose production to support cell wall building. A decrease in *CESA* can trigger cell wall hardening mediated by PME.

B. Potential PIF Mediated Effects on CESA and CESL Expression

Evidence for enhanced cellulose synthesis in response to enhanced leaf growth was seen in *phyB* and *jazQphyB* mutant lines where *CESA4* expression was enhanced (Fig. 8.6) (Campos et al. 2016). In addition, expression of a number of *CSL* genes was also enhanced in these lines (Fig. 8.6). Overall, we see that cellulose synthesis responds to alterations in the upstream *PHYB*/GA/PIF molecular systems while closely interacting with downstream molecular systems such as *PMT*/*PME*/*PMEI* in order to produce sufficient amounts of cellulose to meet the demand for new cell wall material.

III. Regulation of Cortical Microtubule and Microfilament Organization

Anisotropic (polarity-dependent) expansion of the cell wall is a key factor that determines cell shape. The balance between isotropic (polarity-independent) and anisotropic expansion processes determines the shape of an organ such as the leaf (Kim et al. 2002; Baskin 2005; Guerriero et al. 2014). Both turgor pressure and the physical properties of the cell wall determine the extent to which a cell can expand. Although the internal force exerted by turgor pressure on the cell wall is isotropic, because of the localized differences in the physical properties of the cell wall, the net expansion of the cell can be anisotropic, which subsequently determines cell shape and the architecture of the leaf. Anisotropic expansion rates per unit area of cell wall have two components: direction and angle (Baskin 2005).

The primary reason for anisotropic expansion of leaf cell and other cell walls is the arrangement of the cellular cytoskeleton, which is formed by the cortical microtubules and actin microfilaments (F-actin). The cortical microtubules located just beneath the plasma membrane mediate the directionality of the cellulose microfibril alignment (Buchanan et al. 2000; Kim et al. 2002; Baskin 2005; Guerriero et al. 2014). Arrangement of cellulose microfibrils perpendicular to the axis of elongation allows the primary cell wall to maintain strength and extensibility and facilitates anisotropic growth (Baskin 2005; Tenhaken 2015). While the alignment between microfibrils in primary cell walls is somewhat parallel, a stricter organization is seen in secondary cell walls where they exist in parallel arrays. These parallel microfibrils have been shown to arrange in different angles within each layer of secondary wall, thus limiting cells' ability to expand and grow (Baskin 2005; Tenhaken 2015). However, the effect of F-actin on anisotropic cell expansion is rather indirect and does not depend on the directionality of F-actin alignment in the cortex. A network of fine F-actin facilitates the transport of Golgi vesicles containing building material for growth, including cell wall growth (Buchanan et al. 2000; Mathur and Hülskamp 2002; Mathur 2006; Guerriero et al. 2014). In addition, the movement of mitochondria and peroxisomes also takes place along F-actin. Studies have shown the abundance of fine/diffuse F-actin networks to enhance at cell bulges/lobes/protrusions or locations of anisotropic growth; thus vesicle trafficking to the growing area is enhanced (Mathur and Hülskamp 2002; Mathur 2006; Guerriero et al. 2014). On the other hand, dense F-actin networks have been shown to block the movement of vesicles and thereby lead to growth retardation (Mathur and Hülskamp 2002; Mathur 2006; Guerriero et al. 2014). Thus, the resistance of the cell wall to the internal turgor force during anisotropic growth depends on the net effect of microtubule arrangement and microfilament type and abundance.

A large number of genes have been found to regulate synthesis and arrangement of cortical microtubules and F-actin. This section will summarize these molecular mechanisms, identify points of interaction, and present how these molecular mechanisms determine leaf architecture.

A. Genes That Regulate Microtubule Alignment

The lining of cellulose microfibrils mirrors the array of microtubules in the cell cortex because the movement of cellulose synthase and deposition of cellulose is directed by microtubules (Buchanan et al. 2000; Chan 2012). Therefore, genetic regulation of the direction and angle of cortical microtubule alignment can have a drastic effect on anisotropic cell growth and leaf architecture. As mentioned earlier, leaf cell expansion can occur in three directions: length, width and depth, which ultimately affects overall leaf architecture. The direction of leaf cell expansion seems to be regulated by three major genes: *ANGUSTIFOLIA (AN)*, *ROTUNDIFOLIA* (*ROT3*) and *LONGIFOLIA* (*LNG1*, *LNG2*) (Tsuge et al. 1996; Tsukaya 1998, 2002; Kim et al. 2002; Kalve et al. 2014).

Interestingly, *AN* has been shown to facilitate anisotropic growth in leaf-width direction and inhibit expansion in the depth direction, whereas *ROT3* enhances growth in leaf-length direction and inhibits expansion in the depth direction (Fig. 8.7). *LNG1* and *LNG2* have been shown to promote cell expansion in the leaf length direction. *AN* codes for a carboxy terminal binding protein and *an* mutant lines have narrow and thick leaves with significantly altered mesophyll architecture (Fig. 8.7). (Kim et al. 2002). The authors showed that restricted growth in the width direction and enhanced growth in the depth direction in *an* mutant lines was due to: (1) the more regular arrangement of cortical microtubules parallel to the leaf width direction and (2) a reduction in the angle

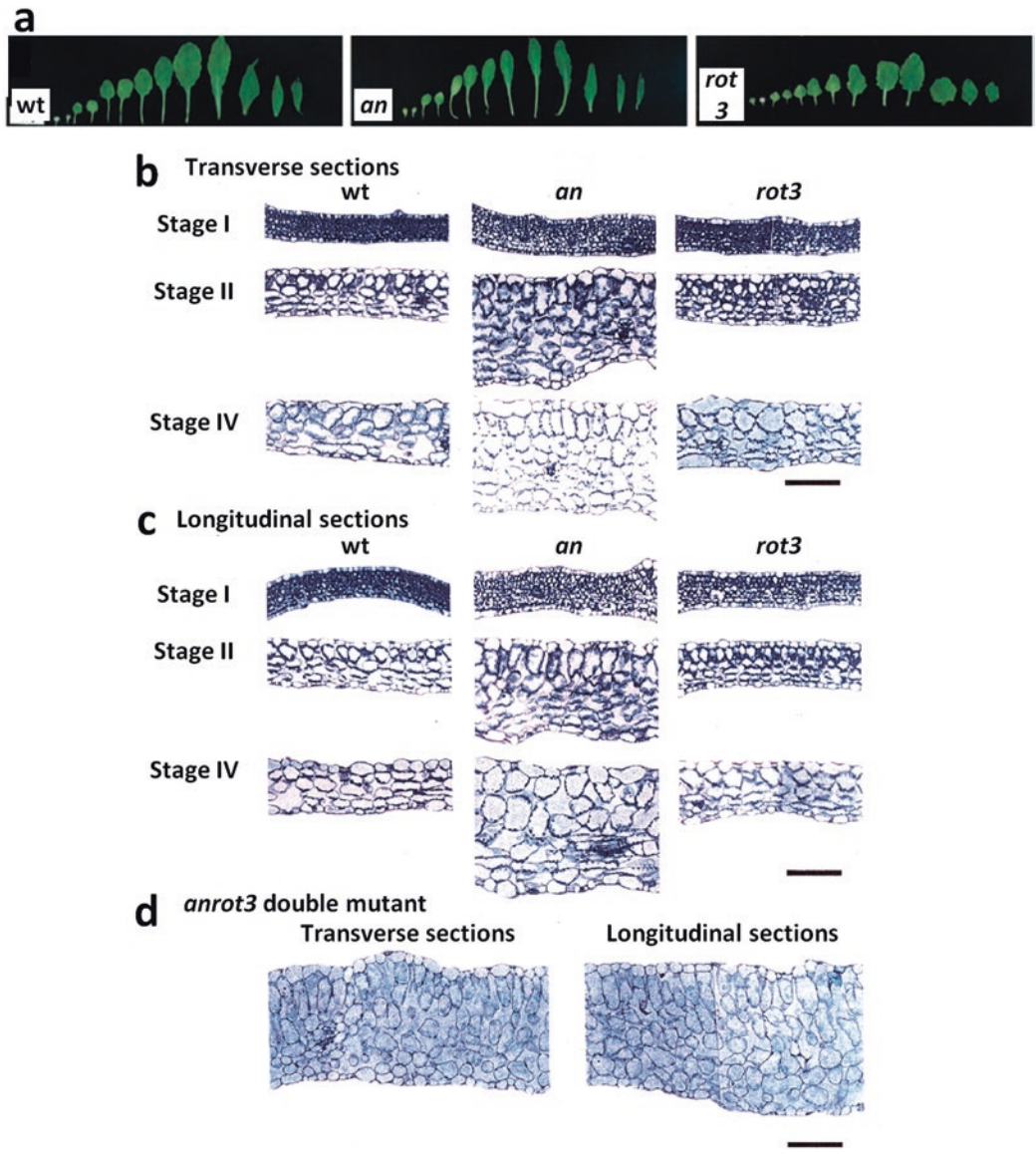

Fig. 8.7. **The effect of suppressed *AN* and *ROT3* gene expression on leaf architecture.** (**a**) The morphology of leaves of *A. thaliana* wild-type (wt), the *an* mutant, and the *rot3* mutant are presented. In (**a**), from the left, are the two cotyledons, eight rosette leaves and three cauline leaves. Leaf cross sections of the fifth leaves of the wild type, *an*, and the *rot3* mutant showing cell development in the (**b**) leaf width direction and (**c**) leaf length direction. (**d**) Transverse and longitudinal sections of leaves of the *an rot3* double mutant. In (**a–d**), leaves were collected when fully expanded. In (**b–d**), the transverse sections reveal a region between the midrib and the leaf margin; longitudinal sections reveal a region in the center of the lamina. The leaf cross sections in horizontal rows are from leaves at the same stage of growth: stage I – leaf length = 1.0 mm, stage II – 5.0 mm; stage III – 10.0 mm, and stage IV – 15.0 mm. Bars = 100 μm. (Reproduced from Tsuge et al. 1996) (Colour figure online)

between cortical microtubules and the plane parallel to the epidermal plane in the transverse section (Kim et al. 2002; Tsukaya 2002). Upregulation of *XTH24*, a xyloglucan endotransglucosylase/hydrolase in an *an* mutant line suggest interactions between AN and XTH resulting in the above cell wall modifications (Kim et al. 2002).

ROT3 has been shown to encode a cytochrome P-450 family steroid hydrolase, *CYP90C1* (Tsuge et al. 1996; Kim et al. 2002; Tsukaya 2002). CYP90C1 was shown to catalyze the conversion of typhasterol to castasterone, one of the last steps of brassinosteroid (BR) biosynthesis (Kim et al. 1998, 2005, 2015; Ohnishi et al. 2006). However, while *rot3* mutant lines showed a significant reduction in growth in length and an increase in breadth (Fig. 8.7), changes in microtubule organization was not observed. BRs have been shown to positively regulate *MICROTUBULE DESTABILIZING PROTEIN40* (*MDP40*) gene expression; *MDP40* is highly expressed in hypocotyls and cotyledons (Wang et al. 2012). *MDP40* has also been shown to co-localize with cortical microtubules and regulate their arrangement to promote hypocotyl cell elongation (Wang et al. 2012). Interestingly, although the *rot3* small leaf phenotype is similar to mutant lines deficient in BRs (*korrigan1*, *dwarf1*, *deetiolated2*), in contrast to *rot3* both cell expansion and cell number is affected in these mutant lines (Fujioka et al. 1997; Choe et al. 2000; Nakaya et al. 2002; Tsukaya 2002). Therefore, *ROT3* mediated cell elongation occurs via mechanisms other than microtubule alignment.

In contrast to *AN* and *ROT3* genes, *LNG1* and *LNG2* have been shown to promote cell expansion in the leaf-length direction independent of *ROT3* expression (Lee et al. 2006). Cold shock proteins characterized as nucleic acid binding proteins were recently found to regulate *LNG1* expression in *A. thaliana* (Yang and Karlson 2012). However, detailed molecular mechanisms through which *LNG* exerts its effects on leaf architecture remain to be found. New data on the role of *PHYB*/GA/PIF mediated regulation of *ROT3*, *LNG1*, and *LNG2*, and potential *ROT3*-regulated genes, are presented in Section IIIB.

B. Regulation of F-Actin Formation and Abundance

The interplay between microtubule arrangement, microfilament type, and abundance generates the interdigitated appearance of leaf epidermal pavement cells (normal epidermal cells) and the genes involved in this process have been studied extensively. It has been shown that anisotropic growth resulting in lobe formation is initiated soon after cell division is completed and is clearly seen after the cell expands several-fold (Frank and Smith 2002). A general pattern has been established for the cortical fine F-actin and microtubule distribution in epidermal pavement cells at various growth stages in the wild-type *A. thaliana* leaves (Fig. 8.8a) (Fu et al. 2002, 2005). During growth of epidermal pavement cells, fine F-actin is abundant and microtubules are scarce in protruding lobe areas (Fig. 8.8a); in contrast, microtubules and dense F-actin is abundant in indentation areas (Mathur and Hülskamp 2002; Mathur 2006). It is known that such coordinated changes in the cytoskeletal material in the cell cortex is regulated mainly through interactions between RhoGTPases (ROPs),

Fig. 8.8. (continued) arranged cortical microtubules confined to the invaginated areas and lobe shoulders (x – region of active ROP2; y – region of active ROP6). Stage III – mature cells with completed lobe extension having only randomly arranged cortical microtubules (Fu et al. 2002, 2005). When the leaf transitions from early to late growth stages, the arrangement of cortical microtubules was shown to change from random to transverse, which is important for expansion along long axis but prevents expansion in the lobe necks (Fu et al. 2002). (**b**–**d**) Scanning electron microscopy images of leaf trichomes (Bar = 200 μm), (**e**–**g**) leaf cross-sections (Bar = 250 μm), (**h**–**j**) scanning electron microscopy images of leaf pavement cells (Bar = 20 μm), and (**k**–**m**) pavement cells in bleached leaves (Bar = 40 μm) are presented for *A. thaliana* wild-type (images at left), and mutant lines with enhanced (images in the middle) and suppressed (images in the right) *ROP2* expression. (**a** is reproduced from Fu et al. 2005; **b**–**m** are reproduced from Fu et al. 2002) (Colour figure online)

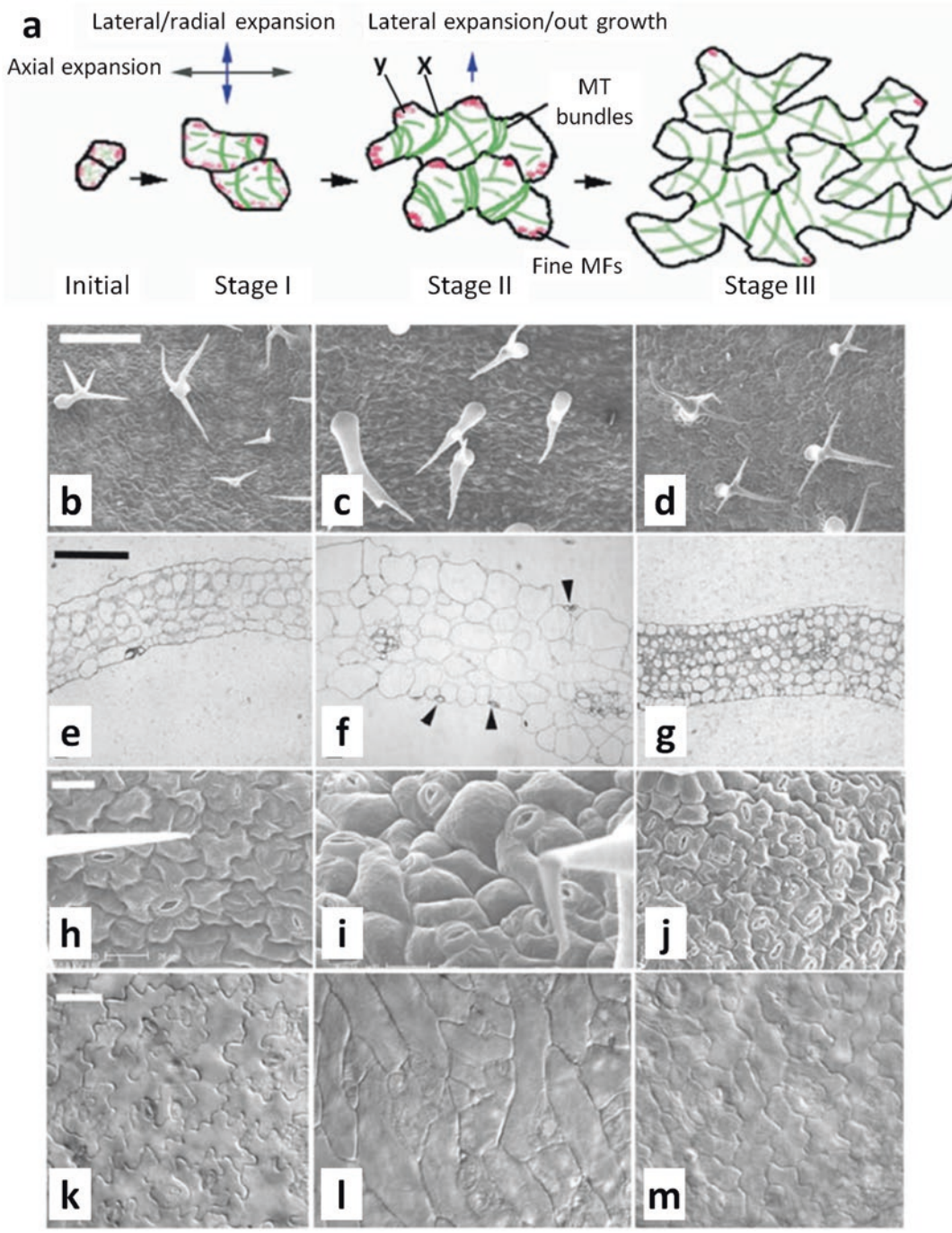

Fig. 8.8. **ROP GTPase modulation of cytoskeleton and morphogenesis of leaf cells.** (**a**) A schematic illustration of *A. thaliana* leaf pavement cell development and associated fine actin filaments (red patches = MFs) and cortical microtubules (green lines = MTs) is presented. ROP-independent actin bundles are not shown. Arrows indicate directions of expansion. Stage I – includes young developing cells at the leaf base prior to lobe formation having only isotropic expansion, with a network of fine F-actin in the cell cortex with greater abundance in the lobe initiation sites, and randomly oriented cortical microtubules. Stage II – expanded cells with developing lobes located between the leaf base and tip area having a network of fine F-actin only in the lobe tips, with transversely

ROP-INTERACTIVE CRIB MOTIF PROTEIN (RIC), *SUPPRESSOR OF CYCLIC AMP RECEPTOR* PROTEIN (SCAR), *WISKOTT–ALDRICH SYNDROME PROTEIN-FAMILY VERPROLIN HOMOLOGOUS PROTEIN* (WAVE), *ACTIN RELATED PROTEIN* (ARP2/3) and actin binding and stabilizing proteins (Fig. 8.9). The center point of this molecular mechanism is held by *RHO-RELATED* GTPase *FROM PLANT* (*ROP*), constituting a family of 11 genes in the *A. thaliana* genome with most of them showing expression in leaves (Fu et al. 2002, 2005; Qian et al. 2009; Craddock et al. 2012) (Fig. 8.9). Fu et al. (2002, 2005, 2009) showed that changes in *ROP2*, *4*, and *6* expression leads to significant changes in leaf architecture in a development stage dependent manner (Fig. 8.8b–m). Genetic manipulation studies of *ROP* genes revealed that *ROP2* and *ROP6* determine epidermal architecture through regulation of the formation and orientation of fine F-actin whereas *ROP6* affects bundling and organization of cortical microtubules (Fig. 8.9) (Fu et al. 2002, 2005, 2009).

Fu et al. (2005) further characterized the *ROP* mediated molecular mechanism for polar cell expansion in epidermal cells and showed that indentations and lobe formation by pavement cells is regulated by the interactions between *ROP-INTERACTIVE CRIB MOTIF PROTEINS* (*RIC*) *RIC1* and *RIC4*. *RIC1* was found to co-localize with cortical microtubules and loss of *ROP2* and *ROP4* enhanced this association (Fig. 8.9) (Fu et al. 2005). In contrast, *ROP6* was found to directly associate with *RIC1* and enhance its interaction with cortical microtubules (Fu et al. 2002, 2005, 2009). *RIC4* co-localized with cortical fine F-actin in the growing lobe regions (Fu et al. 2005, 2009). In summary, these studies showed that *RIC1* and *RIC4* are associated with promoting microtubule and F-actin assembly, respectively (Fig. 8.9). The abundance and assembly of these cytoskeletal components are further regulated via feedback effects on *ROP2-RIC4* interaction executed by *MICROTUBULE ORGANIZATION* (*MOR1*) proteins (Fig. 8.9) (Whittington et al. 2001).

ROP proteins are activated by Rho guanine nucleotide exchange factors (RhoGEFs) in plants and a single RhoGEF (*SPIKE1* or *SPK1*) is present in *A. thaliana* (Qiu et al. 2002; Basu et al. 2008). Studies showed that *SPK1* associates with many ROP proteins including ROP2, 3, 4, and 6 and that it is also capable of interacting with WAVE complex proteins such as SRA1 and NAP1 (Fig. 8.9) (Basu et al. 2008). Activation of SRA1 occurs primarily via ROP activation through SPIKE (Basu et al. 2008). Enhanced expression of *SRA1* and *NAP1* WAVE complex genes also affected leaf morphology through activation of an ACTIN RELATED PROTEIN complex (ARP2/3) (Li et al. 2004; Basu et al. 2005). The *ARP2/3* complex, composed of seven ARP subunits, activates the polymerization of branched F-actin and leads to the assembly of branched F-actin networks (Li et al. 2003; Qian et al. 2009). The *ARP2/3* complex is activated by proteins coded by the *SCAR/WAVE* gene family (Fig. 8.9) (Frank and Smith 2002; Djakovic et al. 2006; Qian et al. 2009). *BRK1* proteins

Fig. 8.9. (continued) interacting factors (PIF) in promoting ROP activity through: 1. upregulation of Pin-formed auxin efflux carrier gene family protein (*PIN*) expression and 2. through suppression of the negative regulator of RhoGTPase (RhoGAP) gene expression; PIF also suppressed *Microtubule associated protein18* (*MAP18*) gene expression required for microtubule bundle formation. Upregulation or downregulation of gene expression is denoted by pointed and blunt ended arrows, respectively. Other abbreviations: *ABP1* – Putative auxin receptor auxin binding protein 1, *TMK* – Transmembrane kinase subfamily of receptor-like kinases, RhoGEF – *Rho-Guanine nucleotide exchange factors* (GEFs), *SPIKE* – a RhoGEF or *DOCK180-type guanine nucleotide exchange factor*, *SRA1 – Rac1-associated protein-1*, *NAP1 – Nck-associated protein*, *BRICK1 – SCAR/WAVE Actin-Nucleating Complex Subunit*

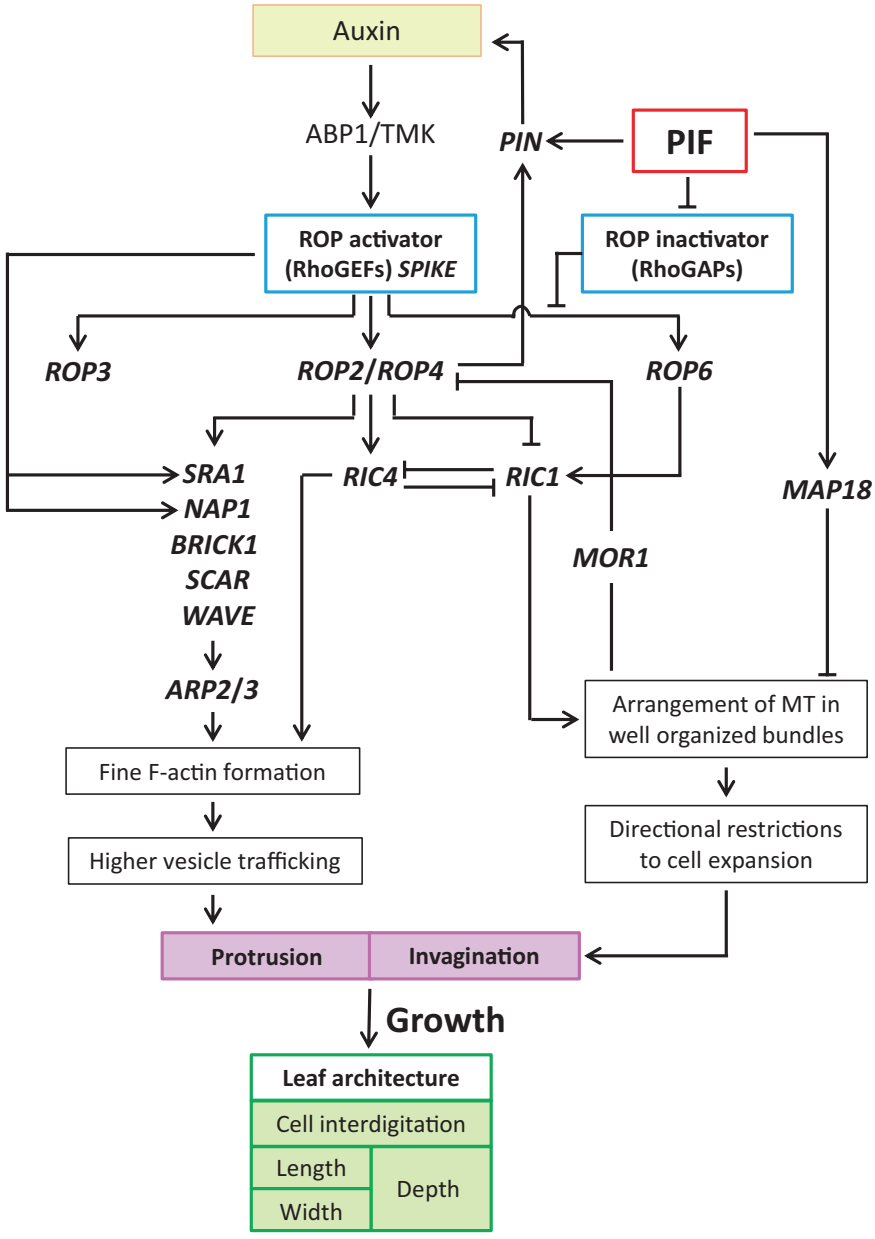

Fig. 8.9. **A summary of gene interactions in the ROP mediated molecular system regulating leaf architecture through the assembly and orientation of cytoskeletal elements.** Corresponding genes encoding key proteins: Rho-related GTPase from plant (ROPs), Rop-interactive crib motif proteins (RIC), Suppressor of cyclic AMP receptor (SCAR), Wiskott–Aldrich syndrome protein-family verprolin-homologous protein (WAVE), and Actin related protein (ARP2/3), regulate fine F-actin network formation and microtubule assembly and orientation. Two pathways, one that operates through ROP2/ROP4 and RIC4, and the other that operates through ROP2/ROP4, SCAR/WAVE and ARP2/3 complex promote fine F-actin network formation. Another system operating via ROP6 and RIC1 promotes assembly of microtubule bundles and their orientation. While regions with fine F-actin promotes growth (protrusions), microtubule bundling creates resistance to growth (invaginations). This interaction leads to anisotropic growth, as seen specifically in pavement cell interdigitation. RIC4 and Mirotubule organization (*MOR1*) proteins help in feedback regulation between fine F-actin network formation and microtubule bundle assembly. Auxin has been found to suppress *Rho guanine nucleotide exchange factors* (RhoGEFs) gene expression; RhoGEFs activate ROP. New evidence (Campos et al. 2016) supports a role for Phytochrome

have been shown to co-localize with WAVE proteins and to associate with SCAR proteins stabilizing and promoting the accumulation of SCAR proteins (Frank and Smith 2002; Djakovic et al. 2006; Qian et al. 2009). Alterations in *ARP* subunit and *BRK1* expression led to marked changes in F-actin polymerization and distribution with subsequent effects on the pavement cell architecture.

It is likely that similar mechanisms exist to regulate anisotropic growth of mesophyll cells. However, only a few studies looked at how ROP mediated changes in pavement cell architecture affected other aspects of leaf architecture, including leaf and cotyledon size and mesophyll architecture (Frank and Smith 2002; Fu et al. 2002; Qiu et al. 2002; Djakovic et al. 2006; Basu et al. 2008). In these studies, a reduction in pavement cell expansion and lobe formation correlated with a reduction in the size of mesophyll cells and consequently leaf size (Frank and Smith 2002; Fu et al. 2002; Qiu et al. 2002; Djakovic et al. 2006; Basu et al. 2008). In addition, there is evidence to suggest that the rate of cell division and expansion of the epidermis can affect the same processes in the inner layers of tissue, but results seem to vary depending on the species examined (Savaldi-Goldstein and Chory 2008; Marcotrigiano 2010). For example, genetically different epidermal layers on either side of the midrib of graft leaf chimeras between *Nicotiana glauca* and *Nicotiana tabacum* were used to show that the rate of cell division in the epidermal cell layer can determine the rate of cell division in the inner layers of the leaf (Marcotrigiano 2010). In addition, expression of BR synthesizing enzymes in an epidermal cell specific manner in brassinosteroid deficient mutant lines enhanced expansion of epidermal cells and subsequently that of mesophyll cells (Savaldi-Goldstein and Chory 2008; Marcotrigiano 2010). These results show that hormonal signaling from the epidermis to the inner layers can coordinate growth in different cell layers in a leaf (Savaldi-Goldstein and Chory 2008; Marcotrigiano 2010). In addition, Kawade et al. (2013) showed that epidermal cell proliferation is dependent on the movement of AN3 protein to the epidermal cells from mesophyll cells where it is synthesized. The detection of reduced cell proliferation in both mesophyll and epidermal cells in *an3* mutants revealed that normal leaf growth is also dependent on signals that travel from the inner mesophyll to the outer epidermal cell layer (Kawade et al. 2013). Thus, inter-cell-layer controls can occur in either direction to coordinate leaf growth. In addition, physical properties of the epidermal cell wall may also determine its capability to bear the force exerted by internal tissues and hence regulate the growth capability of internal tissues (Savaldi-Goldstein and Chory 2008; Marcotrigiano 2010).

C. *Potential PIF Mediated Effects on Fine F-Actin Network and Microtubule Bundle Formation*

Previous studies have shown that epidermal pavement cell interdigitation is promoted by auxin, *PUTATIVE AUXIN RECEPTOR AUXIN BINDING PROTEIN* (*ABP1*), *TRANSMEMBRANE KINASE SUBFAMILY OF RECEPTOR-LIKE KINASES* (*TMK*), RhoGEF, *ROP*, and *PIN-FORMED AUXIN EFFLUX CARRIER GENE FAMILY PROTEIN* (*PIN1*) mediated feedback effects (Xu et al. 2010; Craddock et al. 2012) (Fig. 8.9). RNA-seq data from *phyB* and *jazQphyB* provide supporting evidence for the hypothesis that PIF may promote growth by positively affecting fine F-actin network formation and negatively affecting microtubule bundle formation (Figs. 8.9 and 8.10) (Campos et al. 2016). *MICROTUBULE ASSOCIATED PROTEIN18* (*MAP18*) gene expression, which negatively regulates formation of well-organized microtubule bundles, was suppressed in a *phyB* mutant line (Figs. 8.9 and 8.10a). Well-organized micro-

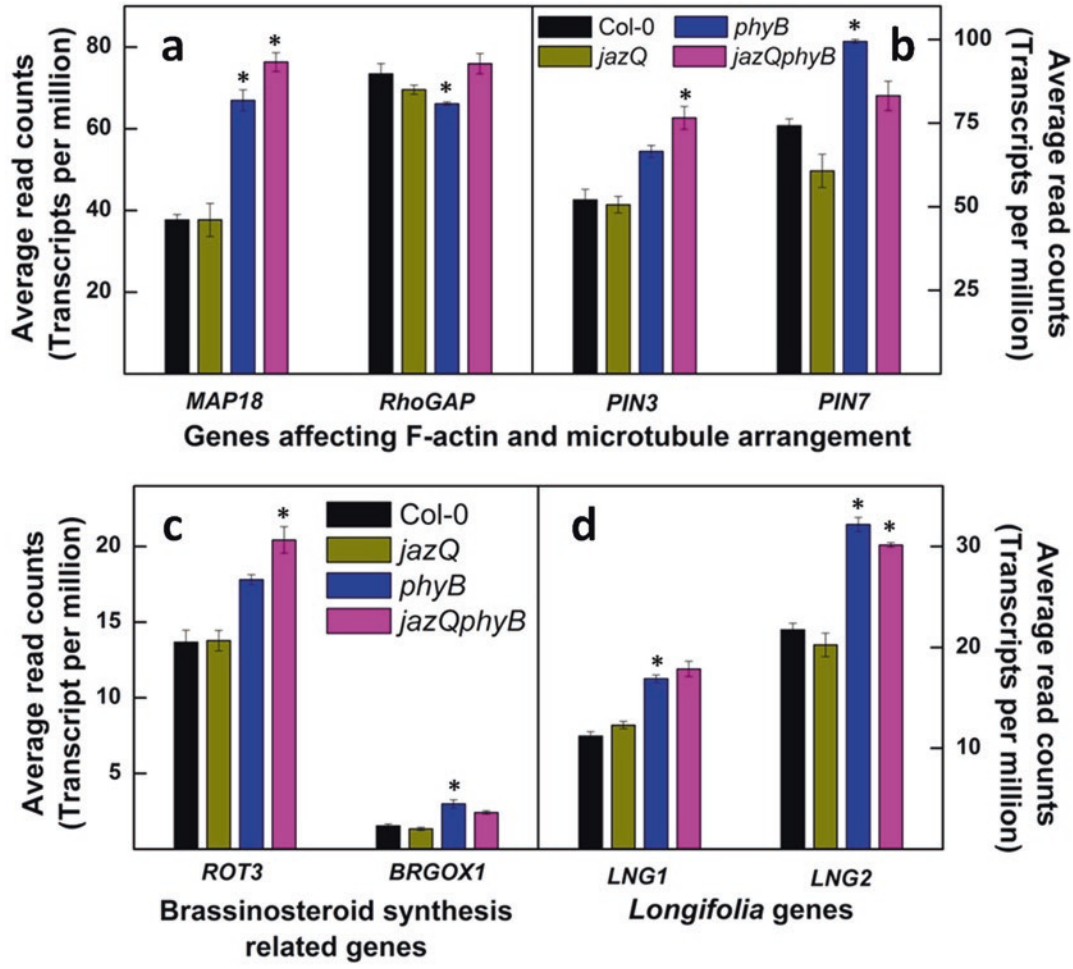

Fig. 8.10. **PIF mediated upregulation of genes associated with cytoskeleton assembly and orientation.** Expression levels of (**a**) *Microtubule-associated Protein18* (*MAP18*) and RhoGAP (AT5G12150, which catalyzes RhoGTPase inactivation), (**b**) *PIN3* and *PIN7* of the Pin-formed (*PIN*) auxin efflux carrier gene family, (**c**) genes of cytochrome P450 proteins (R*otundifolia3* (*ROT3*, *CYP90C1*) and brassinosteroid-6-oxidase (BRGOX1, *CYP85A1*), and (**d**) *Longifolia* genes (*LNG1*, *LNG2*) are presented for *A. thaliana* Col-0 wild-type, and *jazQ*, *phyB*, and *jazQphyB* mutant lines determined by leaf messenger RNA sequencing (Campos et al. 2016). Values represent the mean ± SE and n = 3 plants per line. Statistically different expression levels in comparisons to Col-0 found according to the DESeq algorithm ($P < 0.05$, using a Benjamini-Hochberg adjusted for multiple testing) are marked with asterisks

tubule bundles restrict growth. In addition, the negative regulator of ROP (RhoGAP) expression was also suppressed (Figs. 8.9 and 8.10a). While Rho guanine nucleotide exchange factors (RhoGEFs) activate ROP, RhoGAP inactivates ROP (Moon and Zheng 2003; Xu et al. 2010; Craddock et al. 2012). Only nine RhoGAP genes have been found in *A. thaliana* and data on molecular mechanisms regulating RhoGAP expression are rare (Kost 2010). Data obtained from *phyB* mutant lines show that PIF negatively affects RhoGAP expression (Fig. 8.9). The expression of both PIN3 and PIN7 auxin transporters was elevated in *phyB* (Figs. 8.9 and 8.10b) (Campos et al. 2016). Upregulation of PIN and downregulation of RhoGAP may activate ROP, specifically *ROP2*, which

induces fine F-actin network formation and promotes growth (Fig. 8.9). Therefore, it seems that growth enhancement by PIF (Fig. 8.3) may be mediated, at least in part, by downregulation of well-organized microtubule bundle formation and through promotion of F-actin network formation.

The ability of PIF to alter gene expression of *ROT3* shows that another pathway through which PIF can influence changes in leaf architecture may be through BR synthesis to enhance *MDP40* gene expression that leads to microtubule destabilization. In fact, the expression of *ROT3* (*CYP90C1*) and brassinosteroid-6-oxidase (*CYP85A1*), important cytochrome P450 proteins catalyzing the last steps of BR synthesis, was upregulated in the *phyB* mutant line (Fig. 8.10c). Furthermore, expression of both *LNG* alleles was enhanced in *phyB* (Fig. 8.10d).

In summary, *AN*, *ROT3*, and *LNG1* and *LNG2* are three major genes that regulate microtubule alignment in mesophyll cells and subsequently the direction of leaf cell expansion. There are also two key molecular systems that tightly regulate anisotropic cell expansion to determine the interdigitating architecture of pavement cells. One system operates through (i) *ROP2*/*ROP4* and *RIC4* and (ii) *ROP2*/*ROP4*, *SCAR*/*WAVE,* and *ARP2/3* complexes to promote fine F-actin formation and assembly (Fig. 8.9). The second system acts through *ROP6* and *RIC1* to promote microtubule assembly and orientation. There is clear evidence for coordinated antagonistic regulation of F-actin and cortical microtubule distribution in the protruding lobe and invaginated neck regions of pavement cells (Fig. 8.9). In addition, regulation of epidermal cell expansion by the above two systems also seems to have significant effects on mesophyll architecture and overall leaf growth in a direct or indirect manner. Upstream, the *PHYB*/GA/PIF molecular system seems to enhance growth at least in part via downregulation of well-organized microtubule bundle formation and through promotion of F-actin network formation.

IV. Cross-Linkages Between Different Cell Wall Constituents

In addition to composition, and the orientation of cellulose microfibrils and F-actin formation and abundance, cross-linkages between cell wall constituents also assert strength, and therefore, resistance to cell expansion and growth. Xyloglucans, which form a major portion of hemicelluloses, are cross linked with cellulose microfibrils and pectin, thereby adding rigidity and mechanical strength to the cell wall (Cosgrove 2005; Ochoa-Villarreal et al. 2012; Tenhaken 2015). This interaction between xyloglucan hemicellulosic polymers and cellulose fibers is modulated by expansin and xyloglucan endotransglucosylase/hydrolase (XTH) enzymes (Cosgrove 2005; Ochoa-Villarreal et al. 2012; Tenhaken 2015). Expansins are primarily involved in wall loosening whereas XTHs are more versatile in function. XTH catalyzes the endolytic cleavage of existing xyloglucan-xyloglucan or xyloglucan-other polymer chains, after which reformation of cross-linkages with different xyloglucans or polymers (xyloglucan endotransglucosylase, XET) or with water (xyloglucan endohydrolase, XEH) occurs. XTH's ability to recruit new xyloglucan or other polymer chains to the existing cell wall likely leads to wall strengthening whereas hydrolysis of cross-linkages may lead to wall loosening (Rose et al. 2002; Becnel et al. 2006). Therefore, XTH can regulate plasticity of the cell wall and subsequently cell size and leaf architecture (Nishitani and Tominaga 1992; Rose et al. 2002; Jan et al. 2004; Becnel et al. 2006).

Pectin is synthesized in the Golgi apparatus and secreted to the cell wall and is considered to be a critical element that controls cell wall elasticity and expansion. As mentioned earlier, the degree of pectin methylesterification depends on the action of PMT, PME, and PMEI. A higher degree of demethylation frees carboxyl groups of galacturonic acids to form Ca^{2+} and Mg^{2+} intermolecular

linkages that lead to hardening of pectin and reduce extensibility of the cell wall (Heldt and Piechulla 2010; Kim et al. 2015). The middle lamella, which is responsible for adhesion between adjacent cells, is composed mostly of pectin with a low degree of methylesterification (Caffall and Mohnen 2009; Wolf et al. 2009; Neumetzler et al. 2012). Therefore, as with XTHs, alterations in genes encoding PMT, PME, and PMEI can have a significant effect on cell size and leaf architecture. This section will look at how alterations in *XTH*, *PME*, and *PMEI* gene expression and that of PMTs affect leaf architecture. Expansins will not be discussed in this review.

A. *Xyloglucan Endotransglucosylase/Hydrolase*

The XTH gene family has 33 genes in *A. thaliana* and 29 in *O. sativ*a encoding xyloglucan endotransglucosylase/hydrolase (Yokoyama and Nishitani 2001; Yokoyama et al. 2004). Developmental stage and organ-based expression patterns of *XTH* has been extensively studied and the following genes have been shown to be highly expressed in young to mature rosette leaves of *A. thaliana*: *XTH4*, *6–9*, *16*, *22–24*, *27*, *28*, and *31*, *32*. Interestingly, not all XTHs positively affect cell expansion. For example, overexpression of *XTH3*, *XTH17*, and *XTH24* resulted in the development of smaller leaves as a result of the production of a large number of small cells in the mesophyll or as a result of a reduction in cell number (Verica and Medford 1997; Matsui et al. 2005; Cho et al. 2006; Han et al. 2013). These results point to effects on cell proliferation. On the other hand, many *XTH* genes, when overexpressed, enhance cell expansion and leaf size indicating that these are involved in cell wall loosening (Ogawa et al. 1996; Itoh et al. 2002; Jan et al. 2004; Shin et al. 2006; Liu et al. 2007; Miura and Hasegawa 2010; Hara et al. 2014). Some XTHs, namely *XTH27*, seem to specifically and positively regulate growth of tracheids with no role in leaf expansion (Matsui et al. 2005). Many studies have shown XTH to be involved in stress responses to salinity. For example, salt stress induces *XTH17* and *XTH3* gene expression that results in alterations in cell wall properties, remodelling of stomata, and alterations in mesophyll architecture; the modified leaf architecture increases water retention and survival (Yokoyama and Nishitani 2001; Cho et al. 2006; Chan et al. 2011; Keuskamp et al. 2011; Han et al. 2013). Regulation of *XTH* expression by many growth regulators including SA, JA, and GA is also evident (Yokoyama and Nishitani 2001; Jan et al. 2004; Keuskamp et al., 2011; Campos et al. 2016; unpublished data by Y-S.K., S.M.W., T.D.S., and M.F.T.).

There is evidence to support that not only is *CESA* expression coordinated with pectin methylesterification and demethylesterifcation, it is also coordinated with cell wall loosening by *XTH* expression, specifically with *XTH21* expression (Liu et al. 2007). Suppression of *XTH21* led to a reduction in *CESA2* and *4* expression in *A. thaliana* (Liu et al. 2007). Leaf architecture reported for *XTH21* suppressed *A. thaliana* lines are similar to the leaf characteristics reported for *CGR2* and *CGR3* suppressed lines (Kim et al. 2015; Weraduwage et al. 2016) or *CESA1* silenced *N. benthamina* lines (Burton et al. 2000). The fact that *XTH24* is upregulated in *an* mutants (Tsuge et al. 1996; Kim et al. 2002), and that XTHs are regulated in response to stress and growth regulators, show that XTHs form a key molecular system that modifies cell wall properties and leaf architecture in response to external stimuli; while doing so, it is capable of altering the action of *CESA* to support cell wall modifications and growth.

ERECTA (*ER*) is another gene family that has been found to affect leaf architecture. These are leucine-rich repeat receptor-like kinases known to control a variety of developmental processes including leaf initiation, stem elongation, and leaf elongation in the

length direction (Shpak et al. 2004; Masle et al. 2005; Sánchez-Rodríguez et al. 2009; Villagarcia et al. 2012). Three *ER* family genes have been found in *A. thaliana* and suppression of these genes caused significant changes in leaf shape, size, and mesophyll anatomy (Shpak et al. 2004; Masle et al. 2005; Sánchez-Rodríguez et al. 2009; Villagarcia et al. 2012). In addition, genes of the *ER* family, through their effects on epidermal cell expansion, have been shown to reduce stomatal density and improve transpiration efficiency (Masle et al. 2005; Villagarcia et al. 2012). Interestingly, alterations in expression of *ER* genes have been found to alter cell wall composition while the degree of pectin methylesterification was unaffected (Sánchez-Rodríguez et al. 2009). Thus, it is hypothesized that *ER* regulates leaf architecture by its effects on cell proliferation (Shpak et al. 2004; Masle et al. 2005; Villagarcia et al. 2012). *er* mutants were shown to have fewer, loosely arranged large mesophyll cells in the spongy tissue (Masle et al. 2005; Ferjani et al. 2007). Analyses of cell proliferation and cell expansion rates revealed that the cell enlargement in *er* mutants was "compensated cell enlargement" triggered by reduced rates of cell proliferation (Ferjani et al. 2007). The molecular mechanisms involved in cell-to-cell communication that link cell proliferation and cell enlargement in determinate organs such as leaves is not clear (Ferjani et al. 2007). The exact mechanism through which *ER* genes affect cell wall properties also remains to be found. The effects on cell proliferation suggest a possible involvement of *XTH*, e.g. *XTH24*. Furthermore, ER modulation of epidermal cell expansion indicates potential impacts on genes involved in regulating epidermal cell interdigitation (Fig. 8.6).

In summary, there is strong evidence for the participation *XTH* genes in regulating cell expansion, cell proliferation, and mesophyll and leaf architecture. Coordinated expression of these genes in relation to other molecular systems will be discussed in the following section.

1. *XTH as a Key Downstream Point of Execution of Leaf Architectural Changes, and Its Modulation by CAMTA/SA, JAZ/JA and PHYB/GA/PIF*

As discussed previously, some *XTH* genes regulate wall loosening. Interestingly, expression of *AN* and *PIF* have opposite effects on *XTH24* expression (Figs. 8.11a and 8.12) (Campos et al. 2016). An increase in *XTH24* expression in *an* mutant lines led to narrow and thick leaves. Therefore, wider and shorter cells in the palisade tissue seen in *phyB* are unlikely a result of increased *XTH24*, but may occur through the action of different *XTH*s and other mechanisms such as enhanced PMT activity as described below or through alterations in *LNG* and *ROT3* expression (Fig. 8.10c–d). *LNG* leads to formation of longer leaves and *ROT3* leads to longer, thinner leaves as a result of shorter cells in palisade tissue. We hypothesize that *ROT3* acts on anisotropic cell expansion through *XTH4*, *8*, *9*, *17*, *23* because BRs have been shown to induce their expression (Yokoyama and Nishitani 2001) and because *ROT3*, *BR2OX*, and *XTH4* are all induced in *phyB* (Fig. 8.10c) (Campos et al. 2016).

Interestingly, our data revealed a general PIF mediated upregulation of *XTH* genes (and expansins) in the *phyB* mutant with wider and shorter palisade tissue cells and larger leaf area whereas *XTH31* gene expression was suppressed in *jaz*Q with smaller leaves (Figs. 8.11a, c and 8.12) (Campos et al. 2016). Both *XTH8* and *XTH31* were downregulated in *camta1/2/3* in an SA dependent manner*; camta2/3* produced significantly small cells and leaves (Figs. 8.11b and 8.12) (unpublished data by Y-S.K., S.M.W., T.D.S., and M.F.T.). These data show that PIF may affect *XTH* in a manner opposite to that of JAZ and SA. In other words, PIF would mostly enhance the expression of

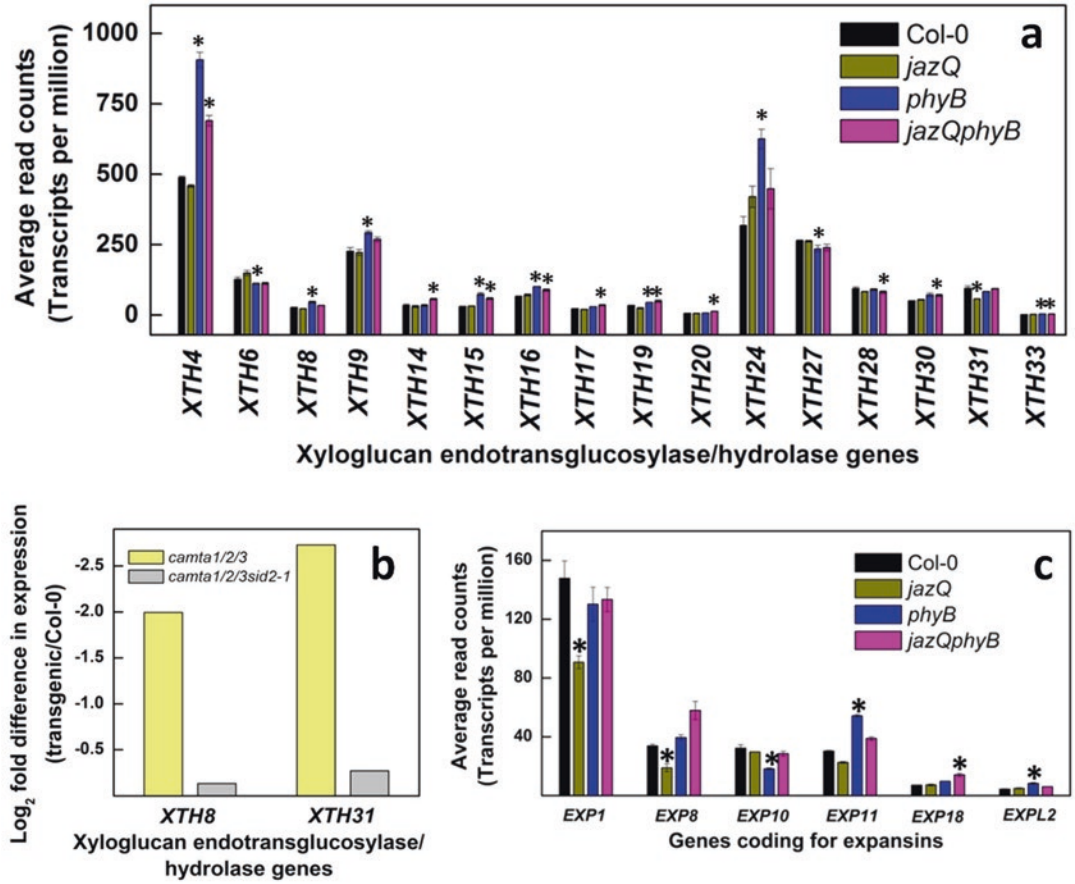

Fig. 8.11. **Comparison of altered expression of genes coding for XTH and EXP in *A. thaliana* mutant lines showing altered leaf architecture.** Expression levels of *Xyloglucan endotransglucosylase/hydrolase* (*XTH*) in *A. thaliana* Col-0 wild-type and (**a**) *jazQ*, *phyB*, and *jazQphyB* mutant lines (Campos et al. 2016), and (**b**) *camta1/2/3*, and *camta1/2/3sid2–1* mutant lines (unpublished data by Y-S.K., S.M.W., T.D.S., and M.F.T.), are shown. (**c**) Expression levels of *Expansin* (*EXP*) genes in *A. thaliana* Col-0 wild type and *jazQ*, *phyB*, and *jazQphyB* mutant lines are presented. Expression levels were determined by leaf messenger RNA sequencing. In (**a**) and (**c**), values represent the mean ± SE and n = 3 plants per line and statistically different expression levels in comparisons to Col-0 found according to the DESeq algorithm ($P < 0.05$, using a Benjamini-Hochberg adjusted for multiple testing) are marked with asterisks. In (**b**), values presented are Log_2 fold differences in expression (transgenic/Col-0) of *XTH* genes as determined by RNA-seq analysis of leaves and negative values indicate indicated a lower level of expression relative to Col-0

XTH, and transcription factors suppressed by JAZ (e.g., MYC) would downregulate the expression of *XTH*, to enhance and suppress leaf growth, respectively (Fig. 8.12). In contrast, transcription factors suppressed by JAZ and SA may act synergistically on *XTH* to suppress leaf growth by suppressing *XTH31* that is common to both pathways and may play an important role in growth suppression during defense or stress responses (Fig. 8.12). This also supports the hypothesis that changes to leaf architecture occurring in response to stress responses take place first at the genetic level at common action points such as changes to *XTH*. Overall, it is clear that *XTH*, which directly regulates cell wall loosening and the capability of cell expansion, is a key downstream execution point of leaf architecture changes common to *AN*,

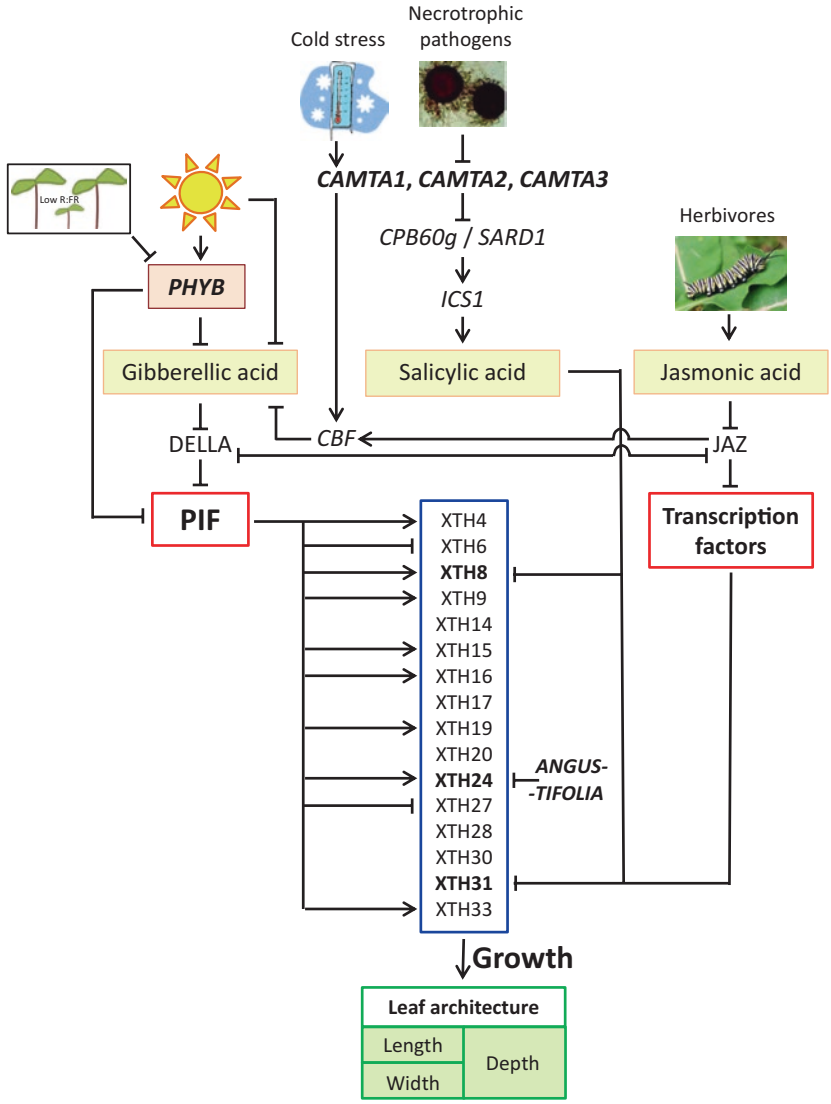

Fig. 8.12. ***CAMTA*/SA, *JAZ*/JA, and *PHYB*/GA/PIF-mediated effects on *XTH* gene expression.** A schematic diagram is presented summarizing the effects of salicylic acid (SA), *JASMONATE ZIM*-domain *(JAZ)* repressors, and phytochrome interacting transcription factor (PIF)-mediated effects on specific target xyloglucan endotransglucosylase/hydrolase (*XTH*) gene expression. Data was derived from messenger RNA sequencing obtained from null mutant lines of *Phytochrome-B* (*PHYB)* and/or *JAZ* gene expression (Campos et al. 2016), and from mutant lines having enhanced SA production as a result of suppressed *Calmodulin binding transcription activator* (*CAMTA*) gene expression (unpublished data by Y-S.K., S.M.W., T.D.S., and M.F.T.). Upregulation and downregulation of gene expression is denoted by pointed and blunt ended arrows, respectively. Other abbreviations: DELLA – PIF transcription factor repressors, *CBF- CRT/DRE Binding Factor*, *ICS1 – Isochorismate synthase*, *CBP60G and SARD1* – transcription factors with CAMTA DNA-binding motifs in promoter regions that positively regulate ICS1

CAMTA/SA, *JAZ*/JA, and *PHYB*/GA/PIF mediated molecular mechanisms.

B. Regulation of Ca^{2+} Mediated Cross-Linking of Pectin

1. Pectin Methylesterase and Pectin Methylesterase Inhibitor

Pectin methylesterases (*PME*) catalyze removal of the methyl moiety from methylated galacturonic acid and release methanol (Pilling et al. 2004; Oikawa et al. 2011). This methanol is given off as a gas and methanol emissions from forests occur when leaves are developing (Hu et al. 2011). Pectin methylesterase is a large gene family constituting more than 67 genes in *A. thaliana* (Markovic and Janecek 2004; Lionetti et al. 2007). A similarly large family of more than 69 genes encoding for PME inhibitor (PMEI) proteins has also been discovered in *A. thaliana* and other plants (Giovane et al. 1995; Jiang et al. 2002; Raiola et al. 2004; Lionetti et al. 2007; Wolf et al. 2009; Volpi et al. 2011). While the action of PME promotes cell wall hardening as explained above, demethylesterified pectin has been found susceptible to fungal endopolygalacturonases and pectin lyase (de Vries and Visser 2001; Lionetti et al. 2007). In fact, PME activity plays a significant role in mediating plant-pathogen interactions (Chen et al. 2000; Wietholter et al. 2003; Lionetti et al. 2007; Raiola et al. 2011). For example, overexpression of *PMEI1* reduces PME activity while enhancing the degree of methylesterified pectin in cell walls and resistance to pectin degrading fungal enzymes; suppression of *PMEI1* enhanced susceptibility (Lionetti et al. 2007; An et al. 2008; Volpi et al. 2011).

Data on the effect of altered *PME* and *PMEI* expression and activity on leaf architecture are scarce. Only a few studies provide evidence to support the idea that enhanced *PMEI* and reduced PME activity promote cell wall extensibility and cell expansion in cotyledons and leaves leading to their increased size (Pilling et al. 2004; Neumetzler et al. 2012; Peaucelle et al. 2012; Müller et al. 2013a, b; Levesque-Tremblay et al. 2015). However, enhanced *PMEI* and reduced PME activity has the opposite effect on growth and differentiation of the shoot meristem, stems, and hypocotyls (Peaucelle et al. 2008, 2012). Recently, a small Golgi-localized protein, *FRIABLE1*, was found to be a negative regulator of *PME* expression in *A. thaliana* (Neumetzler et al. 2012). Overall, based on existing data, negative regulation of PME by PMEI promotes leaf cell expansion and leaf growth owing to reduced cell wall hardening. Although these studies did not observe *PME* mediated alterations of cell adhesion in leaf cells, enhanced *PME* expression was found to promote cell adhesion between cotyledon cells leading to a reduction in cotyledon size (Neumetzler et al. 2012). In addition, alterations in a *SQUAMOSA PROMOTER BINDING PROTEIN–LIKE GENE* (*SBP-BOX*) reduced pectin methylesterase activity, pectin-Ca^{2+} cross-linkages, and cell-to-cell adhesion resulting in large intercellular airspaces in the fruit pericarp in *Solanum lycopersicon* (Orfila et al. 2001; Eriksson et al. 2004; Manning et al. 2006; Caffall and Mohnen 2009). Therefore, it is likely that PME activity plays a role in leaf cell-to-cell adhesion. However, PMTs, such as *CGR2* and *CGR3*, seem to have a stronger effect on cell-to-cell adhesion as discussed below.

2. Pectin Methyltransferase

Pectin methyltransferase (PMT) catalyzes methylesterification of pectin. Out of the 29 putative PMT genes in *A. thaliana*, only the effects of *QUASIMODO1*, *2*, and *3* (*QUA1*, *QUA2*, *QUA3*) (Mouille et al. 2007; Miao et al. 2011), TUMOROUS SHOOT DEVELOPMENT2 (*TSD2*) (Krupkova et al. 2007), and *COTTON GOLGI-RELATED* (*CGR2, 3*) genes (Held et al. 2011; Kim et al. 2015) on cell expansion have been investigated. Interestingly, partial suppression of

QUA1, *QUA2*, or *TSD2* did not enhance cell-cell adhesion, but reduced it as evident by cell detachment in the hypocotyl (Bouton et al. 2002; Krupkova et al. 2007; Mouille et al. 2007). Even though suppression of PMT expression is predicted to result in a decrease in pectin methylesterification and an enhancement of cell-to-cell adhesion, this was not seen during the above studies. Therefore, the function of the above genes as PMTs needs to be further characterized. It may also be that adhesive and expansion capabilities in leaf cells were reduced due to the suppression of the above genes causing the reduced leaf size in the corresponding mutant lines. However, a detailed anatomical study has to be conducted to test this possibility.

Recent studies provide compelling evidence to support the role of *CGR2* and *CGR3* in regulating mesophyll cell expansion and overall leaf architecture in *A. thaliana* (Kim et al. 2015; Weraduwage et al. 2016). Pectin content and pectin methylesterification in leaves were reduced in a double knockout mutant of *CGR2* and *CGR3* genes (*cgr2/3*) and the opposite effect was verified in lines overexpressing *CGR2* (CGR2OX) (Held et al. 2011; Kim et al. 2015; Weraduwage et al. 2016). *cgr2/3* mutant lines produced thin but dense leaf mesophyll with enhanced cell number and reduced air spaces compared to the wild-type (Fig. 8.13a–c) (Kim et al. 2015; Weraduwage et al. 2016). CGR2OX produced thinner leaves compared to the wild-type, but thicker than *cgr2/3*. Cells and intercellular air spaces in CGR2OX leaves were also larger than in the wild-type (Kim et al. 2015; Weraduwage et al. 2016). Both projected and total leaf area were markedly reduced in *cgr2/3* and enhanced in CGR2OX (Fig. 8.13f) (Weraduwage et al. 2016). Above phenotypes in *cgr2/3* were partially resored in cgr2com *by CGR2* complementation. However, despite the changes in leaf expansion, changes in overall leaf shape were not detected (Fig. 8.13f). These data show that *CGR2* and *CGR3* are involved in cell expansion and thereby play a crucial role in determining leaf architecture. The authors hypothesized a reduction in expression of *CGR2* and *CGR3* causes cell wall hardening as a result of reduced pectin methylesterification and a greater degree of Ca^{2+} mediated cross-linking of pectin (Kim et al. 2015; Weraduwage et al. 2016). An increase in cell-to-cell adhesion may have caused the increase in cell density and reduced intercellular air-spaces in the *cgr2/3* mutant while the promotion of mesophyll cell expansion observed with *CGR2* overexpression is probably due to a reduction in cell wall hardening and cell adhesion brought about by an increase in pectin methylesterification. However, given that no change in leaf shape was apparent, expression of *CGR2* and/or *CGR3* does not seem to affect microtubule alignment, but rather have a role in general cell expansion independently from the cytoskeleton.

In summary, data presented above show that PMTs, *PME*, and *PMEI* form an effective molecular system to mediate the degree of pectin methylesterification in order to fine tune cell expansion and adhesion and, consequently, mesophyll and overall leaf architecture. Based on the data available so far, PMTs such as *CGR2* and *CGR3* seem to cause more controllable alterations in cell expansion and leaf architecture compared to PMEI, PME, and other identified putative PMTs. The following section summarizes recent evidence showing how the PMT/*PME*/*PMEI* system can act as a key downstream molecular system common to different upstream signaling pathways targeting changes in leaf architecture.

3. *PMT/PME/PMEI System as a Key Downstream Execution Point of Leaf Architectural Changes and Its Modulation by CAMTA/SA, JAZ/JA, and PHYB/GA/PIF*

In general, PIF positively affects PMEI expression whereas *CAMTA*/SA has a significant inhibitory effect (Figs. 8.14 and 8.15) (unpublished data by Y-S.K., S.M.W., T.D.S., and M.F.T., and by M.L.C., Y.Y.,

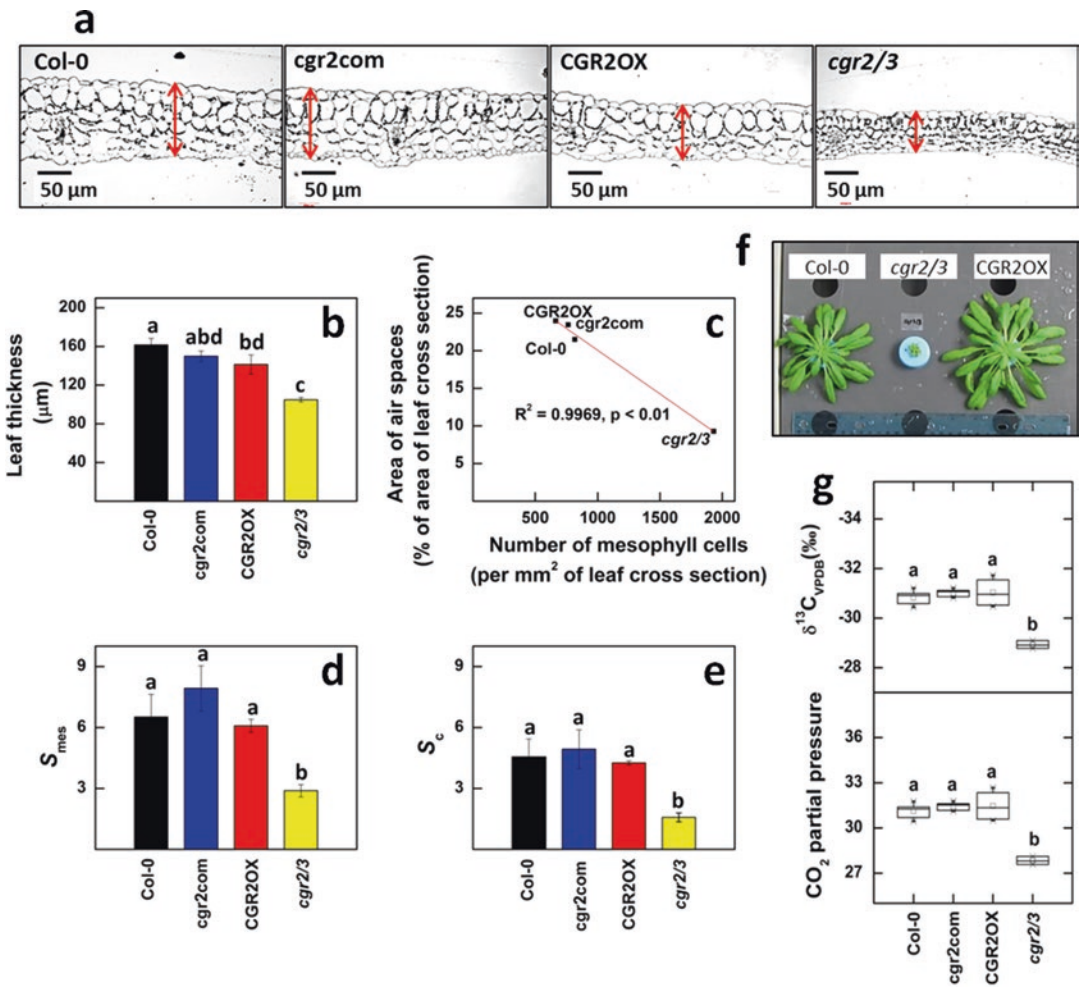

Fig. 8.13. **The effect of altered *CGR2* and *CGR3* gene expression on leaf architecture.** (**a**) Representative micrographs of leaf cross sections, (**b**) leaf thickness, (**c**) the relationship between the number of mesophyll cells and size of the intercellular air spaces in the leaf mesophyll, (**d**) the surface area of mesophyll cells facing intercellular air spaces per unit leaf area (S_{mes}), (**e**) the surface area of chloroplasts facing intercellular air spaces per unit leaf area (S_c), (**f**) size comparison of rosettes, and (**g**) the $\delta^{13}C_{VPDB}$ value calculated as the ratio of ^{13}C to ^{12}C isotopes in leaf tissue relative to a Vienna-Pee-Dee Belemnite standard (VPDB) (top panel), and the CO_2 partial pressure at rubisco calculated using the $\delta^{13}C_{VPDB}$ values (bottom panel), are presented for *A. thaliana* wild-type Col-0 and mutant lines: *cgr2/3* (loss of function double mutant line of *CGR2* and *CGR3*), cgr2com (*cgr2/3* complemented by *CGR2*), and CGR2OX (*CGR2* overexpression line). $\delta^{13}C_{VPDB}$ is described in the Fig. 8.2 legend. In (**a**), leaf thickness is denoted by red double arrows. In (**a–e**), data were obtained from 34-day old leaves. In (**f**), rosettes were photographed 45 days after seeding. In (**b**), (**d**), and (**e**) values represent the mean ± SE and n = 4 plants per line. In (**c**) n = 4 plants per line were used to obtain the mean values for the area of air spaces as a % of area of leaf cross section and the number of mesophyll cells per mm^2 of leaf cross section. Differences between means were tested by carrying out a one-way ANOVA at a = 0.05, followed by a Fisher's Least Significant Difference Test. Statistical differences at $P < 0.05$ are marked with lower case letters. (Reproduced from Weraduwage et al. 2016)

I.T.M., S.M.W., T.D.S., and G.A.H.). This may lead to an inactivation of PMEs in *phyB* and activation in *camta1/2/3* with a corresponding increase and decrease in pectin methylesterification, respectively (Fig. 8.15). This is further supported by the fact that the mesophyll architecture of *cgr2/3* with reduced *CGR2* and *CGR3* expression and

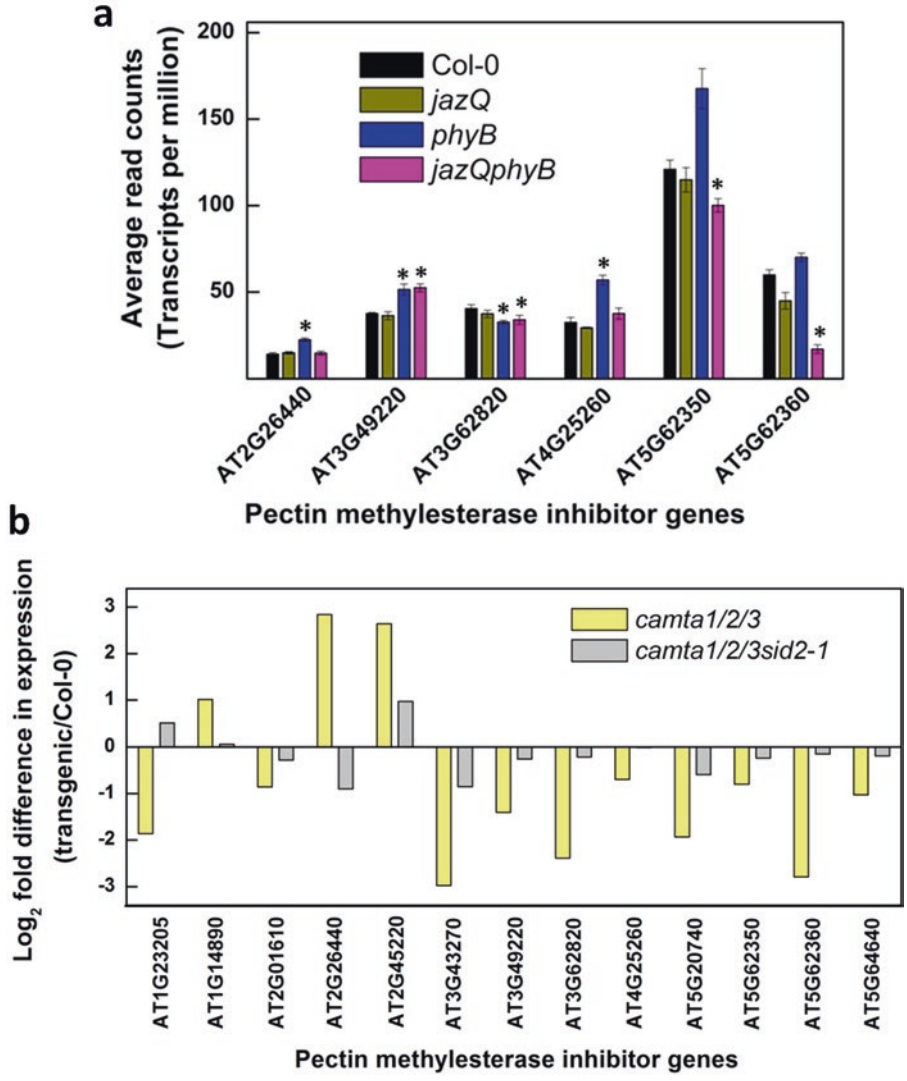

Fig. 8.14. **Comparison of altered expression of genes coding for PMEI in *Arabidopsis* mutant lines showing altered leaf architecture.** Expression levels of *Pectin methylesterase inhibitor* (*PMEI*) in *A. thaliana* Col-0 wild-type and (a) *jazQ*, *phyB*, and *jazQphyB* mutant lines (Campos et al. 2016), and (b) *camta1/2/3* and *camta1/2/3sid2–1* mutant lines (unpublished data by Y-S.K., S.M.W., T.D.S., and M.F.T.), are shown. Expression levels were determined by leaf messenger RNA sequencing. Values represent the mean ± SE and n = 3 plants per line. In (a), values represent the mean ± SE and n = 3 plants per line and statistically different expression levels in comparison to Col-0 found according to the DESeq algorithm ($P < 0.05$, using a Benjamini-Hochberg adjusted for multiple testing) are marked with asterisks. In (b), values presented are Log_2 fold differences in expression (transgenic/Col-0) of *PMEI* genes as determined by RNA-seq analysis of leaves and positive and negative values indicate a higher or a lower level of expression relative to Col-0, respectively

reduced pectin methylesterifiation was similar to *camta2/3* (Figs. 8.2 and 8.13). Significant changes in *PMEI* expression could not be detected in *jaz*Q mutant lines (Fig. 8.14). This may be why *jaz*Q did not show any drastic changes in mesophyll architecture despite having smaller leaves (Fig. 8.3). Thus, the *PMT*/*PME*/*PMEI* system, which regulates the degree of methylation of pectin and subsequently cell wall

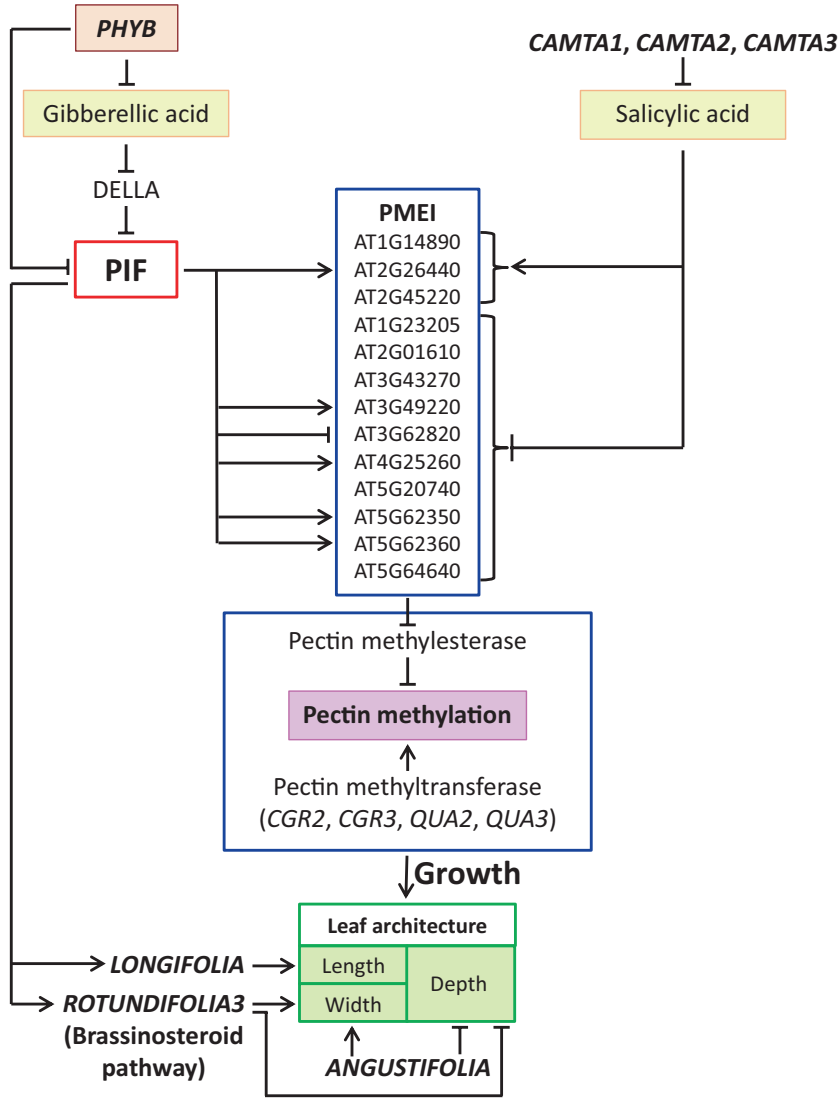

Fig. 8.15. ***PHYB*/GA/PIF and *CAMTA*/SA mediated effects on *PMEI* gene expression and pectin methylesterification.** A schematic diagram is presented summarizing the effects of Phytochrome interacting transcription factor (PIF) and salicylic acid (SA) on specific target *Pectin methylesterase inhibitor* (*PMEI*) gene expression. Data were derived from messenger RNA sequencing data obtained from null mutant lines of *Phytochrome-B* (*PHYB)* gene expression (Campos et al. 2016) and from mutant lines having enhanced SA production as a result of suppressed *Calmodulin binding transcription activator* (*CAMTA*) gene expression (unpublished data by Y-S.K., S.M.W., T.D.S., and M.F.T.). Different *PMEI* genes are denoted by their GenBank accession numbers. The degree of pectin methylation will depend upon: 1. the activity of pectin methylesterase (PME) that demethylates pectin, 2. the expression and activity of PMEI that inhibits PME, and 3. the expression and activity of pectin methyltransferase. A higher degree of methylesterification of pectin has been shown to reduce cell wall hardening and promote cell expansion, while a lower degree of methylesterification promotes cell wall hardening and cell-to-cell adhesion. New evidence was also found to support upregulation of *Longifolia* (enhances cell expansion in the leaf-length direction) and *Rotundifolia3* (enhances cell expansion in the leaf length direction while limiting cell expansion in the leaf-depth direction) gene expression by PIF (Campos et al. 2016). Upregulation and down regulation of gene expression is denoted by pointed and blunt ended arrows, respectively. Other abbreviations: DELLA –PIF transcription factor repressors, *CGR2* and *CGR3 – Cotton Golgi-related 2* and *3*, *QUA2* and *QUA3 – Quasimodo 2* and *3*

extensibility and adhesive properties, is likely a key downstream execution point common to the *PHYB*/GA/PIF and *CAMTA*/SA genetic systems, which seem to have opposite effects on pectin methylesterification (Fig. 8.15). Changes to pectin methylesterification can have more drastic negative effects on leaf architecture than those observed by effects on XTH and expansins alone. These data also emphasize *PMT*/*PME*/*PMEI* as a key downstream molecular system through which changes to leaf architectural changes are executed in response to stress (Fig. 8.15).

V. Broader Implications of Understanding Genes and Molecular Mechanisms That Affect Cell Wall Properties and Leaf Architecture

The architecture of the leaf is designed to ultimately produce an organ that is optimized to act as a solar collector as well as an efficient gas exchanger to maximize photosynthesis. Although a large number of mutations affecting leaf architecture have been studied during the past two decades (Sections II-IV), these mutations have not been studied in the context of positive or negative effects on photosynthesis. In fact, alterations in leaf architecture have profound effects not only on photosynthesis, but also on respiration and overall plant growth (Lambers et al. 2008; Weraduwage et al. 2015, 2016). In the preceding sections, leaf architecture of many mutants including that of *phyB*, *jaz*Q, *camta* and *cgr* mutants and the potential molecular mechanisms involved in bringing about such architectural changes were discussed. Here, we will discuss the impact of such architectural changes on photosynthesis, C partitioning, and plant growth and identify genetic candidates that can be used as tools for crop improvement.

A. Mesophyll Architecture and Its Impact on CO_2 Availability at Rubisco and Area-Based Photosynthesis

CO_2 diffusion into the chloroplast stroma through the cell wall, plasma membrane, and chloroplast envelope takes place along the route that poses the lowest resistance (Terashima et al. 2006). The active area through which CO_2 diffuses in to the chloroplast stroma is the chloroplast surface area facing intercellular air spaces per unit leaf area (S_c), and S_c and internal conductance are positively correlated (Terashima et al. 2006). Lower S_c leads to a reduction in CO_2 concentration at rubisco with subsequent reductions in carboxylation/oxygenation ratio (Terashima et al. 2006). *camta2/3* (Fig. 8.2d), and *cgr2/3* (Fig. 8.13g) with densely packed cells in the leaf mesophyll, and *phyB* and *jaz*Q*phy*B with thin leaves (Fig. 8.3c) showed a reduction in the degree of $^{13}CO_2$ discrimination during CO_2 assimilation and a decrease in average CO_2 partial pressure at rubisco (Weraduwage et al. 2016; unpublished data by Y-S.K., S.M.W., T.D.S., and M.F.T.; unpublished data by M.L.C., Y.Y., I.T.M., S.M.W., T.D.S., and G.A.H). A decrease in average CO_2 partial pressure at rubisco was also accompanied by a reduction in area-based photosynthesis rates in *camta2/3* (unpublished data by Y-S.K., S.M.W., T.D.S., and M.F.T), *phyB* and *jaz*Q*phy*B (Campos et al. 2016), and *cgr2/3* (Weraduwage et al. 2016). The above effects on leaf gas exchange properties were greatest in *camta2/3* and *cgr2/3* where the mesophyll cell density, and hence leaf dry mass per unit leaf area (LMA), was greatest. Further examination of the leaf mesophyll of *cgr2/3* revealed a significant reduction in mesophyll cell surface area facing intercellular air spaces per unit leaf area (S_{mes}) and S_c (Fig. 8.13d–e). Thus, the reduction in CO_2 availability for photosynthesis in *camta2/3* and *cgr2/3* is likely a result of reduced S_{mes}, S_c, and intercellular air spaces.

LMA has been shown to both positively and negatively affect mesophyll conductance

depending on the plant species (Flexas et al. 2008; Soolanayakanahally et al. 2009; Tosens et al. 2012). Milla-Moreno et al. (2016) showed that in *P. balsamifera*, even though an increase in the number of cell layers in palisade tissue can increase leaf thickness and subsequently decrease internal CO_2 conductance, there was a greater positive effect on mesophyll conductance in terms of enhanced S_{mes} and S_c. Thus, in *P. balsamifera*, LMA and mesophyll conductance was positively correlated (Milla-Moreno et al. 2016). However, in *camta2/3* and *cgr2/3* a negative correlation is likely between LMA and mesophyll conductance. A reduction in GA biosynthesis or sensitivity to this growth regulator increases LMA in *A. thaliana* and *S. lycopersicon*; GA supplementation lowers LMA (Dijkstra et al. 1990; Nagel et al. 2001; Poorter et al. 2009). Under light, a decrease in GA occurs as a result of both a reduction in the transcription of genes involved in GA biosynthesis and an increase in gibberellin-2-oxidase that increases GA catabolism (Folta et al. 2003; Hisamatsu et al. 2005; Foo et al. 2006; Achard et al. 2007; Weller et al. 2009; Pierik et al. 2011; Hirose et al. 2012; Colebrook et al. 2014; Mazzella et al. 2014). *phyB* and *jaz*Q*phyB* maintained fairly large intercellular air spaces, but with shorter and wider cells in the palisade tissue and reduced cell layers and subsequently thinner leaves, which resulted in lower LMA and area-based photosynthesis in these lines (Campos et al. 2016). Reduced area-based photosynthesis rates were also reported in *gigantea-2* (*gi-2*) mutant lines with thin leaves and lower LMA (Weraduwage et al. 2015), and in *er* mutants despite having larger intercellular airspaces in the leaf mesophyll (Masle et al. 2005). These observations are attributed to the lack of photosynthetic machinery on a leaf area basis in thinner leaves (Masle et al. 2005; Campos et al. 2016). One difference between sun and shade leaves is that the former produces thicker leaves (longer palisade cells or multiple cell layers, higher LMA) that enables the housing of more chloroplasts per unit leaf area and increased amounts of photosynthetic enzymes per unit leaf area, allowing for the maintenance of higher area-based photosynthesis rates; the opposite is seen in shade leaves (Lambers et al. 2008).

The advantages of producing leaves with a larger surface area (lower LMA) and the disadvantages of producing leaves with higher LMA is discussed in the following sections. In summary, molecular mechanisms affecting cell wall properties and leaf architecture play a significant role in modulating area-based photosynthesis rates through CO_2 diffusion into cells as well as concentrating resources required for photosynthesis per unit leaf area.

B. Mesophyll Architecture and Its Impact on Area-Based Respiration and Daily C Gain

An increase in leaf cell density enhances leaf mass density (LMD, dry mass of leaf per unit volume of leaf tissue) and both leaf thickness and LMD can affect LMA (Lambers et al. 2008; Poorter et al. 2009; Weraduwage et al. 2016). For example, despite having thinner leaves, an increase in leaf cell density resulted in higher LMA in *cgr2/3* (Weraduwage et al. 2016). Analysis of anatomical components that affect LMA has revealed palisade tissue cell properties to be the major contributor to variations in LMA in mature leaves of *Populus balsamifera* (Milla-Moreno et al. 2016).

As mentioned before, thicker leaves usually possess larger LMA as a result of a greater number of cell layers (Lambers et al. 2008; Poorter et al. 2009; Villar et al. 2013; Weraduwage et al. 2015). Changes in leaf architecture such as a larger number of smaller cells with thicker cell walls can also lead to (1) changes in chemical composition such as a higher proportion of lignin and

other cell wall polysaccharides per unit leaf area that can result in higher LMD and LMA and (2) cellular changes such as increased organelle numbers (Cunningham et al. 1999; Lambers et al. 2008). For example, *cgr2/3* cells possess a large number of small chloroplasts per unit leaf area that could also lead to higher LMA; chloroplast thickness was not reported in this study. However, this only translated to higher area-based photosynthesis rates during early growth stages of *cgr2/3* and at latter stages area-based photosynthesis rates were significantly lower as a result of lower S_c (Weraduwage et al. 2016).

Leaves with larger LMA require more energy to maintain a greater number of cells and organelles per unit leaf area and hence incur higher maintenance respiratory costs (Lambers et al. 2008). This was observed in *cgr2/3* in which higher leaf cell density and higher LMA positively correlated with enhanced area-based respiration (Weraduwage et al. 2016). Consequently, photosynthesis to respiration ratios were lower, and coupled with smaller projected leaf area, a significant reduction in daily carbon gain, net assimilated C for growth and overall plant growth was seen in *cgr2/3* (Fig. 8.16). In contrast, in mutant lines such as *gi-2* (Weraduwage et al. 2015) and CGR2OX (Weraduwage et al. 2016), which produced larger, thinner leaves with smaller LMA, area-based respiration was smaller and photosynthesis to respiration ratios were larger and coupled with larger projected leaf area, a significant increase in daily carbon gain, net assimilated C for growth, and overall plant growth was seen (Fig. 8.16). It is also assumed that *phyB* and *jaz*Q*phyB* incur lower construction costs to build their thinner leaves compared to that in *jazQ* (Campos et al. 2016). Overall, the above data indicate that molecular mechanisms affecting cell wall properties and leaf architecture can have a significant impact on area-based respiration in leaves and daily C gain.

C. *Leaf Architecture and Its Impact on Light Capture, Whole-Plant Photosynthesis, and Growth*

Light capture is optimized by having a larger leaf area, and this is seen especially in plants grown under low light (Lambers et al. 2008). Shade plants often produce leaves with smaller LMA (Niinemets 2001; Lambers et al. 2008). This reduces area-based respiration rates, maximizing daily C gain, and allows compensation for lower area-based photosynthesis rates (because of lower LMA as described in Section VA) under low light (Lambers et al. 2008). Although area-based photosynthesis rates were lower, whole plant photosynthesis was enhanced in *A. thaliana* mutants capable of producing large rosettes, e.g., *gi-2* (Weraduwage et al. 2015), *phyB*, and *jaz*Q*phyB* (Campos et al. 2016) and CGR2OX (Fig. 8.16) (Weraduwage et al. 2016) as a result of larger projected leaf area. Projected leaf area represents the effective leaf surface area capable of intercepting light (Honda and Fisher 1978). Model based analyses revealed that in CGR2OX more C is partitioned to leaf area growth (Fig. 8.16). Greater whole plant photosynthesis as a result of larger leaf area coupled with lower area-based respiration as a result of lower LMA led to an enhancement in C available for growth and consequently an increase in overall plant growth in CGR2OX (Fig. 8.16). This was also seen in *jaz*Q*phyB* (Campos et al. 2016).

LMA or 1/specific leaf area has been shown to be a key trait that determines variation in relative growth rates between plants growing in nutrient rich and nutrient poor conditions (Poorter and Remkes 1990; Garnier 1992; Lambers et al. 2008). Also, LMA often increases under water stress as a result of an increase in cell wall thickness and a reduction in cell size (Cutler et al. 1977; Utrillas and Alegre 1997; Van Volkenburgh and Boyer 1985; Fredeen et al. 1991;

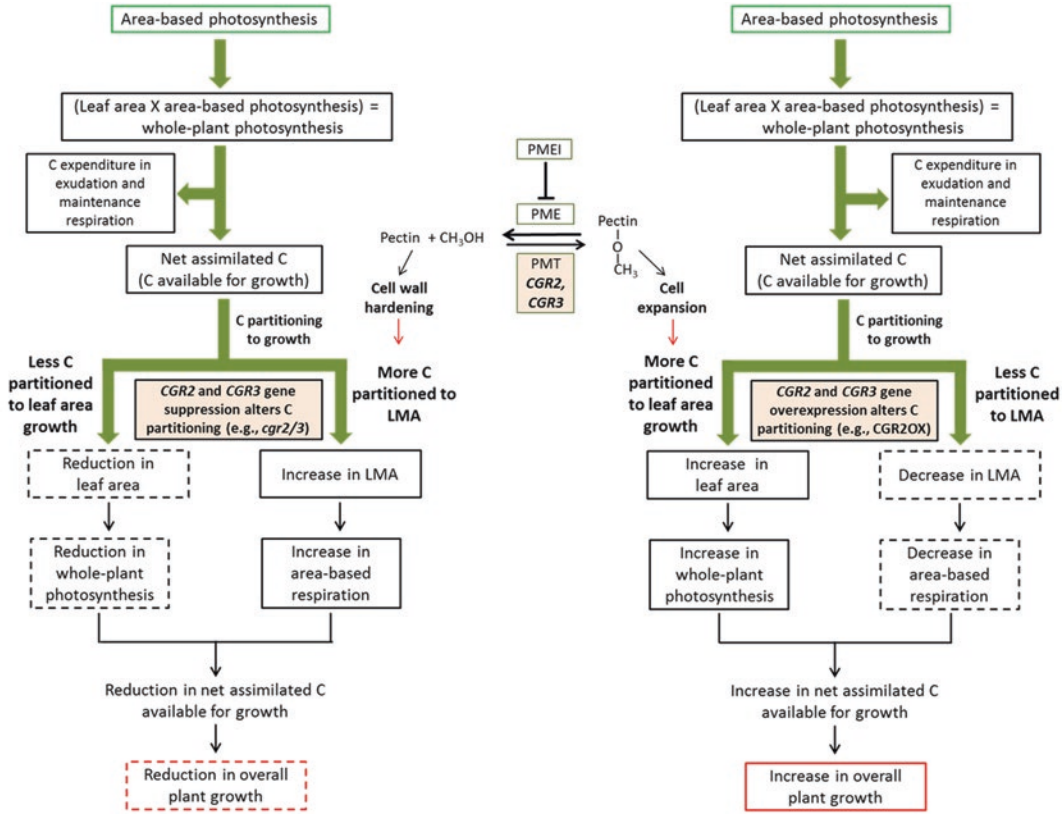

Fig. 8.16. **CGR2 and CGR3 mediated pectin methylesterification regulates the relationship between photosynthesis and plant growth.** A schematic diagram outlining how (**a**) suppression or (**b**) over-expression of *CGR2* and *CGR3* gene expression affects the relationship between photosynthesis and plant growth in *A. thaliana* is presented. Suppression of *CGR2* and *CGR3* reduces the degree of pectin methylation, which leads to an increase in cell-to-cell adhesion, cation-mediated cross linking of galacturonic acid, and consequent hardening of cell walls. *CGR2* over-expression increases the degree of pectin methylation, which allows cell expansion through reduced cell-to-cell adhesion and cation-mediated cross linking. It has been proposed that pectin methyltransferase enzyme, through its ability to directly alter cell expansion, determines the amount of C partitioned to leaf area growth versus growth in terms of LMA. For example, while more C is partitioned to growth in terms of LMA in the *CGR2* and *CGR3* double knockout mutant, in the CGR2 over-expression line more C is partitioned to leaf area growth. An increase in LMA leads to enhanced area-based respiration and a reduction in leaf area contribute to a reduction in whole plant photosynthesis. Collectively, this results in a reduction in C available for growth and consequently a decrease in overall plant growth; an opposite trend is seen in CGR2OX. Thus, *CGR2* and *CGR3*, through their ability to alter the degree of methylated pectin in the cell wall of the mesophyll cells, determine how photosynthate is utilized to grow the plant. In other words, *CGR2* and *CGR3* mediated pectin methylesterification affects the relationship between photosynthesis and plant growth by regulating the proportions of C that are partitioned to leaf area growth and LMA. Final overall growth mainly depends on the expression patterns of *CGR2* and *CGR3* and how much C is partitioned to area growth and LMA and not on area-based photosynthesis. PME: pectin methylesterase; PMEI: PME inhibitor; PMT: pectin methyltransferase. CH_3OH is methanol. (Reproduced from Weraduwage et al. 2016)

Niinemets 2001; Lambers et al. 2008). Thus, under drought stress, enhanced LMA may in part contribute to growth reductions based on factors mentioned above (Niinemets 2001).

D. *Genes Such as CGR2 and CGR3 That Alter Cell Wall Properties Can Modulate the Relationship Between Photosynthesis and Growth*

It has been shown that, while the correlation between area-based photosynthesis and plant growth is not clear, relative growth rate and leaf growth parameters such as leaf area per unit leaf dry mass (inverse of LMA), leaf area per unit plant dry mass, and investment of C in leaf growth are strongly and positively correlated (Shipley 2002; Lambers et al. 2008; Poorter et al. 2009). In dicots, there is a negative correlation between relative growth rate and the root:shoot ratio, highlighting the importance leaf expansion and overall growth (Garnier 1991; Lambers et al. 2008). Similarly, model based analyses of *A. thaliana* leaf growth revealed that while photosynthetic C is required for growth, the magnitude of plant growth depends on the proportions of C partitioned to leaf area growth and LMA (Weraduwage et al. 2015, 2016).

The Arabidopsis leaf area growth model was developed to simulate the C flow from the beginning to end of the *A. thaliana* life cycle while also simulating the utilization of assimilated C in respiration and the partitioning of the remaining C to grow leaves in the form of area and LMA, root growth, and reproduction (Weraduwage et al. 2015). The model can be fitted with measured data to determine partitioning coefficients of C that give rise to the growth patterns of different plants (Weraduwage et al. 2015). The responses of overall plant growth (plant dry weight) to varying magnitude of model inputs such as photosynthesis and partitioning coefficients can also be determined (Weraduwage et al. 2015). For example, the model revealed that enhanced leaf and plant growth in *gi-2* (Weraduwage et al. 2015), and differences in leaf and plant growth and architecture in *cgr2/3* and CGR2OX (Weraduwage et al. 2016), were not a result of differences in area-based photosynthesis rates, but mainly a result of altered C partitioning to leaf area growth and growth in terms of LMA (Figs. 8.16 and 8.17) (Weraduwage et al. 2016). C partitioning to leaf area growth was greater in CGR2OX and *gi-2* and significantly smaller in *cgr2/3*; the opposite trend was seen in terms of C partitioning to LMA (Figs. 8.16 and 8.17). These findings obtained using the Arabidopsis leaf area growth model agree with inferences derived from classical growth models developed by Monsi (1960), Poorter and Lambers (1991), and Tillman (1991) where enhancements in growth rates were shown to positively correlate with biomass allocation to leaves. Therefore, "photosynthesis drives growth through alterations in carbon partitioning to new leaf area growth and leaf mass per unit leaf area" (Weraduwage et al. 2016). It was found that *CGR2* and *CGR3* genes can directly affect the relationship between photosynthesis and growth by directly altering the capacity of cell expansion and cellular organization in the leaf mesophyll, thus creating varying carbon demands for leaf area growth and LMA that will in turn drive C partitioning for these processes (Fig. 8.16). It can be hypothesized that other genes such as *XTH8*, *XTH21*, and *XTH31,* which have a profound effect on leaf architecture (Fig. 8.12) (Ogawa et al. 1996; Itoh et al. 2002; Jan et al. 2004; Liu et al. 2007), may also be able to affect C partitioning between leaf area growth and LMA.

In summary, alterations of leaf and mesophyll architecture through cell wall modifications can have a marked influence on net C assimilation as a result of effects on: 1. CO_2 availability at rubisco, 2. light interception, and 3. respiratory costs. Thus, cell wall plasticity is a key factor influencing photosynthetic processes in plants. Therefore, genes and molecular systems that modulate cell

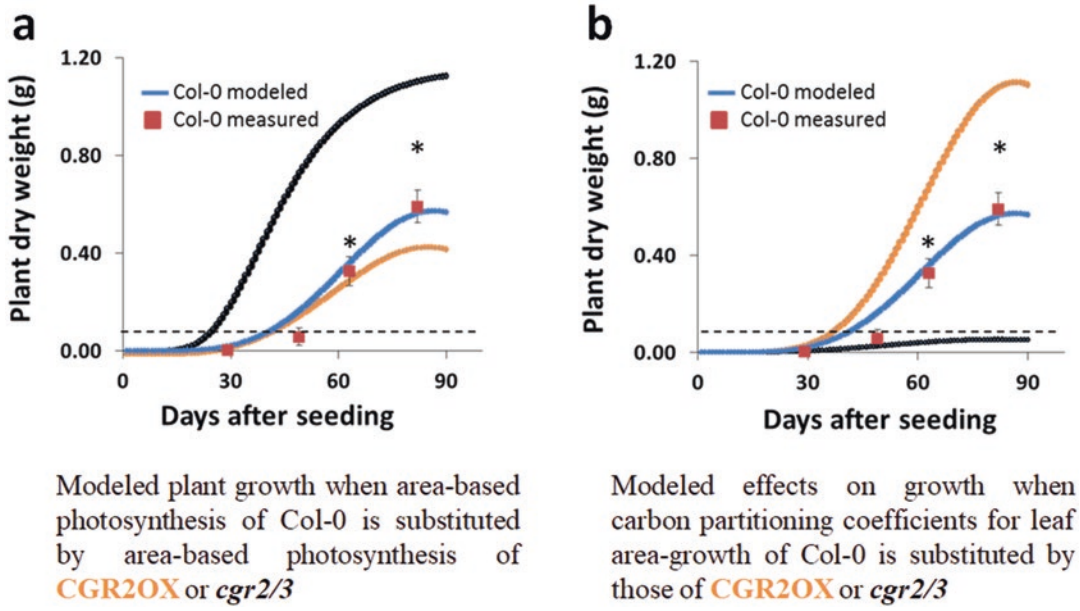

Fig. 8.17. **Comparison of how changes in area-based photosynthesis and C partitioning to leaf growth affects plant growth in *A. thaliana*.** The Arabidopsis Leaf Area Growth Model was used to test how changes in area-based photosynthesis and C partitioning to leaf growth affects plant growth. The model simulates plant growth based on the use of assimilated C in respiration and partitioning of the remaining C to leaf area growth and LMA, root growth, and reproduction. The model is capable of simulating plant growth (blue, black, and orange lines) based on the magnitude of a particular model input, e.g., area-based photosynthesis, C partitioning to different growth processes etc. Measured data from Col-0 wild-type, *CGR2* overexpressed (CGR2OX) or *CGR2*, and *CGR3* suppressed (*cgr2/3*) *A. thaliana* lines were used to test whether the enhancement in growth in CGR2OX was as a result of alterations in area-based photosynthesis or as a result of alterations in C partitioning to leaf area growth (with corresponding reductions of C partitioning to LMA); CGR2OX showed enhanced growth and *cgr2/3* showed suppressed growth (Kim et al. 2015; Weraduwage et al. 2016). In order to do so, area-based photosynthesis (**a**) or partitioning coefficients to leaf area growth (**b**) for Col-0 were replaced by that of CGR2OX (orange line) or *cgr2/3* (black lines). In (**a**) and (**b**), blue lines represent modeled growth for Col-0 and red squares represent measured data points (mean ± SD) for Col-0 at 29, 49, 63, and 82 days after seeding. Black lines represent modeled data for Col-0 when its area-based photosynthesis rates (**a**) or C partitioning to leaf area growth (**b**) are replaced by that of *cgr2/3*. Orange lines represent modeled data for Col-0 when its area-based photosynthesis rates (**a**) or C partitioning to leaf area growth (**b**) are replaced by that of CGR2OX. Asterisks represent measured data for CGR2OX at 63 and 82 days after seeding and dotted lines indicates the upper limit of measured data for cgr2/3. (Reproduced from Weraduwage et al. 2016). Based on the model, the observed enhancement in growth in CGR2OX and reduced growth in *cgr2/3* occurs as a result of small changes in C partitioning to leaf area growth and LMA (Weraduwage et al. 2016). For more information on the Arabidopsis Leaf Area Growth Model, please read Weraduwage et al. (2015, 2016) (Colour figure online)

wall properties can be utilized to optimize leaf architecture to maximize photosynthesis. Genes that code for PMTs, specifically *CGR2* and *CGR3*, and XTH through their role in regulating cell wall plasticity and mesophyll architecture can direct C partitioning between leaf area growth and LMA and thereby, directly affect the relationship between photosynthesis and plant growth. Thus, understanding genes and corresponding molecular mechanisms that affect cell wall properties and leaf architecture is of utmost importance to select candidate genes for crop improvement.

VI. Conclusions

Based on results from key studies carried out during the past two and a half decades, several major molecular systems that interact to regulate cell wall composition (*CESA*/*CSL*, PMT/*PME*/*PMEI*, *XTH*), anisotropic cell expansion (*ROP* and associated genes, *LNG*, *ROT3*, *AN*, *XTH*), and isotropic cell expansion (*XTH*, PMT/*PME*/*PMEI*) to cause profound changes in leaf architecture were identified. Upstream signaling systems such as *CAMTA*/SA, *JAZ*/JA, and *PHYB*/GA/PIF can cause significant changes in leaf architecture by interacting with a large number of midstream and downstream molecular pathways. *PHYB*/GA/PIF can affect leaf architecture by altering expression of genes belonging to a variety of molecular pathways including: ROPs, *LNG* and *ROT3*, *CESA*/*CSL*, XTH, *EXP*, and *PMEI*. *AN* interacted with *PHYB*/GA/PIF at downstream *XTH* genes. Recent evidence suggests that the effects of *CAMTA*/SA on leaf architecture may be occurring through *XTH* and *PMEI* and that of *JAZ*/JA through *XTH* and *EXP*. The major downstream execution points of leaf architecture changes common to *CAMTA*/SA, *JAZ*/JA, and *PHYB*/GA/PIF mediated signaling pathways were the *XTH* and PMT/*PME*/*PMEI* systems. *XTH* expression is also affected by both *ROT3* and *AN*. Overall, growth promotion by *PHYB*/GA/PIF and growth suppression by *JAZ*/JA and *CAMTA*/SA seems to occur via modulation of *XTH* and *PMEI* of the PMT/*PME*/*PMEI* systems in opposite directions. It is also clear that changes in leaf architecture that occur in response to stress responses take place first at the genetic level at common action points such as *XTH* and PMT/*PME*/*PMEI*.

C for growth for most organisms on earth is supplied through photosynthesis and the leaf is the primary photosynthetic organ in plants. Alterations in leaf architecture have a profound impact on (1) CO_2 availability at rubisco and area-based photosynthesis, (2) area-based respiration, and (3) light capture and whole-plant photosynthesis, with ultimate effects on daily C gain growth. In addition, molecular systems such as the PMT/*PME*/*PMEI*, and specifically *CGR2* and *CGR3* genes (PMTs) that alter cell wall properties, can modulate the relationship between photosynthesis and growth by directly altering the capacity of cell expansion and cellular organization in the leaf mesophyll thereby creating differential carbon demands for leaf area growth and LMA that can in turn drive C partitioning for these processes. Therefore, our understanding of genes and molecular mechanisms that affect cell wall properties and leaf architecture will facilitate the identification of novel genetic model systems that can be utilized to improve photosynthesis and growth of crop plants.

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Chapter 9

Significance of C_4 Leaf Structure at the Tissue and Cellular Levels

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Summary

The CO_2 concentrating mechanism (CCM) in C_4 plants requires a complex coordination of both leaf anatomical and biochemical traits. While there are key traits common across the 60 plus C_4 lineages, there is also significant structural and biochemical variation. Traditionally, C_4 plants are described as one of three biochemical subtypes based on the primary enzyme used for C_4 acid decarboxylation: NADP-malic enzyme (NADP-ME), NAD-malic enzyme (NAD-ME), and phospho*enol*pyruvate carboxykinase (PCK). However, there may be biochemical flexibility and overlap between these subtypes. C_4 plants typically rely on Kranz-type anatomy that partitions the C_4 cycle into the mesophyll (M) cells and the majority of C_3 cycle into the bundle-sheath (BS) cells. However, within the succulent Chenopods some NAD-ME type C_4 plants use one of two single-cell arrangements to partition and compartmentalize the C_4 and C_3 cycles. Here we discuss key leaf anatomical traits at the tissue, cellular, and sub-cellular level that influence the efficiency and effectiveness of C_4 photosynthesis. Specifically, we discuss preconditioning of leaf traits that increase the evolvability of C_4 photosynthesis, the evolutionary transition of organelles from C_3 to a C_4 leaf, gas and metabolite movement within the leaf, the positioning and maintenance of organelles in M and BS cells, and the movement of M chloroplasts.

I. Introduction

As widely discussed in the literature, the minimum requirements of the CO_2 concentrating mechanism (CCM) in terrestrial C_4 plants are (a) cell-specific capture of bicarbonate (HCO_3^-) via phospho*enol*pyruvate carboxylase (PEPC) into 4-carbon organic acids; (b) the movement of these C_4 acids to compartmentalized sites of decarboxylation where the CO_2 partial pressure is increased around Rubisco and the majority of the Calvin-Benson cycle (C_3 cycle), with complementary adjustments of photosystem and electron transport activities; (c) novel cell-specific organelle metabolite transporters; (d) symplastic connections of the spatially separated sources and sinks of the C_4 acids; and (e) barriers to CO_2 diffusion between the site of HCO_3^- fixation by PEPC and the sites of CO_2 release and refixation by Rubisco (Edwards et al. 2001; Edwards and Voznesenskaya 2011).

In terrestrial plants, C_4 photosynthesis has independently evolved over 60 times across the angiosperms (Edwards et al. 2010; Sage et al. 2011a). While there are key traits, as described above, common across these C_4 lineages, there is also significant structural and biochemical variation (see reviews by Edwards et al. 2001; Edwards and Voznesenskaya 2011). Traditionally, C_4 plants have been categorized as one of three biochemical subtypes based on the primary

location and enzyme used for the C_4 acid decarboxylation: chloroplastic NADP-malic enzyme (NADP-ME), mitochondrial NAD-malic enzyme (NAD-ME), and cytoplasmic phospho*enol*pyruvate carboxykinase (PCK). However, the delineation between subtypes may not be as clear as previously proposed (Kanai and Edwards 1999; Furbank 2011; Wang et al. 2014a). The majority of C_4 plants across this biochemical diversity rely on Kranz-type anatomy that partitions the C_4 cycle into the mesophyll (M) cells and the majority of C_3 cycle into the bundle-sheath (BS) cells (Kanai and Edwards 1999; see Fig. 9.1). However, within the succulent Chenopods there are some NAD-ME type C_4 plants that use one of two single-cell arrangements to partition and compartmentalize the C_4 and C_3 cycles, as discussed below (Voznesenskaya et al. 2001; Voznesenskaya et al. 2002; Edwards et al. 2004). In the remaining sections, we outline leaf anatomical traits at the tissue, cellular, and sub-cellular level that are key factors influencing the efficiency and effectiveness of C_4 photosynthesis.

II. The C_4 Leaf

The CCM of all known C_4 plants relies on the spatial separation of the C_4 and C_3 cycles. This allows the initial cytosolic C_4 cycle reactions of CO_2 hydration to HCO_3^- by carbonic anhydrase (CA) and the carboxylation of phospho*enol*pyruvate (PEP) with HCO_3^- by PEPC to occur adjacent to the inter-cellular airspace. Close proximity of the CA and PEPC reactions to the inter-cellular airspace helps minimize the potential limitation of CO_2 supplied from the atmosphere. Alternatively, the C_3 cycle and the CCM accumulated CO_2 is typically compartmentalized deep within the leaf to minimize potential leakage of CO_2 back into the inter-cellular airspaces. In both the Kranz and single-cell type C_4 plants, there are several leaf structural changes that help maximize the supply of CO_2 to the initial reactions of the C_4 cycle while enhancing the capacity of the CCM to elevate the partial pressure of CO_2 around Rubisco.

A. Leaf Structures in Kranz-Type C_4 Plants

The Kranz-type anatomy comes in many forms but is generally defined as two concentric layers of cells where the C_4 cycle occurs in the outer layer of M cells and the inner layer of the BS cells (Fig. 9.1f, g, h). The BS cells are tightly packed around the vascular tissue or water-storage cells and, because of high M coverage of the BS cells, the BS cells have minimal direct exposure to the inter-cellular air space (Edwards and Voznesenskaya 2011; Sage et al. 2014). High vein density in Kranz-type C_4 plants, i.e., short inter-veinal distances (IVD), is generally associated with one layer of M cells surrounding, and in direct contact with, the BS cells (Fig. 9.1f, g, h). This leads to a low ratio of M/BS cells within a C_4 leaf and a high M surface area exposed to the inter-cellular air space (S_m). Additionally, the M cells in Kranz-type C_4 plants typically have fewer chloroplasts and lower chloroplast coverage of the cell walls adjacent to the inter-cellular air spaces compared to closely related C_3 plants (Stata et al. 2014). The high S_m and the reduced number of chloroplasts in the M cells presumably increases M cell CO_2 conductance (g_m), defined in C_4 plants as the movement of CO_2 from the inter-cellular airspace to the site of CO_2 hydration by CA and initial carboxylation by PEPC in the M cytosol (Ubierna et al. 2017).

B. Leaf Structure of Single-Cell C_4 Plants

Single-cell C_4 photosynthesis has been described in four terrestrial species within the Chenopodiaceae family (*Bienertia sinuspersici*, *B. cycloptera*, *B. kavirense,* and *Suaeda aralocaspica*; Voznesenskaya et al. 2001, 2005; Sage 2002; Edwards et al. 2004; Erlinghaeuser et al. 2016; Koteyeva et al.

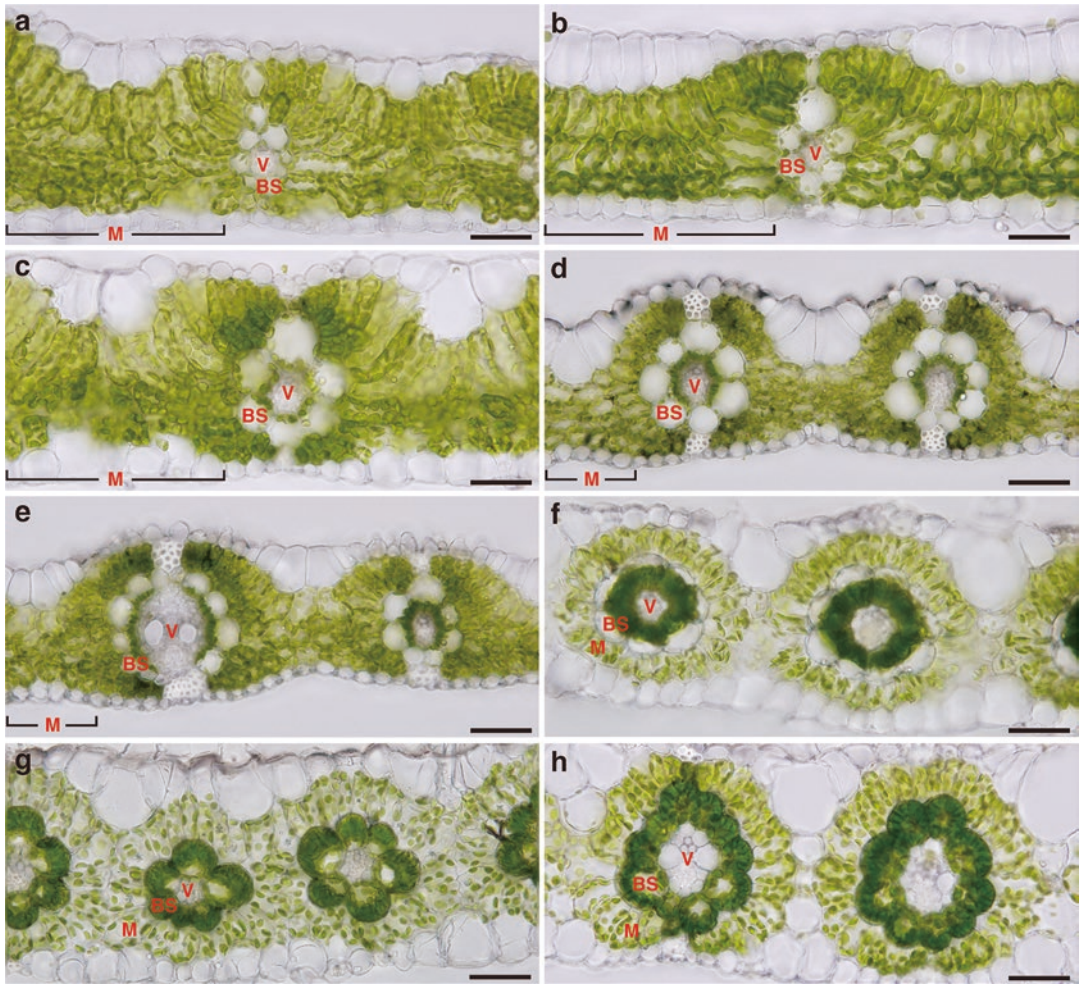

Fig. 9.1. Light micrographs of the cross sections of leaf blades. (**a**) *Panicum bisulcatum* (C_3). (**b**) *P. trichanthum* (C_3). (**c**) *P. laxum* (= *Steinchisma laxum*, proto-Kranz). (**d**) *P. decipiens* (= *Steinchisma decipiens*, C_2). (**e**) *P. milioides* (= *Steinchisma hians*, C_2). (**f**) *P. miliaceum* (NAD-ME type C_4). (**g**) *P. ramosum* (PCK type C_4). (**h**) *P. virgatum* (NAD-ME type C_4). In C_3 species, the inter-veinal distance is long and there are many M cell layers between vascular bundles. The BS cells of C_3 plants have few chloroplasts (**a**, **b**). In proto-Kranz species, BS cells are enlarged and contain slightly more chloroplasts, although the inter-veinal distance is long (**c**). In C_2 species, the inter-veinal distance becomes considerably shortened and centripetally-arranged green chloroplast rings are obvious in BS cells (**d**, **e**). In typical Kranz-type C_4 leaves, a layer of M cells encircles a cylinder of BS cells. Large BS chloroplasts are arranged centripetally (**f**) or centrifugally (**g**, **h**). Upper side of each leaf section is the adaxial side. *BS* bundle sheath cell, *M* mesophyll cell, *V* vascular bundle. Scale bars = 50 μm (Colour figure online)

2016). Prior to the discovery of the single-cell C_4 plants, it was assumed that a two-cell Kranz-type system was required in terrestrial plants for an effective CCM. However, the single-cell C_4 plants can have high rates of NAD-ME type C_4 photosynthesis with an efficient CCM (King et al. 2012; Stutz et al. 2014). These halophytic species utilize dimorphic chloroplasts within a single-cell to meet the essential requirements needed for an effective CCM in a terrestrial C_4 plant (see Fig. 9.2 and Voznesenskaya et al. 2001; Edwards and Voznesenskaya 2011; Offermann et al. 2011; von Caemmerer et al.

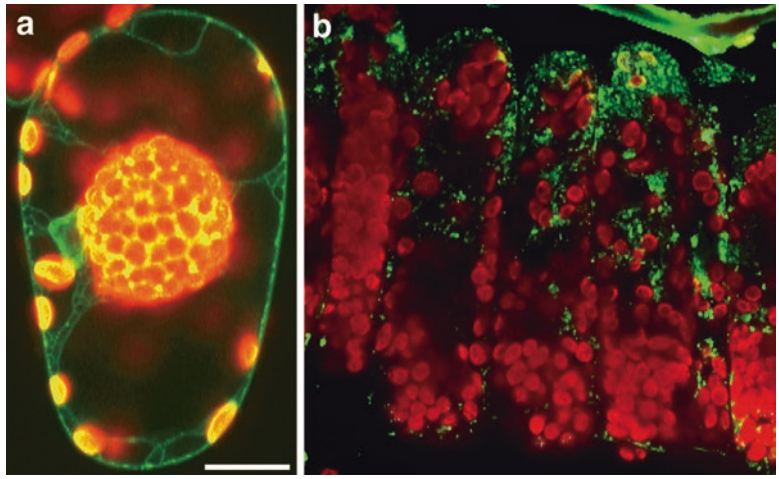

Fig. 9.2. The single cell C_4 systems. Confocal microscopy of fluorescent staining in live *Bienertia sinuspersici* (**a**) and *Suaeda aralocaspica* (**b**) chlorenchyma cells. Chlorenchyma cells were stained with 3,3′-dihexyloxacarbocyanine iodide to illuminate the reticular structures of the cytoplasmic strands (green) and the red chlorophyll autofluorescence indicates the positioning of chloroplasts. Images courtesy of Elena Voznesenskaya (unpublished). Scale bars = 20 μm (Colour figure online)

2014a). For example, *B. sinuspersici* has Bienertiod anatomy where peripheral chloroplasts, appressed to the plasma membrane, support the C_4 CCM and a central chloroplastic compartment, made of chloroplasts and mitochondria, is the site for C_4 acid decarboxylation and Rubisco fixation (Fig. 9.2a). Alternatively, in *S. aralocaspica*, the spatial separation of the C_4 and C_3 cycles is caused by localization of the dimorphic chloroplasts at opposite ends of elongated cells that function analogously to M and BS chloroplasts in Kranz type C_4 species (Fig. 9.2b). The large cell size and the distance between the cytoplasm and chloroplasts involved in the C_4 and C_3 cycles, respectively, provides the resistance to CO_2 diffusion needed for an effective CCM (von Caemmerer et al. 2014a). These features of the single-cell plants are effective in maintaining high rates of C_4 photosynthesis and an efficient CCM under a number of growth and measurement conditions (Stutz et al. 2014).

Collectively, the ability of C_4 photosynthesis to effectively capture, concentrate, and assimilate atmospheric CO_2 requires a "highly sophisticated suite of structural adaptations that not only establish the necessary compartmentalization required by the C_4 CCM, but also produce the subcellular intricacy needed for efficient C_4 photosynthesis" (Sage et al. 2014). The aim of the remainder of this chapter is to outline some of the recent developments on understanding the evolution, function, and significance of C_4 leaf structure at the tissue and cellular levels.

III. Evolution of C_4 Leaf Structure

A. *Leaf Anatomical Traits: Preconditions for C_4 Evolution*

The evolvability of C_4 photosynthesis has been questioned for many years as it frequently appears in certain lineages but is absent from others (Edwards et al. 2010; Sage et al. 2011a). The evolution of C_4 photosynthesis has been discussed in many recent publications (e.g., Sage et al. 2012) describing the biochemical and anatomical transitions from the C_3 ancestral state to the

derived C_4 state via the initial activation of the BS cells and the subsequent compartmentalization of the glycine decarboxylase complex (GDC) of the photorespiratory pathway in the BS cells (see section III.B). This restriction of the GDC decarboxylation reaction in the BS cells increases the probability of refixation of photorespired CO_2 and increases the photosynthetic efficiency under environmental conditions that favor the Rubisco oxygenation reaction (Sage et al. 2011b, 2012). However, the ammonia released during the GDC decarboxylation leads to a nitrogen imbalance between the M and BS cells, which has been suggested to be an important driver of the evolution of the CCM in C_4 plants. For example, differential expression of enzymes involved in organic and amino acid metabolism, which are also key components of the C_4 CCM, were thought to be important for re-balancing nitrogen metabolism between the two cell types (Heckmann et al. 2013; Edwards 2014). This differential expression of GDC and nitrogen metabolism is thought to be an important step in the biochemical evolution of the CCM in C_4 plants. The fitness advantage of the CCM in C_4 plants under environmental conditions that increase Rubisco oxygenation and the energetic cost of the photorespiratory pathway suggests strong selective pressure for the "inevitability of C_4 photosynthesis" (Edwards 2014). A phylogenic analysis across the grasses indicates that the evolvability of C_4 photosynthesis strongly increases when the proportion of leaf M to BS tissue decrease, either by decreased IVD or larger BS cell volume (Christin et al. 2013). In ancestral C_3 plants these traits were likely selected to minimize leaf desiccation and hydraulic failure while maintaining rates of A_{net} in hot, arid, windy and low CO_2 environments.

It has been suggested that the low CO_2 partial pressure in the atmosphere over the last 30 Myr was a key factor driving the evolution of C_4 photosynthesis (Edwards et al. 2010; Osborne and Sack 2012; Sage et al. 2012). In addition to decreases in atmospheric CO_2 and decreased soil water availability other factors such as decreased boundary layer conductance and increased incident solar radiation lead to an increased rate of E at a given value of g_s (Osborne and Sack 2012). A reduction in g_s to minimize water loss under these conditions would likely limit CO_2 availability and reduced rates of photosynthesis. Therefore, the large potential evaportraspiration under windy, high light, arid, and/or low CO_2 environments would select for leaf traits such as low IVD and enlarged BS for stomata to remain open (high g_s) without leaf desiccation and hydraulic failure (Sack and Holbrook 2006; Osborne and Sack 2012; Griffiths et al. 2013).

1. *Low Interveinal Distance*

Several of the anatomical traits that are viewed as preconditions for the evolution of C_4 photosynthesis likely played an important role in leaf water relations in ancestral C_3 plants (Osborne and Sack 2012; Griffiths et al. 2013). For example, C_4 plants typically have low IVD in Kranz-type C_4 plants (Fig. 9.1), allowing the CCM to efficiently concentrate CO_2 in the BS cells. The evolution of the "proto-Kranz anatomy" includes low IVD, an important early precondition for the evolution of C_4 photosynthesis (see section III.B.2 and Sage et al. 2014). Generally g_s decreases at low leaf water potential to conserve leaf water and minimize the threat of hydraulic failure and a low IVD in ancestral C_3 lineages was likely selected for to maintain g_s under high evaporative demands. For example, this type of scenario might have occurred as C_3 grasses transitioned from the closed understory of the forest floor to the more open environments (Osborne and Sack 2012) or in eudicots during hot summers months with periods of limiting soil water availability and low atmospheric humidity or salinity stress (Sage et al. 2014).

Leaf water potential is largely driven by the balance of E and the total leaf water con-

ductance (K_{leaf}; leaf water flow rate/evaporative demand; Sack and Holbrook 2006). A low IVD or high vein density increases the paths for water to flow within the leaf and decreases the path length for water to move outside the xylem and exit the leaf (Brodribb and Feild 2010; McKown et al. 2010; Sack and Scoffoni 2013). Therefore, a lower IVD and higher K_{leaf} can maintain g_s and consequently A_{net}, providing a selective advantage for C_3 plants growing in a high evaportraspiration environment. As previously mentioned, a decrease in IVD is one of two means to increase the portion of BS tissue in a leaf, increasing the probability of evolving C_4 photosynthesis. The decrease in IVD is thought to be a key anatomical trait for the initial steps in evolving C_4 photosynthesis that likely first evolved in ancestral C_3 species in response to selection for maintaining leaf water potential under high evaporative demand. This is supported by anatomical surveys in both monocot and dicot lineages that show IVD in the C_3 sister taxa are similar to the C_4 taxa (Muhaidat et al. 2007; Christin et al. 2013; Sage et al. 2014).

2. *Decrease in Mesophyll to Bundle Sheath Cell Area*

The second means of increasing the proportion of leaf tissue occupied by BS is by an increase in BS cell volume (Muhaidat et al. 2007; Osborne and Sack 2012; Christin et al. 2013; Griffiths et al. 2013; Sage et al. 2014). This is also a proposed precondition for the evolution of C_4 photosynthesis and many closely related C_3 sister taxa have enlarged BS cells (Muhaidat et al. 2007; Christin et al. 2013; Griffiths et al. 2013). This likely provides an increase in water storage capacity to maintain the hydration of M cells and to buffer the leaf water potential against fluctuating evaporative demands (Sage 2001). The increase in BS cell volume appears to be an adaptive trait for controlling leaf hydraulic fluxes, particularly from outside the xylem to the M cells, and ultimately maintaining g_s, and is often seen in C_3 lineages adapted to arid environments (Brodribb and Feild 2010).

B. *Sub-cellular Changes During Evolutionary Transition from C_3 to C_4 Leaf Anatomy*

The structure and function of M and BS cells are thought to have changed in a stepwise fashion during the evolution from C_3 to C_4 plants through C_3-C_4 intermediate plants. The evolutional intermediaries from C_3 to C_4 plants are divided into three main phases termed proto-Kranz, C_2 photosynthesis, and C_4-like photosynthesis (Sage et al. 2012; Sage et al. 2014).

1. *Preconditional Sub-cellular Anatomy in C_3 Plants*

In C_3 plants, M cells play a prominent role in photosynthesis, whereas the BS cells of C_3 plants have few chloroplasts randomly distributed along the cell walls (Figs. 9.1a, b and 9.3a) and had been thought to contribute very little to photosynthesis. However, it has become clear that BS cells of C_3 plants are photosynthetically active, because they contain well-developed grana, stroma thylakoids, starch grains, and Rubisco proteins (Yamane et al. 2003). Notably, BS chloroplasts in C_3 rice leaf tissues at an immature stage accumulate large amounts of starch translocated from other organs and subsequently provide a source of carbon and energy during leaf development (Miyake and Maeda 1976). This specialization of BS chloroplasts into carbohydrate accumulating compartments differentiates the BS as a starch sheath. Therefore, the starch sheath in C_3 ancestors is considered a preadaptation for the evolution of C_4 photosynthesis from C_3 photosynthesis allowing for the recruitment of BS chloroplasts for synthesizing and storing assimilatory carbohydrates in C_4 plants (Miyake 2016). Starch accumulation in BS chloroplasts during leaf development

is also found in other grass species regardless of photosynthetic type (Miyake and Maeda 1978) and further surveys are needed to reveal how this anatomical and biochemical syndrome of C_3 ancestors may have contributed to C_4 evolution.

Changes in ultrastructural modifications of M and BS cells began at early stages in the evolutionary transition from C_3 to C_4 plants. Some C_3 species have anatomical preconditioning traits that were already present before the transition to the proto-Kranz phase. Such features include greater leaf vein density and larger BS cells (Muhaidat et al. 2011; Griffiths et al. 2013) as mentioned before. An increase in BS cell volume may results in the physiological activation of these cells. Ultrastructural observations of C_3 grasses from 3 subfamilies (Ehrhartoideae, Panicoideae, and Pooideae) revealed that a significantly higher proportion of BS mitochondria are positioned at the centripetal (close to the vascular tissue) rather than in the centrifugal (close to M cells) position as opposed to BS chloroplasts that are preferentially positioned at the centrifugal position (Hatakeyama and Ueno 2016). This subcellular localization of BS organelles is also thought to be an anatomical preadaptation prior to the C_4 evolution.

2. *Proto-Kranz Anatomy*

The term 'proto-Kranz' was initially used by Muhaidat et al. (2011) as an initial phase in C_4 evolution. The structural characteristics of proto-Kranz species appear in the BS cells; increased cell size, larger mitochondrial size, increased mitochondrial numbers, and localization of mitochondria along the inner walls adjacent to the vascular bundle. Immunolocalization analysis revealed that the mitochondrial photorespiratory enzyme complex GDC is highly accumulated in the centripetal BS mitochondria in addition to M mitochondria (Muhaidat et al. 2011; Sage et al. 2014). BS chloroplasts are distributed at the inner vascular side and the outer peripheral side adjacent to intercellular spaces (Fig. 9.1c). It is thought that the photorespiratory cycle operates separately in M and BS cells. Since BS mitochondria show subcellular localization, the oxygenation and carboxylation reactions of Rubisco should occur ectopically in BS cells. The oxygenation reaction of Rubisco in the outer BS chloroplasts produces glycolate, which is further converted to glycine in peroxisomes. The glycine migrates to the inner BS mitochondria for decarboxylation by GDC. The decarboxylated CO_2 accumulates in BS cells and enhances the carboxylation activity of Rubisco in inner BS chloroplasts.

3. *Sub-cellular Structure of C_2 Photosynthetic Plants*

The term 'C_2 photosynthesis' refers to a carbon concentration mechanism with a photorespiratory glycine shuttle between M and BS cells. 'C_2' is named for the two carbons in the glycine molecule, and this intercellular glycine shuttle concentrates CO_2 into BS cells similarly to the C_4 photosynthesis. In C_2 species, GDC is generally expressed only in BS mitochondria but not in M mitochondria (Muhaidat et al. 2011; Sage et al. 2011b; Sage et al. 2013). Therefore, glycine which is originally delivered by the oxygenation reaction of Rubisco in M and BS chloroplasts diffuses to BS mitochondria for decarboxylation by GDC, and the released CO_2 is preferentially fixed by Rubisco in BS chloroplasts. To establish C_2 photosynthesis, enlarged mitochondria are localized along the inner periphery of BS cells (Fig. 9.3b). Although there is no strict rule for BS chloroplast positioning in the C_2 species, most chloroplasts are positioned between mitochondria and vacuole at the inner vascular side (see Figs. 9.1d, e and 9.3b and Khoshravesh et al. 2016). This arrangement may be advantageous for chloroplasts to refix the CO_2 released from mitochondria. Chloroplast number in BS cells is higher in many C_2 species compared to C_3 and proto-Kranz species (Stata et al. 2016). In addition, the BS cell size of C_2 species is larger in contrast to the

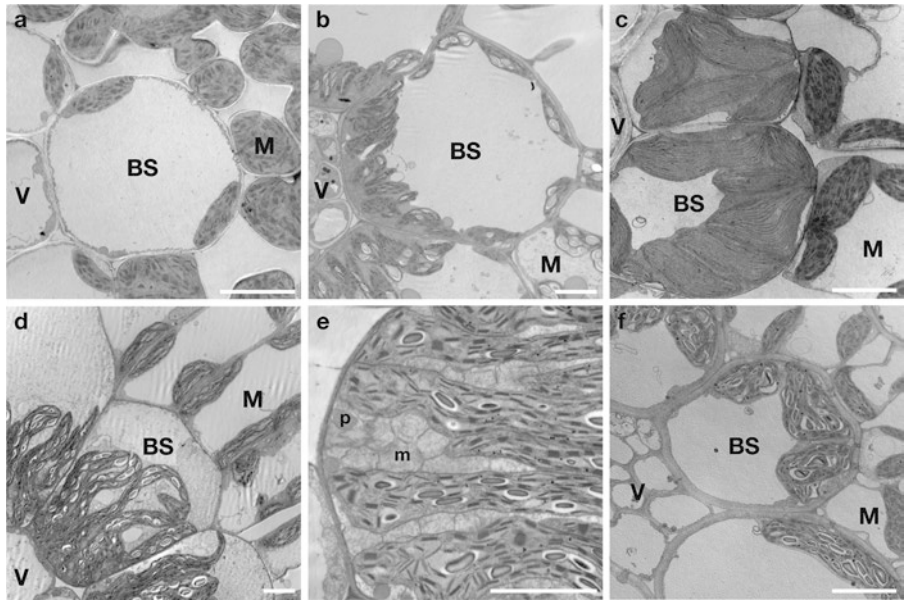

Fig. 9.3. Electron micrographs of leaf blade cross sections. (**a**) rice (*Oryza sativa*, C_3). BS cells have few chloroplasts facing toward the intercellular air space. (**b**) *Panicum milioides* (C_2). BS chloroplasts are distributed in the periphery of the cell, although many chloroplasts are arranged at the centripetal position with mitochondria. (**c**) maize (*Zea mays*, NADP-ME type C_4). BS chloroplasts are centrifugally arranged and lack well-developed grana. (**d**, **e**) proso millet (*Panicum miliaceum*, NAD-ME type C_4). BS chloroplasts are centripetally arranged and placed in contact with many mitochondria and peroxisomes. (**f**) Rhodes grass (*Chloris gayana*, PCK type C_4). BS chloroplasts are centrifugally arranged. *BS* bundle sheath cell, *M* mesophyll cell, *m* mitochondrion, *p* peroxisome, *V* vascular bundle. Scale bars = 5 μm. (Provided by Dr. Takao Oi, Nagoya University)

relatively smaller size of M cells. The enlargement of BS cells increases light interception and the potential to generate the required ATP for the more metabolically active BS cells (Bellasio and Lundgren 2016).

4. *Sub-cellular Structure of C_4-like Plants*

The anatomical and physiological properties of C_4-like species are intermediate between C_2 and C_4 species. In the C_4-like species, the C_4 photosynthetic enzymes such as PEPC and NADP-ME are expressed in a cell-specific manner and, therefore, the C_4 photosynthetic cycle partially functions between M and BS cells to concentrate CO_2 in BS cells (Cheng et al. 1988; Ku et al. 1991). The activity of the C_3 cycle including Rubisco is reduced in M cells of C_4-like plants, but still partially accumulates. Thus, M cells of C_4-like species undergo the oxygenation reaction of Rubisco and the photorespiratory glycine shuttle remains to deal with glycine generated in M cells. The size and number of BS chloroplasts in C_4-like species are also intermediate between those of C_2 and C_4 species. Many BS mitochondria are located along the inner periphery cell membranes adjacent to the vascular bundle, similar to those of C_2 species (Holaday et al. 1984; Brown and Hattersley 1989; Araus et al. 1990). At later evolutionary steps from C_4-like to C_4 species, the activities of C_4 photosynthetic enzymes became enhanced and Rubisco synthesis restricted to BS cells. Subsequently, optimization of the C_4 system both in terms of biochemistry and anatomy evolved to enhance photosynthetic efficiency (Munekage and Taniguchi 2016).

In addition, the ultrastructure of M cells changed during the evolution of C_4 plants. Chloroplast number and coverage of the M cell periphery in C_4 and C_4-like species of

Flaveria are approximately half relative to the C_3, proto-Kranz, and C_2 *Flaveria* species (Stata et al. 2016). The decline in these chloroplast parameters during C_4 evolution appears to be roughly proportional to the up regulation of the C_4 pathway. In contrast, chloroplast size and exposure of cytoplasm to the cell perimeter in M cells have increased during C_3 to C_4 evolution, although there are some exceptions (Stata et al. 2016). These structural changes involving chloroplasts may be a selective pressure for the C_4 pattern of M cells, and seem to be correlated with the decreased expression of genes involved in plastid biogenesis (Stata et al. 2016). A gene family that influences the establishment of chloroplast size and compartmentation has been identified in C_3 *Arabidopsis* (Larkin et al. 2016) and, therefore, the molecular mechanism determining the cellular volume allocated to chloroplasts in C_4 M and BS cells will likely soon be elucidated. Another factor inducing anatomical changes during C_4 evolution is photorespiration. Reducing GDC activity in M cells of C_3 rice can lead directly to M cell-specific anatomical changes typically associated with C_4 (Lin et al. 2016): Chloroplast area and coverage of the cell wall periphery were significantly reduced in M cells of the GDC gene knockdown lines. These alterations correspond to some of the events at the later stages of C_4 evolution when GDC activity is decreased in M cells.

IV. Tissue Structure and Function

A. *Leaf Structural Influence on Gas and Metabolite Movement within a C_4 Leaf*

1. *Mesophyll CO_2 Conductance*

It has been well documented that the internal conductance of CO_2 from the inter-cellular airspace to the initial site of carboxylation (mesophyll conductance; g_m) can significantly influence rates of A_{net} in C_3 plants. In many C_3 species, g_m is similar in magnitude to g_s and can change in response to variation in short and long term environmental conditions (Flexas et al. 2013, 2016). The tradeoff between g_s and g_m can have important implications for leaf intrinsic transpiration efficiency, defined as A_{net}/g_s, because a large g_m will allow more inter-cellular CO_2 to reach the site of carboxylation (chloroplast in a C_3 plant and the mesophyll cytoplasm in a C_4 plant) at a given g_s. Theoretically, a large g_m could offset a reduction in g_s and consequently reduce E without restricting CO_2 availably for photosynthesis. Variation in g_m is thought to be influenced by leaf anatomical traits as well as facilitated diffusion through aqua-porins or other membrane proteins (Flexas et al. 2008). Leaf structural features such as S_m, the thickness and porosity of M cell walls, and, in C_3 species, the percentage of S_m covered by chloroplast have all been demonstrated to influence g_m (Evans et al. 1994; Evans and von Caemmerer 1996). In C_4 plants, g_m will be influenced by similar structural factors except that the initial carboxylation of HCO_3^- by PEPC occurs in the M cytoplasm instead of via Rubisco in the chloroplast as in C_3 plants. Therefore, all else being equal CO_2 diffusion, the pathway for g_m is potentially shorter in a C_4 compared to a C_3 plant, suggesting that g_m may be higher in a C_4 species. C_4 plants generally operate at low internal CO_2 partial pressures and contain fewer M chloroplasts, which suggests that g_m to the cytosol in a C_4 plant would be higher compared to g_m in a C_3 species.

In C_3 plants the primary methods to estimate g_m, measurements of CO_2 isotope exchange and chlorophyll fluorescence, are not suitable for estimating g_m in C_4 plants and there is thus little information on how g_m differs between C_4 and C_3 plants. Early estimates of g_m in C_4 plants (see Table 9.1) were made by combining the initial slope of an A_{net} versus intercellular CO_2 partial pressure relationship with *in vitro* maximum PEPC

Table 9.1. Summary of the methods and assumptions used in estimating mesophyll conductance (g_m) in C_4 plants

Initial slope
Calculates g_m = (slope*V_{pmax})/(V_{pmax}-slope*K_p) where *slope* is initial slope of an *A*-C_i curve, V_{pmax} is the measured *in vitro* maximum rate of leaf PEP carboxylation, and K_p is Michaelis-Menten constant of PEPC for HCO_3^-. This equation is derived from the C_4 photosynthesis model (von Caemmerer 2000) and assumes that bundle-sheath CO_2 conductance (g_{bs}) is small, that g_m is independent of [CO_2], and that *in-vitro* V_{pmax} accurately represents *in-vivo* V_{pmax}.
In-vitro V_{pmax}
Determines g_m from differences between apparent *in vivo* and *in-vitro* V_{pmax} using models and measurements of C_4 photosynthesis (von Caemmerer 2000) and ^{13}C discrimination (Farquhar and Cernusak 2012). Assumes difference between i*n vitro* and *in vivo* V_{pmax} is driven by CO_2 or HCO_3^- availability using the CA-saturated or CA-limited models of C_4 photosynthesis, respectively (Ubierna et al. 2017).
^{18}O Discrimination ($\Delta^{18}O$)
Combines measurements of leaf gas exchange and oxygen stable isotope composition of CO_2 and transpired water to estimate the CO_2 partial pressure at the site of oxygen exchange between leaf water and CO_2 (Barbour et al. 2016; Ubierna et al. 2017). Assumes that estimates of the isotopic composition of water at the site of evaporation is the same as the water at the site of exchange with CO_2 and that this CO_2/H_2O is in complete isotopic equilibrium.

The initial slope method originally described by Pfeffer and Peisker (1998) estimates g_m with combined gas exchange and *in vitro* measurements of PEPC activity, the *in-vitro* V_{pmax} derives g_m by retrofitting models of C_4 photosynthesis and ^{13}C discrimination with gas exchange, kinetic constants, and *in-vitro* V_{pmax} measurements, and the ^{18}O method combines measurements of gas exchange with models and measurements of leaf ^{18}O discrimination

activity (V_{pmax}; Pfeffer and Peisker 1998) or based on leaf anatomical measurement (Pengelly et al. 2010). Barbour et al. (2016) recently applied combined measurements of leaf gas exchange and stable oxygen isotope composition of CO_2 and transpired water to estimate g_m, as initially described by Gillon and Yakir (2000), on several C_4 species (Table 9.1). C_4 g_m has also been estimated by retrofitting models of C_4 photosynthesis (von Caemmerer 2000) and ^{13}C photosynthetic discrimination (Farquhar and Cernusak 2012) with gas exchange, kinetic constants, and *in vitro* estimation of V_{pmax} (Ubierna et al. 2017; see Table 9.1 for more details).

The methods described above for estimating g_m in C_4 plants all require different assumptions. For example, the initial slope method assumes that g_m is independent of CO_2 but in C_3 species g_m has been reported to change with CO_2 (Flexas et al. 2007, 2008; Hassiotou et al. 2009; Tazoe et al. 2011). The anatomical estimates of g_m rely on assumptions related to cell wall thickness, porosity, and membrane permeability. The oxygen isotope method assumes that water at the sites of oxygen exchange between leaf water and CO_2 is isotopically the same as the water at the site of evaporation, and that the CO_2 and water are in full isotopic equilibrium (Barbour et al. 2016; Ubierna et al. 2017). Finally, the "*in vitro* V_{pmax}" method described by Ubierna et al. (2017) assumes that the primary factor controlling differences between *in vitro* and *in vivo* V_{pmax} is caused by g_m limitations to substrate (HCO_3^-) availability and not due to other factors (see Table 9.1 for further details on limitations and assumptions).

In C_4 plants, a lower S_m compared to C_3 plants suggests less area available for CO_2 diffusion to the initial site of carboxylation (Longstreth et al. 1980; Dengler et al. 1994; Evans and von Caemmerer 1996). However, because the initial carboxylation reaction occurs in the cytosol in C_4 plants, the path length for CO_2 diffusion is less and this may decrease g_m relative to C_3 plants. Additionally, C_4 M cells typically have few chloroplasts, which would allow CO_2 to more easily diffuse to the cytoplasmic space. However, recent publications have reported that g_m in several C_4 plants (*Flaveria bidentis*, *Setaria viridis*, *Zea mays*, and *Miscanthus* x *giganteus*) is similar to the higher end values reported for C_3 plants and has a similar temperature response (see Table 9.2; Barbour

Table 9.2. Leaf mesophyll CO_2 conductance (g_m) comparison between C_3 plants and C_4 grasses

Species	g_m (μmol CO_2 m^{-2} s^{-1} Pa^{-1})	Method	References
C_3 plants			
Herbaceous monocots	5.0 ± 0.8	Various (species = 2)	Flexas et al. (2012)
Herbaceous dicots	5.0 ± 1.2	Various (species = 7)	Flexas et al. (2012)
Woody deciduous angiosperms	2.7 ± 1.1	Various (species = 11)	Flexas et al. (2012)
Woody evergreen angiosperms	1.3 ± 0.2	Various (species = 6)	Flexas et al. (2012)
Woody evergreen gymnosperms	1.2 ± 0.1	Various (species = 2)	Flexas et al. (2012)
C_4 various plants			
Zea mays	≈ 10.8	Initial slope	Pfeffer and Peisker (1998)
Zea mays	17.8 ± 0.4	^{18}O discrimination	Barbour et al. (2016)
Setaria viridis	6.6 ± 0.1	^{18}O discrimination	Barbour et al. (2016)
Flaveria bidentis	7.2 ± 0.1	^{18}O discrimination	Barbour et al. (2016)
Flaveria bidentis	≈ 6.9 ± 2	^{18}O discrimination	Osborn et al. (2017)
Zea mays	3.9 ± 0.3	^{18}O discrimination	Ubierna et al. (2017)
Setaria viridis	7.9 ± 1.5	^{18}O discrimination	Ubierna et al. (2017)
Miscanthus x giganteus	2.9 ± 0.5	^{18}O discrimination	Ubierna et al. (2017)
Zea mays	3.4 ± 0.5	*in vivo* V_{pmax}	Ubierna et al. (2017)
Setaria viridis	2.9 ± 0.5	*in vivo* V_{pmax}	Ubierna et al. (2017)

Values are reported as the mean ± standard error. General methodology for estimating g_m in C_3 plants can be found in Flexas et al. (2008) and for C_4 grasses as described in Table 9.1

et al. 2016; Osborn et al. 2017; Ubierna et al. 2017). Additional studies are needed to characterize how g_m in C_4 plants changes in response to growth conditions and with variation in leaf anatomical traits.

2. *Leakiness and Metabolite Flux*

During C_4 photosynthesis, sufficient CO_2 is concentrated around Rubisco to typically minimize rates of photorespiration; however, the efficiency of this CCM is influenced by the balance between the rates of the C_4 and C_3 cycles as well as the conductance of CO_2 between the BS and M cells or the corresponding cellular compartments in the single-cell C_4 system (g_{bs}; Fig. 9.4). Unfortunately, g_{bs} cannot be measured directly and it is not clear how changes in leaf anatomy at the tissue, cellular, or subcellular level influence g_{bs}. It is reasonable to assume that g_{bs} is in part influenced by the BS surface area per unit leaf area (S_{bs}) and the BS cell wall thickness (von Caemmerer and Furbank 2003; Ma et al. 2016). Indirectly g_{bs} can be inferred through models of C_4 photosynthesis (von Caemmerer 2000) and estimates of leakiness (ϕ), defined as the fraction of CO_2 that is pumped into the BS cells that subsequently leaks back out (Farquhar 1983; Hatch et al. 1995; Kromdijk et al. 2014). Values of ϕ are typically estimated through comparing models and measurements of leaf CO_2 isotope exchange (Kromdijk et al. 2014; von Caemmerer et al. 2014b) and it has been well documented that manipulation of key enzymatic steps of the C_4 and C_3 cycles can dramatically impact ϕ (von Caemmerer et al. 1997, 2005; von Caemmerer and Furbank 2003; Cousins et al. 2006, 2007). However, it has been more difficult to identify specific anatomical traits that directly impact ϕ. This is further complicated by the fact that ϕ is influenced by both g_{bs} and the biochemical capacity of the C_4 and C_3 cycles. Therefore, the potential

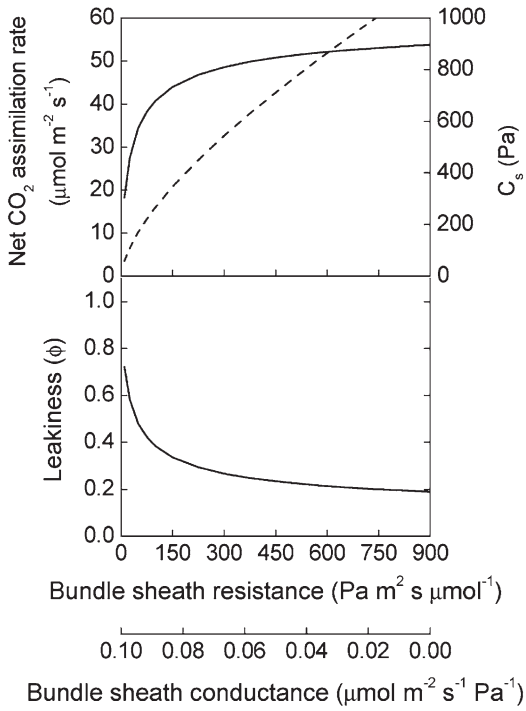

Fig. 9.4. Modeling the net rate of CO_2 assimilation (A_{net}), bundle sheath CO_2 partial pressure (C_s, Pa), and leakiness (ϕ) as a function of bundle sheath CO_2 resistance ($1/g_{bs}$) or conductance (g_{bs}). (Modeling as described in von Caemmerer 2000)

change in g_{bs} due to differences in the BS cells may be compensated for by changes in leaf metabolism in order to maintain ϕ under different growth conditions. For example, leaf anatomy and biochemistry changed in *Miscanthus* grown under different nitrogen and light availability, but there was little change in ϕ (Ma et al. 2016). Additionally, ϕ was relatively constant in the single-cell C_4 plant *B. sinuspersici* grown under different light conditions (Stutz et al. 2014).

Different growth conditions have been shown to influence the carbon isotope composition ($\delta^{13}C$) of C_4 leaves, which has been suggested to correspond to changes in ϕ (e.g., Meinzer et al. 1994; Williams et al. 2001). However, estimates of ϕ from direct measurements of $\Delta^{13}C$ compared to estimates from leaf $\delta^{13}C$ do not always correlate (von Caemmerer et al. 2014b; Ellsworth and Cousins 2016). This is in part because $\delta^{13}C$ is influenced by changes in stomatal conductance and post-photosynthetic fractionation that can be independent of changes in ϕ (see Fig. 9.5; von Caemmerer et al. 2014b; Ellsworth and Cousins 2016). Taken together, leaf anatomy and biochemistry in C_4 plants can acclimate to growth conditions to maintain ϕ and the efficiency of the CCM. However, additional work is needed to determine how BS cell wall properties, particularly changes in suberin, cellulose, hemicellulose, mixed linkage glucans, and S_{bs} influence g_{bs} and ultimately ϕ.

3. *Plasmodesmata and Metabolite Flux*

Facilitating metabolite movement between the C_4 and C_3 cycles in Kranz-type C_4 plants requires a high flux through the plasmodesmata (PD) in the interface between the M and BS cells (Weiner et al. 1988; Furbank et al. 1989; Jenkins et al. 1989a,b). The necessity of numerous PD for this intracellular movement is heightened by the fact that the M and BS cell wall interface is thickened and reinforced with suberin in many C_4 plants (Furbank et al. 1989; Mertz and Brutnell 2014; Sage et al. 2014). These features are thought to be important to decrease g_{bs} and rates of CO_2 leakage from the BS cells (von Caemmerer and Furbank 2003). However, this impermeability of the M/BS cell wall decreases the capacity for metabolite movement between these cell types through the apoplastic space and therefore requires high densities of PD to maintain A_{net}. In fact, the flux of organic acids from the M to the BS cells must be higher than A_{net} as the CCM in C_4 plants typically over-cycles by *ca*. 20% (von Caemmerer and Furbank 2003; Danila et al. 2016). There is little known about how PD control or limit metabolite movement between cells, but the flux is likely determined by 1) path length, 2) percentage of the M-BS interface covered with

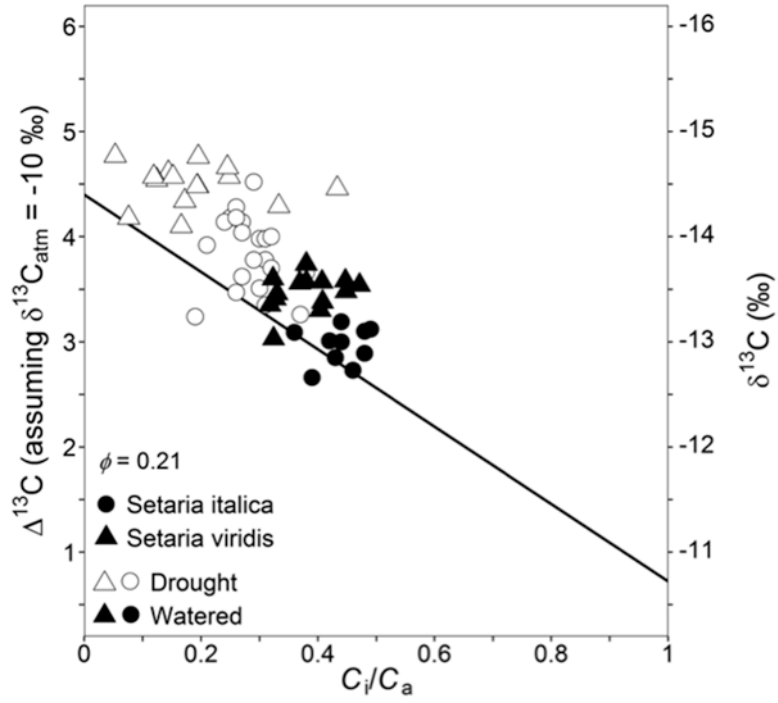

Fig. 9.5. The relationship between leaf carbon isotope discrimination ($\Delta^{13}C_{leaf}$), leaf carbon isotope composition ($\delta^{13}C_{leaf}$), and C_i/C_a in *Setaria italica* and *S. viridis* grown under well-watered or drought conditions (reprinted with permission from Ellsworth and Cousins 2016). The *y*-axes show $\Delta^{13}C_{leaf}$ calculated from $\delta^{13}C_{leaf}$ and the corresponding $\delta^{13}C_{leaf}$ with the *x*-axis showing the measured C_i/C_a. The $\delta^{13}C$ of the CO_2 in the growth chambers was −10‰. The line represents the theoretical relationship between $\Delta^{13}C_{leaf}$, $\delta^{13}C_{leaf}$, and C_i/C_a assuming leakiness (ϕ) was 0.21 (von Caemmerer et al. 2014b)

PDs, and 3) the diffusion co-efficient for a given metabolite (Wang et al. 2014b). Recent work by Danila et al. (2016) using new techniques to quantify PD distribution showed that PD and associated pit fields are higher in the M-BS interface in C_4 compared to C_3 plants. This ability to effectively quantify PD density will provide needed information to enhance the accuracy of modeling C_4 photosynthesis; however, additional information on the regulation and diffusion co-efficient of key C_4 and C_3 metabolites is still needed for more robust modeling (Wang et al. 2014b) and is a major limitation to the engineering of C_4 traits into C_3 plants (Covshoff and Hibberd 2012).

V. Cell

A. Differential Positioning of Organelles in M and BS Cells of C_4 Plants

1. Mesophyll Chloroplasts

There are many differences in intracellular structure between M and BS cells of C_4 plants. Generally, C_4 M cells have fewer chloroplasts compared to closely related C_3 species. The C_4 M chloroplasts do not appress to the M plasmalemmal surface in contrast to C_3 chloroplasts and, therefore, cytoplasm exists between plasmalemma and outer chloroplast membranes (Stata et al.

2014). These properties of C_4 M chloroplasts are thought to be an essential adaptation for efficient C_4 function; including primary carbon assimilation by PEPC in the cytoplasm and further transport of the C_4 dicarboxylate products into chloroplasts. In addition, the intracellular dispersion of the relatively low number of M chloroplasts has an advantage in light transmission to neighbor M chloroplasts and inner BS chloroplasts (Yoshimura et al. 2004; Stata et al. 2014).

2. *Bundle Sheath Chloroplasts*

BS chloroplasts are generally larger than M chloroplasts and are more numerous per cell (Dengler and Nelson 1999; Yoshimura et al. 2004). In addition, NADP-ME type C_4 species with few exceptions differ in the absence of well-developed grana in BS chloroplasts (Fig. 9.3c). Although M chloroplasts of all C_4 species are randomly distributed along cell membranes, BS chloroplasts are typically located in a specific position, either centripetal or centrifugal. In Poaceae, BS cells of NADP-ME- and PCK-type C_4 species have centrifugal chloroplasts, while BS chloroplasts of NAD-ME-type C_4 species are located in the centripetal position (see Figs. 9.1f, g and 9.3c, d, f and Gutierrez et al. 1974). However, some NAD-ME-type C_4 species having centrifugal BS chloroplasts were found within the genus *Panicum* (Poaceae; see Fig. 9.1h and Ohsugi and Murata 1980; Ohsugi et al. 1997). The dicotyledonous C_4 species fall into two groups, NADP-ME- and NAD-ME-type, whereas PCK-type dicotyledon has not been found (Gutierrez et al. 1974). All BS cells of C_4 dicots have centripetal chloroplasts irrespective of the C_4 subtypes. These intracellular arrangements of BS chloroplasts are thought to have physiological significance. The centrifugal position of BS chloroplasts is advantageous for metabolite exchange between M and BS cells because of the shortened distance between the two. However, CO_2 generated by decarboxylation in BS cells can potentially leak more readily back into M cells. To potentially overcome this disadvantage, C_4 species with centrifugally arranged BS chloroplasts have generally developed a suberized lamella in BS cell walls (Hattersley and Browning 1981; Ohsugi et al. 1988). The suberized lamella is impermeable to CO_2 and water, and is thought to reduce leakage of decarboxylated CO_2 from BS cells. However, there are some exceptional cases that counter this hypothesis (Prendergast et al. 1987; Eastman et al. 1988). It is proposed that the centripetal orientation of BS chloroplasts maximizes the length of the CO_2 diffusion pathway between BS and M cells, and minimizes CO_2 leakage from both of the cells (Hattersley and Browning 1981). This hypothesis is plausible, but a study of leaf anatomy and carbon discrimination in hybrids between C_4 *Panicum* species with centripetal or centrifugal chloroplasts argues that the chloroplast disposition and suberization are not directly related to their expression, and suberized lamellae may instead contribute to carbon isotope discrimination (Ohsugi et al. 1997). Studies on molecular mechanisms for BS suberization are developing (Mertz and Brutnell 2014) and the physiological significance of suberization will likely be elucidated in the near future.

3. *Bundle Sheath Mitochondria*

There is a noticeable difference in mitochondrial morphology among the three C_4 subtypes. Generally, NAD-ME- and PCK-type C_4 species have large and numerous mitochondria in BS cells compared to those in their M cells and in NADP-ME-type of C_4 plants (see Fig. 9.3e and Hatch et al. 1975; Yoshimura et al. 2004; Voznesenskaya et al. 2006). Also, development of mitochondrial cristae membrane structure is highest in NAD-ME-type, intermediate in PCK-type, and lowest in NADP-ME-type species (Hatch et al. 1975). In addition to these structural differences, the metabolic capabilities are also different in a cell-specific man-

ner; for instance, C_4 photosynthetic enzymes and relevant metabolite transporters are differentially accumulated in BS mitochondria of NAD-ME-type C_4 plants during cell differentiation (Taniguchi and Sugiyama 1997). Moreover, the number of BS mitochondria per cell in NAD-ME-type C_4 species increases during leaf development, although that of M mitochondria decreases (Dengler et al. 1986). In addition, the internal membrane structures of M mitochondria in C_4 plants are less developed relative to the counterparts in BS cells (Hatch et al. 1975). The large number and highly developed membranes of BS mitochondria should contribute the enhancement of metabolic and transport processes of mitochondria (Hatch et al. 1975). The BS mitochondria of NAD-ME- and PCK-type C_4 species exist in close association with BS chloroplasts located in a centripetal or centrifugal position depending on plant species. The BS mitochondria of NAD-ME- and PCK-type C_4 species are the site of decarboxylation in the C_4 photosynthetic cycle of these two biochemical subtypes. The access of mitochondria to chloroplasts is thought to be beneficial in capturing the released CO_2 by chloroplasts and exchanging metabolites between the organelles. Indeed, Miyake et al. (1985) reported that BS mitochondria of the NAD-ME-type C_4 plant *Portulaca oleracea* adhere tightly to chloroplasts in some restricted regions and still attach to the chloroplasts in the original regions even after shrinkage of mitochondria. This tight association between chloroplasts and mitochondria is also observed in some C_3 M cells (Sage and Sage 2009; Busch et al. 2013; Oikawa et al. 2015; Betti et al. 2016; Hatakeyama and Ueno 2016). The organelle association in C_3 cells may be related to the photorespiratory pathway: GDC releases CO_2 and NH_4^+ in the mitochondria and the tightly-associated chloroplasts contribute to the recovery of the CO_2 and NH_4^+ and prevention of CO_2 efflux into the extracellular space (Sage and Sage 2009; Busch et al. 2013; Betti et al. 2016). The connecting points are thought to facilitate the large metabolite fluxes across membranes of both organelles. At present, studies on membrane contact sites in plants are proceeding rapidly (Perez-Sancho et al. 2016). Identification of membrane tethering molecules and functional characterization of metabolite and/or signal transport will likely be revealed in the near future.

B. Maintenance Mechanism for the Localization of BS Chloroplasts

1. In Case of Kranz-Type C_4 Plants

The centripetal or centrifugal position of BS chloroplasts in C_4 plants is acquired during cell maturation (Miyake and Yamamoto 1987; Kobayashi et al. 2009). Young plastids are evenly distributed along the cell walls of BS cells in the immature regions of elongating leaves. During cell maturation, the chloroplasts migrate toward the vascular bundle and establish a centripetal position in the case of NAD-ME-type C_4 monocots (Kobayashi et al. 2009). The establishment of the chloroplast arrangement does not require light and, therefore, seems to be a programmed phenomenon related to the stage of tissue development (Miyake and Yamamoto 1987). Other organelles such as mitochondria, microbodies, and the nucleus also migrate with chloroplasts. The intracellular arrangement of M and BS chloroplasts in mature leaf tissues can be disrupted by centrifugal force but recovered within 1 or 2 h (Kobayashi et al. 2009). Pharmacological analyses revealed that the actomyosin system and *de novo* protein synthesis but not tubulin or light are necessary for positioning of both C_4 chloroplasts in cell development and reorientation back to the original positions (Miyake and Nakamura 1993; Kobayashi et al. 2009). It is likely that actin filaments serve as a leader or a rail for guiding and anchoring chloroplasts at proper positions. The motility and positioning of chloroplasts are mediated by actin filaments and/or micro-

tubules in several green algae and higher C_3 plants (Wada et al. 2003; Takagi et al. 2009; Kong and Wada 2014). The distribution of actin filaments in BS and M cells of C_4 plants has been observed with immunofluorescent labeling of actin (Kobayashi et al. 2009). Fine actin filaments encircle the M and BS chloroplasts, forming a basket-like structure as in C_3 *Arabidopsis* leaf cells (Kandasamy and Meagher 1999). Configuration changes of actin filaments during light-dependent redistribution of chloroplasts occur dynamically in C_3 plants (Takagi 2003). Fine actin bundles associate with chloroplasts that migrate toward the lateral cell wall under high-intensity light (Sakai and Takagi 2005). These chloroplasts become considerably resistant to centrifugal force, and it is speculated that the chloroplasts are anchored strongly with actin filaments (Sakai and Takagi 2005; Takagi et al. 2009).

2. *In Case of Single-Cell C_4 Plants*

Local positioning of chloroplasts is also observed in some plant species having a single-cell C_4 system as mentioned in section II.B (Fig. 9.2). These plants have chlorenchyma cells with polarized dimorphic chloroplasts, which are interconnected by cytoplasmic channels. This unique compartmentation of organelles in single-cell C_4 plants is established during maturation of chlorenchyma cells and maintained by the cytoskeletons, microtubules and actin filaments (Chuong et al. 2006; Park et al. 2009). The two cytoplasmic domains in the single cell of *Bienertia* species are very stable irrespective of variable light conditions; only under very prolonged exposure to low light does the central cytoplasmic compartment shift toward the periphery of chlorenchyma cells (Lara et al. 2008; Park et al. 2009). The molecular mechanism for maintaining the polarized distribution of chloroplasts in the Kranz-type and single cell-type C_4 plants should be elucidated, and identification of mutants that show deficits in the specific arrangement in these unique plant species may be one of the solutions.

C. *Aggregative Movement of C_4 Mesophyll Chloroplasts*

The orientation of chloroplasts in C_4 M cells can be adjusted in response to environmental stresses. It was originally reported that severe water stress induced centripetal re-arrangement of M chloroplasts in two C_4 species, maize and *Amaranthus cruentus*, which show decreased CO_2 assimilation rate and distortion of intercellular spaces (Lal and Edwards 1996). Following this initial research, the intracellular movement of C_4 M chloroplasts was studied intensely in finger millet (*Eleusine coracana*), an NAD-ME-type C_4 plant (see Fig. 9.6 and Yamada et al. 2009). When mature leaves of finger millet are exposed to extremely strong light (more than 3000 μmol photons m^{-2} s^{-1}), most M chloroplasts move to the BS side within 2 h, whereas the centripetal arrangement of BS chloroplasts is unchanged (Yamada et al. 2009). This aggregative movement of M chloroplasts also occurs at normal light intensities in response to environmental stresses, such as drought, salinity, or hyperosmosis (Yamada et al. 2009). Moreover, the re-arrangement of M chloroplasts is observed in field-grown C_4 plants that are exposed to multiple stresses such as strong light, high temperature, and drought at mid-day of midsummer, and is partially reversed at nighttime when plants exhibit some recovery from photoinhibition. The aggregative movement of M chloroplasts in response to environmental stresses is unique to M chloroplasts, and the intracellular arrangement of nuclei and mitochondria is not changed in M and BS cells (Yamada et al. 2009).

Chloroplast movement of C_3 plants in response to light intensity is well known as chloroplast photorelocation movement and has been extensively studied at molecular level (Wada et al. 2003; Kong and Wada 2014). Under low-intensity light, chloroplasts

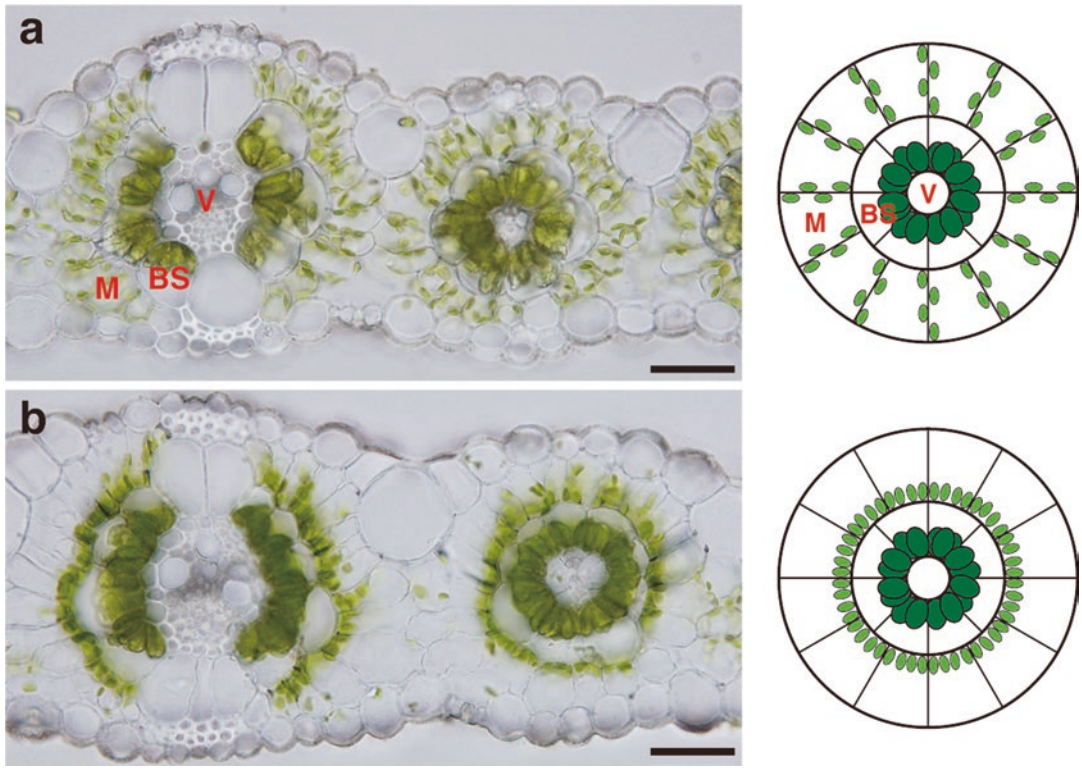

Fig. 9.6. Aggregative movement of C_4 mesophyll chloroplasts. (a) Light micrograph of a transverse section of a leaf blade from control finger millet, an NAD-ME type C_4 plant. BS chloroplasts are located in a centripetal position, whereas M chloroplasts are randomly distributed along the cell walls. (b) Leaf segments were de-aerated in 30 μM ABA solution and floated on the solution under blue light (500 μmol photons m^{-2} s^{-1}) irradiation for 8 h. Most M chloroplasts are aggregatively distributed toward the BS side. The schematic model of the chloroplast arrangement is shown on the right side of each photograph. *BS* bundle sheath cell, *M* mesophyll cell, *V* vascular bundle. Scale bars = 50 μm (Colour figure online)

move parallel to the direction of light path to maximize photoreception (called "accumulation movement"), whereas under high-intensity light, they move toward anticlinal cell walls to avoid over excitation (called "avoidance movement"). Although C_4 M chloroplasts also show accumulation (Taniguchi et al. 2003) and avoidance movement (Inoue and Shibata 1974; Maai et al. 2011b), prominent aggregative movement occurs in C_4 plants that receive severe stresses. The aggregative movement of M chloroplasts is controlled by actin filaments and also induced in a light-dependent fashion upon incubation of leaf segments with abscisic acid (ABA; Yamada et al. 2009). The plant hormone ABA can accumulate and function as a physiological signal transducer in response to environmental stresses (Zhang et al. 2006). Plant-specific photoreceptors (phototropin, phytochrome, or neochrome) participate in signal transduction for the photorelocation movement of C_3 plants (Wada et al. 2003; Kong and Wada 2014). Blue light irradiation essentially induces the rearrangement of C_4 M chloroplasts, which is analogous to the avoidance movement of C_3 chloroplasts (Maai et al. 2011b). In the presence of ABA, most of the M chloroplasts exhibit the aggregative movement in response to blue light (Fig. 9.6b). Therefore, it is thought that blue light induces the avoidance movement of C_4 M chloroplasts and ABA can shift it to the aggregative movement.

Although few studies have centered on chloroplast movement in C_4 plants (Königer and Bollinger 2012), we have confirmed that the aggregative movement of M chloroplasts is common in C_4 plants despite varied responses among plant species (T. Tsukaguchi and M. Taniguchi, unpublished data). The C_3 chloroplasts do not show such aggregative movement and therefore C_4 plants have acquired the mechanism during the evolutionary process from C_3 to C_4 plants. Although the physiological significance of C_4 M chloroplast movement is unknown, it is speculated that the chloroplast movement contributes to protection against photodamage through mutual shading of chloroplasts. Another possibility is maintenance of C_4 photosynthetic activity under environmental stress conditions due to shortening C_4 photosynthetic pathway and re-fixing CO_2 leaked from BS cells (Yamada et al. 2009; Maai et al. 2011a). Generally, C_4 M chloroplasts are susceptible to environmental stress compared with BS chloroplasts (Hasan et al. 2005; Hasan et al. 2006; Omoto et al. 2009) and both chloroplasts show different changes in membrane structure and lipid composition in response to environmental stress (Omoto et al. 2016). C_4 chloroplasts have acquired cell-specific strategies for dealing with stress; M cell-specific chloroplast aggregative movement, and BS cell-specific scavenging of reactive oxygen species (Omoto et al. 2013) and regulation of chloroplast lipid content.

The aggregative repositioning of M chloroplasts also occurs in water-stressed *Portulaca grandiflora* and *P. oleracea*, succulent C_4 species, in which Crassulacean Acid Metabolism (CAM)-like metabolism is enhanced on stress (Guralnick et al. 2002; Lara et al. 2003, 2004; D'Andrea et al. 2014). In addition, chloroplasts of some succulent CAM plants form large clumps under combined light and drought stress (Kondo et al. 2004). Exogenously applied ABA can induce the chloroplast clumping in a light-dependent manner, suggesting that C_4 and CAM plants may possess a common mechanism for chloroplast aggregative movement that is triggered by ABA. Moreover, it was reported that C_3 *Arabidopsis* mutants impaired in light-induced chloroplast movement have defects in ABA signaling (DeBlasio et al. 2005; Rojas-Pierce et al. 2014). This indicates that ABA can even affect chloroplast photorelocation movement in C_3 plants, but it is not clear whether the underlying mechanism by which ABA influences chloroplast movement is the same as that in C_4 plants or not. ABA also functions as an inducer of transition from C_3 to C_4 photosynthetic mode. Submerged form of amphibious sedge, *Eleocharis vivipara*, developes C_3-like traits without Kranz anatomy, but new emerged culms from plants grown in an ABA solution show C_4-like traits with Kranz anatomy (Ueno 1998).

VI. Conclusions

Most C_4 plants rely on Kranz-type anatomy to spatially separate primary CO_2 assimilation and re-assimilation of CO_2 released by the decarboxylation reaction. The evolutionary acquisition of Kranz structure is becoming further clarified by exploring and analyzing intermediate species and the intercellular C_2 photorespiratory pathway to concentrate CO_2 in the internal BS cells. This cellular differentiation appears to be driven by the preconditioning of leaf structural traits, such as decreased IVD and enlarged BS cells. Significant progress has been made in determining the genes involved in the formation of the C_4 leaf structure such as BS cell-specific plastid development (Kinsman and Pyke 1998), genetic variants with altered BS cells (Fladung 1994), and the gene locus involved in Kranz structure formation (Tolley et al. 2012). Additionally, transcription factors, Scarecrow and SHORT-ROOT, have been shown to be part of genetic regulation of Kranz anatomy development (Slewinski 2013). Moreover, systems biol-

ogy has become a powerful approach to reveal developmental control of the C_4 leaf structure (Fouracre et al. 2014).

The intracellular localization of organelles is a key to evolution and optimization of C_4 photosynthesis. Therefore, it is important to elucidate the molecular mechanism of how (a) plants sense the positions of organelles in the cell, (b) the direction of organelle movement is decided, and (c) the cellular arrangement of organelles is maintained. Furthermore, additional research is needed to determine how leaf anatomy and structure influence the photosynthetic properties of C_4 plants. Particular areas of interest include how C_4 leaf structure influences mesophyll CO_2 conductance, CO_2 leakage from the BS cells, and metabolite flux between M and BS cells through PD. It is likely that future work at both the cellular and tissue levels will help elucidate functional characteristics of C_4 plants for future crop improvement.

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Chapter 10

Functional Anatomical Traits of the Photosynthetic Organs of Plants with Crassulacean Acid Metabolism

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Summary

Crassulacean acid metabolism (CAM) is a photosynthetic adaptation to water and/or CO_2 limited environments that has evolved in 400 genera from 36 families of higher plants. Despite the taxonomic and ecological diversity of CAM, plants with this photosynthetic specialization share a number of common anatomical traits that impinge on the physiological processes underpinning photosynthetic CO_2 assimilation and water use. Thick, succulent leaves and/or stems are typical for terrestrial CAM plants. The large cells within these succulent tissues serve to accommodate the overnight vacuolar accumulation of malic acid that defines CAM and also increase water storage capacity. Significant morphological and anatomical diversity exists among leaf and stem succulents that impact on water-use strategies and thus the predisposition towards CAM. We provide an overview of CAM diversity in terms of leaf and stem anatomy, leaf venation and stomatal patterning. We consider the physiological implications of these anatomical traits in terms of water use and leaf hydraulic properties as well as the impacts on CO_2 uptake and carbon gain. We also discuss which anatomical traits are likely to be important determinants for the mode and level of CAM that might be engineered into non-CAM species as a means of improving plant water use efficiency.

I. Introduction

Among the three modes of photosynthesis found in higher plants, the C_3 pathway is ancestral and most common, accounting for approximately 90% of all higher plant species. Crassulacean acid metabolism (CAM) is a specialized mode of photosynthesis, found in ~ 6% of higher plants, that evolved from C_3 photosynthesis in response to water and CO_2 limitation (Yamori et al. 2014). While C_3 photosynthesis uses the three-carbon molecule 3-phosphoglycerate (3-PGA) for the fixation of CO_2 captured by Rubisco during the day, CAM generates a four-carbon organic acid from the fixation of CO_2 at night. In CAM, this nocturnal carboxylation reaction is catalyzed outside the chloroplast by phosphoenolpyruvate carboxylase (PPC), which uses the 3-carbon substrate phosphoenolpyruvate (PEP) supplied by the glycolytic breakdown of carbohydrate formed during the previous day (Fig. 10.1). PPC is activated at night by phosphorylation which is catalysed by a dedicated phoshoenolpyruvate carboxylase kinase (PPCK; Nimmo 2000). The nocturnally accumulated malic acid is stored overnight in a large central vacuole and, during the subsequent day, malate is decarboxylated to release CO_2 at an elevated concentration for Rubisco in the chloroplast. The decarboxylation of malate may be catalysed by NAD-malic enzyme (NAD-ME), NADP-ME or phosphoenolpyruvate carboxykinase (PEPCK) depending on the plant species (Holtum et al. 2005). Transgenic silencing of mitochondrial NAD-ME has indicated this as the major decarboxylase in the model CAM species *Kalanchoë fedstchenkoi* (Dever et al. 2015; Hartwell et al. 2016) and pyruvate produced from decarboxylation is subsequently processed to PEP either in the cytosol or the chloroplast (Kondo et al. 2001; reactions summarized in Fig. 10.1).

The diel separation of carboxylases in CAM is accompanied by an inverse (compared to C_3 and C_4) day/night pattern of stomatal closure/opening. The main periods of stomatal opening (night, Phase I) and stomatal closure (middle part of day, Phase III) may be punctuated by periods of variable duration where stomata open for direct uptake of CO_2 at the start (Phase II) and end (Phase IV) of the day, giving rise to four

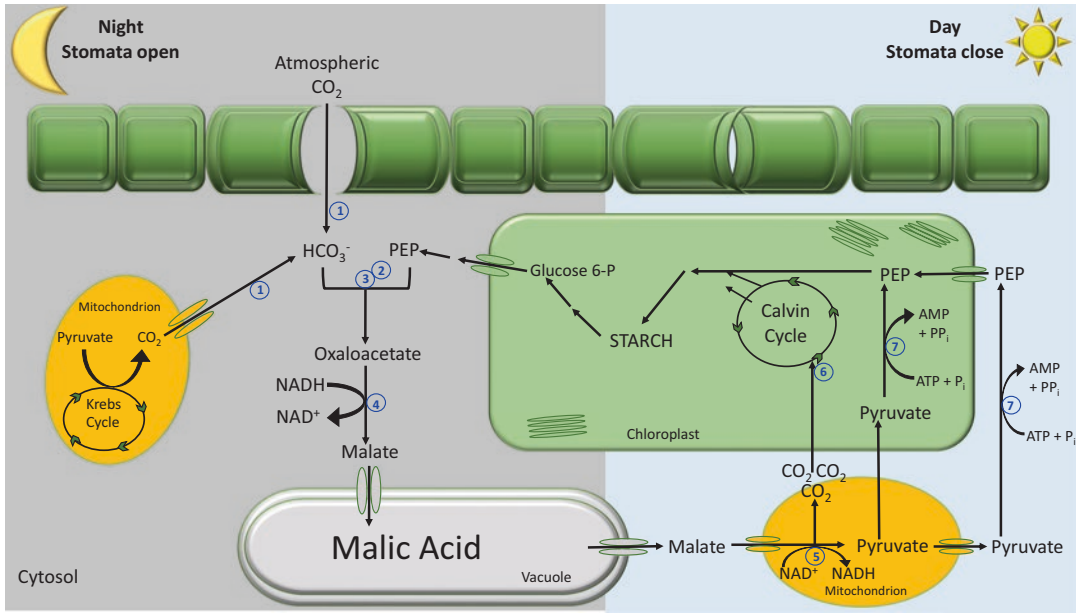

Fig. 10.1. Summary outline of the nocturnal and daytime metabolic reactions of crassulacean acid metabolism (CAM). At night, atmospheric CO_2 enters through open stomata and is converted to HCO_3^- via the enzyme carbonic anhydrase (CA). Respiratory CO_2 produced by the mitochondria can also be converted to HCO_3. Phosphoenolpyruvate (PEP) is produced via the glycolytic breakdown of storage carbohydrate (e.g. starch). PEP and HCO_3 are substrates for phosphoenolpyruvate carboxylase (PPC) that is activated (phosphorylated) by a dedicated kinase (PPCK). The product oxaloacetate is converted to malate via nicotinamide dinucleotide (NAD) malate dehydrogenase (MDH). The malate is pumped into the central vacuole and stored overnight as malic acid. During the day while stomata are closed, malate exits the vacuole and, in the case of NAD malic enzyme (NAD-ME) type CAM plants such as *Kalanchoë fedstchenkoi*, enters the mitochondrion where it is decarboxylated to produce pyruvate. The CO_2 released is re-fixed by Rubisco and processed via the Calvin cycle. Pyruvate is converted to PEP via the enzyme pyruvate phosphate dikinase (PPDK), a reaction which can occur in the cytosol and/or chloroplast. Carbohydrate (starch) is recovered by the gluconeogenic processing of PEP. Enzyme-catalyzed steps are represented by blue numbering where 1 = CA, 2 = PPC, 3 = PPCK, 4 = MDH, 5 = NAD-ME, 6 = Rubisco, and 7 = PPDK

'typical' phases of leaf gas exchange in the CAM mode (Osmond 1978; Fig. 10.2). Closing stomata for much of the day and opening stomata predominantly at night when temperature is generally lower and humidity higher than during the day, enhances water-use efficiency (WUE: i.e., the ratio of moles of CO_2 fixed to moles of water lost by transpiration). The WUE of CAM plants can be 6-fold higher than that of C_3 plants and 3-fold higher than C_4 plants under comparable conditions (Borland et al. 2009).

The water conserving properties of CAM have highlighted the potential of succulent CAM genera like *Agave* and *Opuntia* as dedicated bioenergy feedstocks that can be grown on semi-arid land with minimal inputs (Owen and Griffiths 2014; Cushman et al. 2015). The CAM pathway has also been identified as a target for synthetic biology to engineer improved water use efficiency into C_3 crops that are grown for food, feed and as sources of bioenergy (Borland and Yang 2013; Borland et al. 2014; DePaoli et al. 2014). In principle, CAM-into-C_3 engineering is realistic because all of the enzymes required for CAM appear to be homologs of ancestral forms found in C_3 species (West-Eberhard et al. 2011). The existence of facultative CAM species, which can change photosynthetic physiology from C_3 to CAM in response to drought or salinity

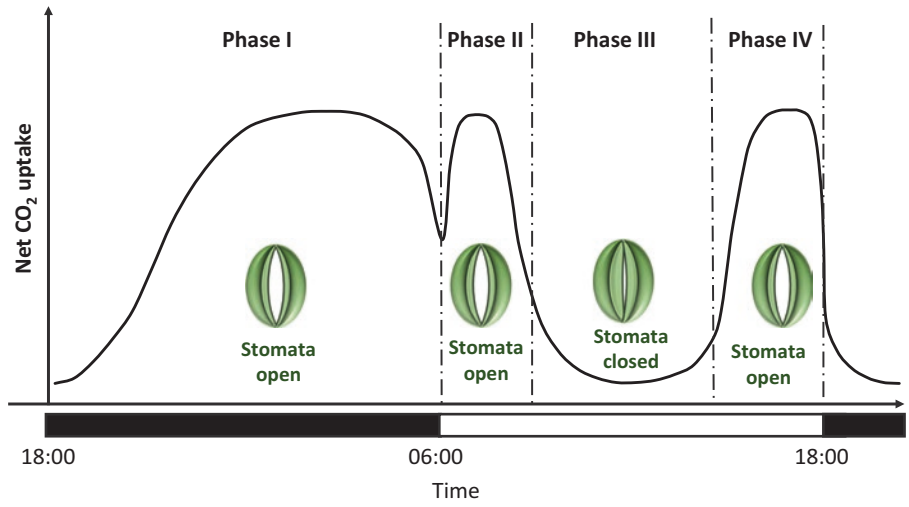

Fig. 10.2. The 4 phases of crassulacean acid metabolism (CAM) that occur over a 24 h dark-light cycle. At night (Phase I), stomata open in response to the draw-down in internal [CO_2] as HCO_3^- is fixed by PPC. The transition of PPC from the dephosphorylated form (with low activity) at the start of the night to the phosphorylated (active) form as the night progresses, allows continued CO_2 uptake from the atmosphere until the vacuoles begin to reach their limit for holding malic acid leading to decreased CO_2 uptake as dawn approaches. At the start of the photoperiod (Phase II), stomata remain open and a surge of net CO_2 uptake occurs as Rubisco becomes the principle carboxylase. During the middle part of the photoperiod (Phase III), large amounts of CO_2 are released inside the tissues as malic acid leaves the vacuoles and is decarboxylated. This elevation of internal [CO_2] is believed to be a key stimulus for stomatal closure and throughout Phase III internally generated CO_2 is fixed in the chloroplasts behind closed stomata. Towards the end of the photoperiod (Phase IV), the internal store of malic acid is exhausted and the internal concentration of CO_2 falls allowing stomata to open and CO_2 to be fixed directly from the atmosphere by Rubisco (Based on Osmond 1978).

(Winter and Holtum 2014), also implies that there are no metabolic incompatibilities between the operation of CAM and C_3 photosynthesis at the organismal level. Moreover, CAM is a single-cell carbon concentrating mechanism, meaning that, in principle, plants will concentrate and assimilate carbon in the same cell. Therefore, engineering the CAM pathway into C_3 species does not require differentiated mesophyll and bundle sheath cell types, each with their own specialized metabolic adaptations, as is the case with C_4 photosynthesis (Reeves et al. 2017; see Chap. 9). However, the photosynthetic organs of CAM species do possess particular anatomical characteristics that impinge on the physiological processes underpinning photosynthetic CO_2 assimilation and water use. Within the context of the convergence of the CAM phenotype across diverse taxonomic groups, the aim of this chapter is to present an overview of the anatomical traits typically associated with the photosynthetic organs of CAM plants, to discuss the physiological consequences of these anatomical traits and to consider the implications for bioengineering CAM into non-CAM species.

II. Convergence of CAM Across Diverse Phylogenies

Convergent evolution (when unrelated lineages independently evolve similar morphological and/or functional features) is thought to underpin the repeated emergence of CAM from C_3 ancestors in response to similar ecological selection pressures (Edwards and Ogburn 2012). CAM has evolved in more than 400 distinct genera from over 36 families (Yang et al. 2015) and is found in terrestrial, epiphytic, and aquatic species. Terrestrial

CAM plants live in semi-arid, seasonally arid or other water limited environments (Herrera 2009). They have independently evolved similar physiological characteristics to adapt to water-deficient conditions thus enhancing WUE relative to C_3 plants (Borland et al. 2014; Yang et al. 2015). For example, *Kalanchoë laxiflora*, *Lithops hookeri*, *Phalaenopsis equestris* (orchid) and *Ananas comosus* (pineapple) are terrestrial obligate CAM plant species belonging to four different orders (Saxifragales, Caryophyllales, Asparagales, and Poales) (Fig. 10.3a). Specifically, all of these species open their stomata at night for net CO_2 uptake and keep stomata closed for much of the day. The daytime closure of stomata is possible due to the decarboxylation of vacuolar stores of malic acid that generates CO_2 for assimilation via Rubisco and the Calvin cycle while transpirational water loss is curtailed. In addition to the terrestrial plants, CAM photosynthesis is also present in some aquatic plants such as species in the genera of *Isoëtes*, *Crassula*, *Littorella* and *Sagittaria* (Griffiths 1992; Keeley 1998). Both *Littorella* and *Sagittaria* have evolved CAM traits despite these genera being very distantly related (i.e., part of the dicot Lamiales and monocot Alismatales orders, respectively; Fig. 10.3a). In contrast to the terrestrial CAM plants, the convergent evolution of CAM in aquatic plants is driven by an adaptation to alleviate reduced availability of CO_2. The slow diffusion of CO_2 in water as well as competition for CO_2 from other aquatic photosynthetic species employing C_3 photosynthesis during the day means that CO_2 availability can be limiting in aquatic environments (Keeley 1998; Klavsen et al. 2011). Thus, the primary selective advantage of CAM in aquatic plants is to provide an internal source of CO_2 during the day when external levels of CO_2 are potentially limiting for photosynthesis (Keeley 1998).

Phenotypic and functional convergence is underpinned by changes in genomic sequences (Pfenning et al. 2014). Although CAM convergence is widely recognized at the physiological level, our knowledge about the molecular basis underlying the convergent evolution of CAM photosynthesis is limited. Recently, the evolution of the key carboxylation enzyme PPC was studied by Deng et al. (2016) using phylogenetic analysis of the PPC gene family in 60 species. A CAM-specific isoform of PPC was found to be shared among five different CAM species in the monocot lineage (Fig. 10.3b). This supports the hypothesis that convergence in PPC gene evolution contributes to emergence of the CAM syndrome. To test this hypothesis, future studies on convergent evolution of PPC and other CAM genes should include both monocot and dicot lineages. A more comprehensive genome-wide comparative analysis of the newly published orchid (Cai et al. 2015) and pineapple genome sequences (Ming et al. 2015) as well as the genomics data generated from the ongoing *Kalanchoë* genome project (Yang et al. 2015, 2017) will provide new insights into the genomic basis of CAM convergence.

III. Succulence and Diversity in Anatomy and Morphology of CAM Species

A. The Succulence Syndrome

Despite the taxonomic and ecological diversity that underpins CAM, plants with this photosynthetic specialization share a number of common anatomical traits. Thick, succulent leaves and/or stems are typical for terrestrial CAM plants (Sayed 1998). At the cellular level, succulence is manifest as multiple numbers of large cells with greatly enlarged vacuoles that occupy 90% or more of the cell volume. The enhanced vacuolar storage capacity of succulent leaves/stems is a key factor for accommodating the nocturnal storage of malic acid that defines CAM (Martin and Siedow 1981). Positive relationships have been demonstrated between succulence and the magnitude of CAM expression and

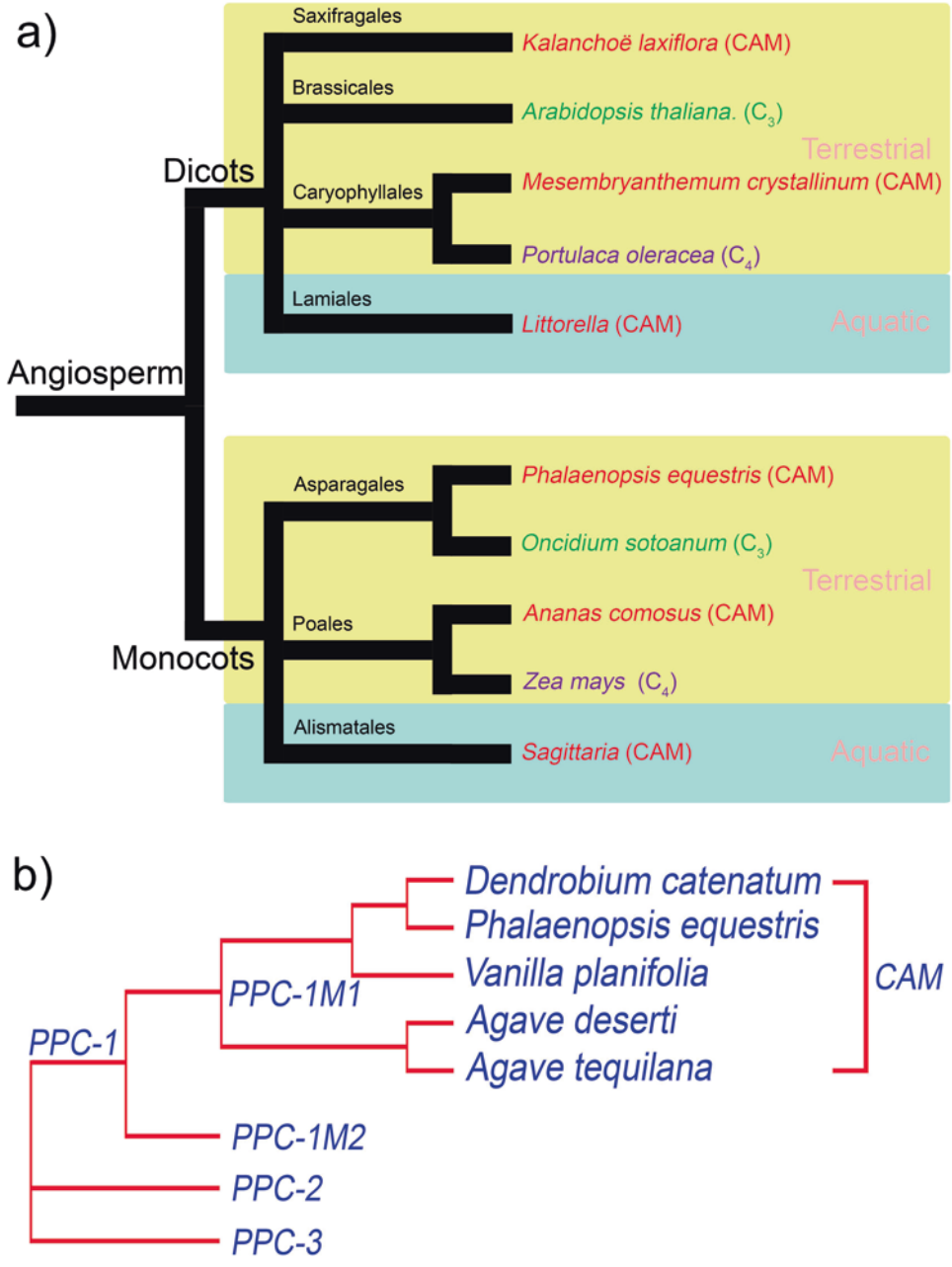

Fig. 10.3. CAM convergence at physiological and molecular levels. (**a)** Physiological convergence of CAM species from both dicot and monocot lineages. Species names of C_3, C_4, and CAM plants are shown in green, purple and red fonts, respectively. The yellow box indicates terrestrial plants whilst the blue box indicates aquatic plants. (**b)** Molecular evolution of the PPC gene family can be divided into three subfamilies: PPC-1, PPC-2 and PPC-3. Within the subfamily PPC-1, the clade PPC-1 M1 is shared by five CAM species in the CAM lineage, including three orchid species (*Dendrobium catenatum*, *Phalaenopsis equestris* and *Vanilla planiolia*) and two *Agave* species (*Agave deserti* and *A. tequilana*). (Adapted from Deng et al. 2016) (Colour figure online)

between leaf thickness and CAM-like ^{13}C discrimination in diverse phylogenetic lineages that include the Crassulaceae and tropical tress of the genus *Clusia* (Teeri et al. 1981; Winter et al. 1983; Kluge et al. 1991; Borland et al. 1998; Holtum et al. 2004; Vargas-Soto et al. 2009). Across nine species of *Clusia* with photosynthetic characteristics ranging from obligate C_3 through C_3-CAM intermediate to constitutive CAM, the species with more succulent and thicker, denser leaves (lower specific leaf area) engaged in a greater amount of dark CO_2 uptake than the thinner-leaved species (Barrera-Zambrano et al. 2014).

In addition to enhancing the nocturnal storage capacity for malic acid, succulence increases water storage and is often linked with general adaptation to water-limited habitats (Ogburn and Edwards 2010). In most leaf or stem succulents, water is stored either in or immediately adjacent to photosynthetic tissues, indicating an intimate relationship between succulence and daily carbon uptake and growth. It has been suggested that possession of leaf or stem succulence might have predisposed ancestral CAM taxa towards the evolution of this photosynthetic specialization in water-limited habitats (Sage 2002). However, great morphological and phylogenetic diversity exists among leaf and stem succulents (Eggli and Nyffeler 2009) and it is worth considering how anatomical variants of the succulence trait in photosynthetic organs might impact water-use strategies and thus the predisposition towards CAM.

B. Succulent Traits Have Been Incorporated into a Variety of Different Leaf Anatomies Across CAM Lineages

One type of leaf succulence can be classified as 'all-cell succulence' where all cells contain chloroplasts and store water simultaneously. Examples of CAM families with this type of succulence include the Crassulaceae and Aizoaceae and within the genus *Kalanchoë,* in which the largely undifferentiated mesophyll is made up of tightly compacted cells (Fig. 10.4a; Balsamo and Uribe 1988; Nelson et al. 2005). In contrast, storage succulence is a situation in which specialised, achlorophyllous water storage cells are adjacent to, but clearly differentiated from, the photosynthetic cells (e.g., as found in the CAM genera *Aloe* and *Lithops;* Eggli and Nyffeler 2009). In *Aloe*, large non-photosynthetic cells (or hydrenchyma) with a water storage function are found in the central core of the leaf, and are surrounded by a layer of tightly packed photosynthetic mesophyll cells (chlorenchyma) (Fig. 10.4c; Ni and Tizard 2004). In other CAM genera, there is more heterogeneity across the leaf, with several distinct layers of chloroplast-containing mesophyll, as that found in the genera *Peperomia* and *Clusia* (Fig. 10.4b; Nelson et al. 2005; Barrera-Zambrano et al. 2014). In the leaves of tropical trees of the genus *Clusia*, the photosynthetic tissues are differentiated into a layer of palisade mesophyll comprised of tightly compacted and elongated cells and a layer of spongy mesophyll that is generally less tightly packed (Barrera-Zambrano et al. 2014; Fig. 10.4b). Water storage tissue or hydrenchyma is found just below the epidermis in *Clusia* as is also the case for *Peperomia* (Kaul 1977). The anatomical complexity of *Clusia* leaves is compounded by the arrangement of chlorenchyma cells surrounding the mid-vein, which are often tightly compacted, rather like the mesophyll cells of *Kalanchoë* (Lüttge 2008a; Fig. 10.4b).

C. CAM Is Found in Specific Cell Types Within the Leaf

In many succulent CAM species, the leaf mesophyll tissues are usually not strongly differentiated into palisade and spongy layers (Nelson et al. 2005; Nelson and Sage 2008). However, those CAM species that do possess differentiated mesophyll cells present the potential for variations both in cell size and for accommodating differential distribution of PPC and Rubisco proteins within the leaf. A comparative study between C_3 and CAM

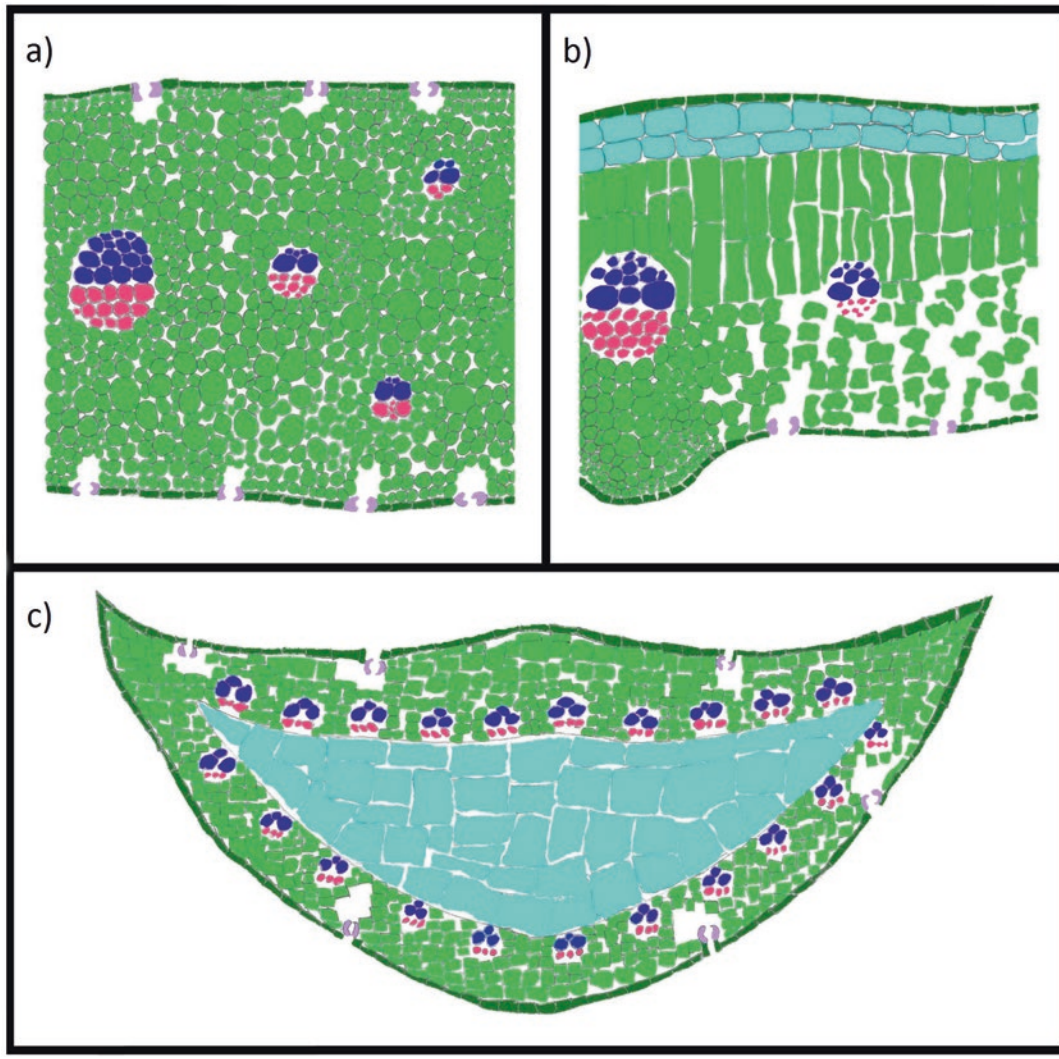

Fig. 10.4. Morphological diversity of succulent leaf forms in CAM plants. (**a**) In *Kalanchoë*, tightly packed and largely undifferentiated cells make up the mesophyll. A 3-dimensional arrangement of veins is found both above and below the central plane running through the middle of the leaf. These 3D veins are suspected to be necessary for the transport of water and photosynthate into highly succulent tissue. Stomata are located within the epidermis on both surfaces of the leaf. (**b**) In *Clusia*, a differentiated leaf anatomy is comprised of a palisade mesophyll layer, a spongy mesophyll layer and a layer of hydrenchyma below the upper epidermis. It has been suggested that CAM activity is more prominent in the tightly packed cells of the palisade mesophyll and the chlorenchyma surrounding the veins than in the spongy mesophyll in *Clusia*. Stomata are located on the abaxial leaf surface. (**c**) In *Aloe*, the mesophyll is made up of tightly compacted chlorenchyma cells surrounding a central core of hydrenchyma cells that function in water storage. The veins of *Aloe* are found in a 3D arrangement and stomata are distributed throughout the epidermis

Clusia species demonstrated not only that leaves were thicker in CAM performing species, but also that cells of the palisade mesophyll were significantly larger and constituted a greater % of leaf thickness in species that undertake a greater amount of nocturnal net CO_2 uptake (Barrera-Zambrano et al. 2014; Fig. 10.5). In addition, the abundance of PPC protein in CAM species of *Clusia* was found to be three times higher in cells of the palisade

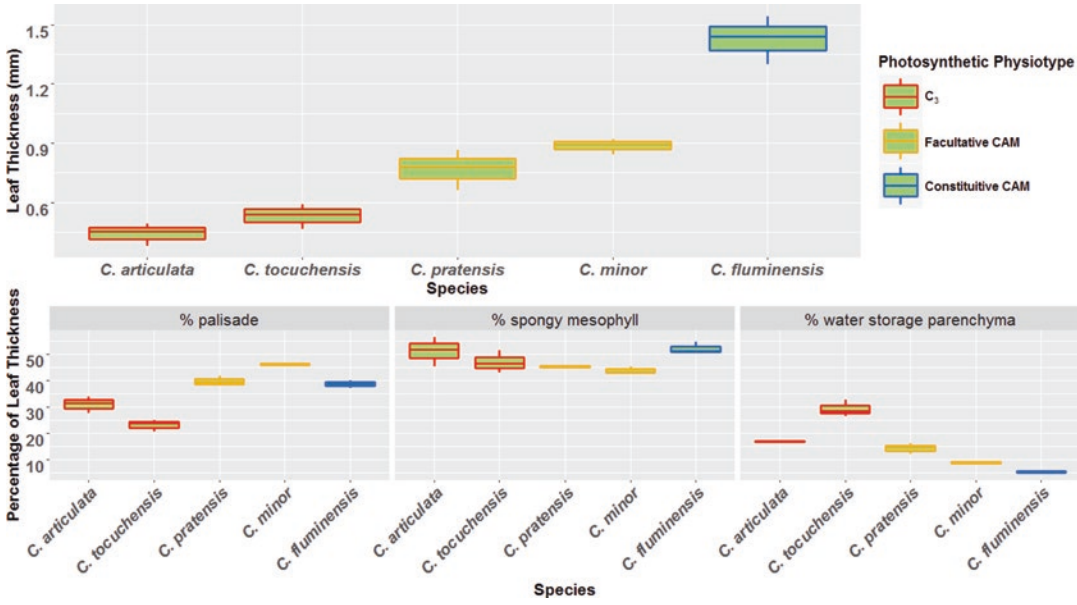

Fig. 10.5. Leaf anatomical traits are related to the capacity for CAM in *Clusia.* (**a**) Species that undertake a greater amount of night time CO_2 uptake have thicker leaves. The putative C_3 and obligate C_3 species *C. articulata* and *C. tocuchensis* have the thinnest leaves. Facultative CAM species, *C. pratensis* and *C. minor* have an intermediate leaf thickness. The obligate CAM species, *C. fluminensis* has the thickest leaves. Three separate groups for obligate C_3, facultative CAM and constitutive CAM are supported by ANOVA and post hock Tukey-Kramer test. (**b**) The proportion of the leaf made up by the palisade mesophyll layer is higher for species that undertake more night time CO_2 uptake. The proportion of the leaf made up by the spongy mesophyll (**c**) and hydrenchyma (**d**) (water storage parenchyma) does not show a direct relationship with the capacity for nocturnal CO_2 uptake. For all species, measurements were made on a single mature leaf from each of 3 different plants, with 3 technical measurements made for each leaf. Data are presented using box and whisker plots where the upper and lower edge of box represents the 75% and 25% quantile respectively, and the middle line is the median value. Upper and lower whiskers extend to the largest or smallest value, respectively

mesophyll compared to the spongy mesophyll (Barrera-Zambrano et al. 2014). Furthermore, a study of the facultative CAM species *Clusia minor* suggested that the tightly packed chlorenchyma cells that surround the leaf mid vein can perform CAM when the rest of the leaf is C_3 (Lüttge 2008b). An uneven distribution of CAM activity across the differentiated leaves of *Clusia* may explain how this photosynthetic specialization is able to evolve in different morphological backgrounds and could have important implications for bioengineering CAM into non-CAM crops (see Sect. 7).

The compartmentalization of CAM into specific cell types could, in principle, facilitate the evolution of CAM and C_4 photosynthesis in the same leaf (Sage 2002). However, it has been observed that very few species possess the capacity for both C_4 and CAM, despite evidence showing that both modes of photosynthesis probably evolved from similar clades in the phylogeny of angiosperms (Edwards and Ogburn 2012). This is likely to be because of metabolic and anatomical incompatibilities between CAM and C_4 photosynthesis (Sage 2002). Anatomical incompatibilities are due to the fact that CAM requires large succulent cells, specialised for intracellular storage functions, whereas C_4 leaves require cells that can efficiently undertake intercellular transport of metabolites (Sage 2002). *Portulaca* is the only genus known to show both CAM and C_4 characteristics and this has probably been possible due to the evolution of CAM in specific cell

types (Guralnick et al. 2008). In *P. grandiflora* leaves, for example, CAM activity is localised to the central water storage tissue, whereas C_4 activity occurs in the mesophyll and bundle sheath cells surrounding the vasculature (Winter and Holtum 2014). The evolution of CAM, which is believed to have originated before C_4 in *Portulaca*, occurred in cells most predisposed to succulent characteristics (i.e., large cells with big vacuoles; Christin et al. 2014). This allowed the subsequent evolution of C_4 Kranz anatomy in the mesophyll, adding to the diversity of leaf forms in which CAM is found.

D. CAM in Photosynthetic Stems

Many species have evolved CAM in organs besides the leaf, such as stems (Hastilestari et al. 2013; Kocurek et al. 2015; Winter and Holtum 2015). Across the plant kingdom there is a spectrum of CAM photosynthetic physiologies in stems. Some species, such as *Kalanchoë pinnata* and *Clusia rosea* which show strong CAM in the leaves, are able to carry out weak CAM in their stems, while *Jatropha curcas* can show weak CAM in both stems and leaves (Kocurek et al. 2015; Winter and Holtum 2015). The overnight accumulation of low levels of malate in these stems is probably an adaptation to recover endogenous CO_2 that would otherwise be lost via nocturnal respiratory processes (Kocurek et al. 2015).

In other species, the stem dominates carbon gain, having taken over from the leaf as the major organ for photosynthesis. CAM has emerged polyphyletically in several taxa of stem succulents that include the Cactaceae, Asclepiadaceae, Apocynaceae, Asteraceae, Didieraceae, Euphorbiaceae and Vitaceae phylogeny (Eggli and Nyfeller 2009; Hastilestari et al. 2013; Kocurek et al. 2015). In the majority of stem succulents with CAM, an external photosynthetically active chlorenchyma surrounds an internal water storing hydrenchyma of mainly non-green cells built up from cortex and pith of the stems (Eggli and Nyffeler 2009). An iconic example of CAM in stems evolving to have an analogous role to CAM in leaves is found within the genus *Opuntia*, which belongs to the Cactaceae (Pimienta-Barrios et al. 2012; Mason et al. 2015). During the evolution of *Opuntia*, leaves have reduced in size or disappeared altogether. In their place the stems have evolved into thick, flat, succulent organs called cladodes that use strong CAM to take up CO_2 almost exclusively at night. A retardation in the developmental rate of woody tissues (allometric neoteny) has been proposed as the main mechanism for the development of stem succulence in cacti (Altesor et al. 1994).

Some stem succulents in the Opuntioideae, Euphorbiaceae and Didieraceae that perform CAM will also undertake seasonal production of less succulent leaves. The production of these leaves allows a photosynthetic division of labour between stems and leaves on the same plant that serves to optimize carbon gain and water use in response to changing environmental conditions (Lüttge 2008b). In *Euphorbia tirucalli*, the C_3 performing leaves are shed in response to drought and the stem increases the amount of malate accumulated overnight (Hastilestari et al. 2013). In most leaf-producing stem succulents, CAM activity is generally highest in the stems (Lüttge 2008b), which most likely reflects the higher vacuolar capacity for nocturnal storage of malic acid in the more succulent stems compared to leaves. As found in leaf succulent CAM species, a full range of CAM photosynthetic physiologies may be found in stem succulents, ranging from weak scavenging of respiratory CO_2 to substantial night-time net CO_2 uptake.

E. CAM in Other Photosynthetic Organs

Perhaps the most extreme example of CAM evolving outside of the leaf is in the epiphytic orchids (Kerbauy et al. 2012). The origin of an epiphytic lifestyle is often associated with the evolution of CAM that serves to conserve

water in the variable and potentially drought-prone epiphytic habitat (Silvera et al. 2009). Within the epiphytic orchids, there are examples of CAM in every different photosynthetic organ. CAM is found in leaves, pseudobulbs (enlarged succulent internodal regions of the stem), photosynthetic roots and even flowers (Martin et al. 2010; Kerbauy et al. 2012). Furthermore, during their evolutionary history, some species have lost their leaves altogether. In these species, such as *Bulbophyllum minutissimum* and *Campylocentrum tyrridion*, the role of leaf photosynthesis as the major source of fixed carbon has been replaced by CAM pseudobulbs (Winter et al. 1983) or CAM photosynthetic roots (Winter et al. 1985), respectively. While CAM is usually considered an adaptation in the leaf, it can occur in any photosynthetically active succulent tissue, either to recover CO_2 lost from respiration or to increase water retention across the entire plant body.

In summary, a great deal of morphological and anatomical variation in photosynthetic organs exists across CAM species. The fact that CAM is found in such diverse forms has implications for the evolution of this carbon concentrating mechanism as it means that succulent traits that facilitate CAM can originate in a number of different anatomical 'starting points'.

IV. Physiological Consequences of Succulence

A. Water Use

Succulence and CAM are commonly considered as traits that are characteristic of plants found in desert habitats, but in reality both traits are largely lacking in plants of extremely xeric environments (Schmida 1985). Leaf and stem CAM succulents are more commonly found in semi-deserts, semi-arid scrub or rainforests, the latter exemplified by over 10,000 species of epiphytic orchids and bromeliads that can be subject to variable and potentially limiting water supply (Zotz and Hietz 2001). In ecophysiological terms, succulence represents a mechanism to avoid drought, rather than being physiologically tolerant of extreme water deficits, and succulent CAM species rarely develop water potentials less than −1 MPa while nearby C_3 shrubs may approach −4 MPa or lower (Ogburn and Edwards 2010). Moreover, in many species of cacti and agave, the roots rapidly dehydrate and shrink to lose contact in soil water potentials between −0.03 and −0.3 MPa (Nobel 1988; North et al. 2004).

The general mechanism by which succulents avoid drought at the cellular level can be described by the physiological trait of hydraulic capacitance (C), the change in volume of a cell or tissue per unit change in water potential (Nobel 1999). Capacitance is closely related to cell wall elasticity. Succulent cells or tissues, such as the specialized hydrenchyma cells, tend to have high values of C (Ogburn and Edwards 2010). Thus, hydrenchyma cells can take up or lose large volumes of water for a given change in C relative to cells or tissues with lower values of C. This ability to maintain turgor during tissue desiccation is one factor explaining the tendency of succulents to have relatively high tissue water potentials, even when droughted (Ogburn and Edwards 2010).

The modulus of elasticity (e), which provides an estimate of cell wall rigidity, is closely related to the inverse of capacitance (Nobel 1999) and has important implications for the movement of water between neighbouring tissues. In the case of storage succulence, where tissues within water-storing leaves and stems are divided into large celled, achlorophyllous hydrenchyma and smaller-celled chlorenchyma, there can be a tendency for hydrenchyma cells to lose water and buckle during desiccation and for chlorenchyma cells to stay hydrated at their expense (Schmidt and Kaiser 1987). Direct measurements on some tissue succulents have shown

that cell wall thickness of chlorenchyma cells can be twice as high as that of the hydrenchyma cells (Goldstein et al. 1991). Thus, if hydrenchyma tissues have cell walls with lower values of e (i.e., less rigid cell walls), they will better maintain turgor, and hence higher water potentials, when compared with the more rigid chlorenchyma cells for a given amount of drying across all tissues (Goldstein et al. 1991). This differential decrease in water potentials provides a driving force for water flow from hydrenchyma to chlorenchyma that requires no expenditure of energy. Thus, if water availability decreases, the water potential of photosynthetically active tissue is buffered. Such preferential hydration of chlorenchyma at the expense of hydrenchyma has been documented for a range of CAM succulents that include *Carnegiea gigantea, Opuntia basilaris* and *Peperomia magnoliaefolia* (Barcikowski and Nobel 1984; Schmidt and Kaiser 1987). Modelling of hydrenchyma water storage as an electrical analog of capacitance is consistent with reports that these succulent cells deliver between 34% (*Ferocactus*) and 37% (*Agave*) of daily transpiration demand (Smith et al. 1987; Schulte et al. 1989). The role of the hydrenchyma as an internal water reservoir is particularly important during CAM-idling, a situation in which stomata remain closed during both day and night and the plant internally recycles respiratory CO_2 via PPC under severe drought stress. CAM-idling has been well documented for cacti of the American semideserts (Szarek et al. 1973; Holthe and Szarek 1985). During CAM-idling, any water lost by cuticular transpiration can be replaced in the chlorenchyma by reserves in the hydrenchyma, and thus plants can survive for up to several months via CAM idling.

The vacuolar storage capacity for malate, a feature that is positively correlated with succulence and CAM activity, has been linked with plant water uptake from the soil in several species. Nocturnally accumulated malate functions as a solute in vacuoles of the chlorenchyma, increasing osmotic pressure and providing a stronger driving gradient for soil water uptake. This effect has been demonstrated in the leaf succulents *Kalanchoë daigremontana* (Smith and Lüttge 1985), *Clusia minor* (Herrera et al. 2008), and *Senecio medley-woodii* (Ruess and Eller 1985). In contrast, diel malate fluctuations were found to be relatively unimportant in driving soil water uptake in *Agave deserti* (Smith et al. 1987; Tissue et al. 1991). Since malate is consumed during the day in CAM plants and osmotic potential becomes higher again, the water so gained becomes thermodynamically more available to the tissues (Lüttge 2004).

B. Division of Labor Between Hydrenchyma and Chlorenchyma: Implications for CAM and Water Use

In most stem succulents that have been examined, the non-photosynthetic central hydrenchyma does not appear to participate in the diel oscillations of organic acid levels that define CAM (Lüttge et al. 1989). In the peripheral stem chlorenchyma, nocturnal malate accumulation can increase osmotic pressure within these cells so that they take up water from the hydrenchyma and thus turgor pressure of the chlororenchyma increases (Lüttge and Nobel 1984). Dynamic diel cycles of radial internal water distribution in the stem succulent cacti are such that water moves more readily towards the water storage tissue at dusk and towards the chlorenchyma at dawn. These assertions are supported by detailed quantitative assessments of water relation parameters such as cell osmotic pressure and turgor pressure and through use of hydrogen isotopes ^3H and ^2H (tritium and deuterium, respectively) to assess mixing of water between the two tissues (Goldstein et al. 1991; Tissue et al. 1991). Such diel changes in water relation parameters appear to determine diel timing of growth cycles such that in cladodes of

Opuntia growth is maximal at midday when turgor is still high while malate mobilisation also provides a source for production of carbohydrates to fuel the carbon and energetic demands of growth (Gouws et al. 2005). This contrasts with the situation for many C_3 species where growth of the photosynthetic organs generally occurs at night (Gouws et al. 2005). In CAM plants, this uncoupling of leaf expansion growth from nocturnal carbohydrate degradation has been proposed as a means of reconciling potential conflicts of demand between accumulation of carbohydrate reserves required for PPC-mediated CO_2 uptake at night and partitioning of resources for growth during the day (Borland et al. 2009, 2016).

In some leaf succulents in which the hydrenchyma is formed by layers of cells below the epidermis, there is a negative relationship between thickness of the hydrenchyma layer and CAM expression, a situation reported within the genus *Peperomia* (Sipes and Ting 1985). Leaves of the C_3-CAM intermediate *P. obtusifolia* possessed a thicker hydrenchyma (i.e., 63% of total mesophyll cross-section) compared with *P. macrostachya* a constitutive CAM species where hydrenchyma thickness represented 19% of total mesophyll (Fondom et al. 2009). The same study also indicated that the drought-induced switch to CAM in *P. obtusifolia* was accompanied by a shrinkage of the hydrenchyma and palisade mesophyll but this species still presented a higher water use efficiency compared with the constitutive CAM *P. macrostachya* under the same conditions of drought (Fondom et al. 2009). It was thus suggested that, even when *P. obtusifolia* performed CAM, the hydrenchyma could act to conserve water.

In a comparative study of leaf anatomy across nine species of tropical trees of the genus *Clusia*, the presence of a thick layer of hydrenchyma was particularly evident in two C_3 species, with the thickness of this layer determined principally by cell size and number of cell layers (Barrera-Zambrano et al. 2014). It is tempting to speculate that by acting as a means of buffering against water shortage, the hydrenchyma layer that is present in these obligate C_3 *Clusias* (section Anadrogyne of the Clusiaceae) might obviate the need for CAM. However, the presence of hydrenchyma in a strong CAM species of *Clusia* (*C. alata*) indicates that the presence of hydrenchyma and CAM are not mutually exclusive within the *Clusia* genus. It is possible that the thickness of hydrenchyma is determined more by phylogeny than photosynthetic mode since *C. grandiflora*, a C_3 species within Section Chlamydoclusia of the Clusia phylogeny had a reduced hydrenchyma compared to the other C_3 species examined in this study but which was of comparable depth to the hydrenchyma in the CAM species *C. rosea*, also located within this section (Barrera-Zambrano et al. 2014). A more comprehensive survey of leaf anatomy encompassing species within all sections of the *Clusia* genus would be informative in terms of the evolutionary origins of tissue succulence and CAM within this photosynthetically diverse genus.

C. CO_2 Uptake and Carbon Gain

Leaf and stem succulence is generally accompanied by an increase in mesophyll cell size that leads to low internal air space (IAS) as a result of the tightly packed cells (Smith and Heuer 1981). A reduced IAS and the concomitant reduction in the length of mesophyll cells that are exposed to the IAS (L_{mes}/area) will increase resistance to CO_2 efflux from the leaf (Nelson et al. 2005). It has been proposed that, alongside the daytime reduction in stomatal conductance, a reduced IAS improves the carbon economy of CAM during the day-time decarboxylation of malate (Phase III of CAM, Fig. 10.2) because net efflux of CO_2 from the leaf will be curtailed (Maxwell et al. 1997). Moreover, a low internal conductance to CO_2 will reduce the diffusion out of mesophyll cells and this could enhance the recapture of respiratory CO_2 at night (Phase I of CAM)

via PPC (Griffiths 1992). Recapture of respiratory CO_2 at night is thought to have been an early step in the evolutionary process by which CAM evolved from C_3 photosynthesis. Hence, it has been suggested that low IAS may be a trait that was selected for to enhance the efficiency of CAM rather than simply being the unavoidable consequence of the large, tightly packed cells that characterize succulent leaves or stems (Maxwell et al. 1997). This assertion is supported by a study of 18 CAM plants belonging to 13 families and six C_3 and four C_4 plants that found a close association between the degree of succulence, IAS and L_{mes}/areas and with all 3 traits being substantially lower in the CAM species (Nelson et al. 2005). Thus, tight cell packing appears to be a trait that is common in all CAM lineages and that reflects evolutionary convergence of leaf anatomy within the CAM functional type.

While a reduced IAS and L_{mes}/area would seem to improve the carbon economy of CAM by minimizing net CO_2 efflux from the leaf, a reduction in leaf internal conductance to CO_2 will curtail direct uptake of atmospheric CO_2, particularly during the latter part of the photoperiod when stomata may re-open and direct Rubisco-mediated CO_2 uptake occurs, i.e., Phase IV in Fig. 10.2 (Griffiths 1992; Nelson and Sage 2008). In 'weak CAM' plants, which rely heavily on Phase IV uptake of CO_2, (as opposed to 'strong CAM' in which Phase I nocturnal uptake of CO_2 dominates diel carbon gain), a low IAS could limit photosynthetic efficiency since diffusion through mesophyll limits carbon availability for Rubisco (Evans and von Caemmerer 1996; Maxwell et al. 1997; Nelson and Sage 2008). Thus, it would appear that photosynthetic divergence between weak and strong CAM is mediated by % IAS and L_{mes}/area that collectively present a functional threshold for predominantly Rubisco- or predominantly PPC-mediated net CO_2 uptake (Nelson and Sage 2008). The compromise between maximizing day- or night-time uptake of CO_2 was exemplified by a comparison of two *Kalanchoë* species (*K. daigremontiana* and *K. pinnata*) that differed in the magnitude of leaf succulence and % IAS (Griffiths et al. 2008; von Caemmerer and Griffiths 2009). The more succulent species (*K. daigremontiana*) was more committed to the conventional CAM cycle, with higher rates of acid accumulation and dark net CO_2 uptake as well as a higher stomatal conductance at night. In contrast, the less succulent *K. pinnata* showed a more C_3-like expression with a higher proportion of integrated 24-h net CO_2 uptake mediated directly by Rubisco during Phases II and IV (Griffiths et al. 2008). A perceived incompatibility between the optimal anatomy for high nocturnal PPC activity and the internal structure ideal for C_3 photosynthesis may account for the bimodal distribution of weak and strong CAM plants that is indicated by carbon isotope ratios ($\delta^{13}C$) across various families known to contain both C_3 and CAM species (Winter and Holtum 2002; Crayn et al. 2004; Silvera et al. 2005).

V. Vasculature and Hydraulic Traits of Photosynthetic Organs of CAM Plants

A. Venation Patterns

The typically succulent leaves and stems of CAM species could potentially present a high cell hydraulic path length for water flow or the transfer of sugars between vascular bundles and metabolic tissues. However, it seems that highly succulent species have circumvented limitations to hydraulic connectivity by evolving 3D venation (Balsamo and Uribe 1988; Cutler 2004; Ogburn and Edwards 2013). Most leaves have their vasculature arranged in two dimensions, with all of the veins arranged in a flat plane that ramifies through the central portion of the mesophyll. However, some succulent CAM lineages, like the monocot *Aloe*, have 3D

vasculature, with veins running through the leaf mesophyll both above and below the central hydrenchyma tissue (Fig. 10.4c). Such 3D vein architecture is also found in dicots where the veins have a fractal-like appearance with a primary vein (the midrib) giving rise to smaller secondary veins, which in turn give rise to tertiary veins, and so on. In the CAM species *Kalanchoë daigremontiana*, the fifth order veins branch at different angles, moving into the adaxial (upper) and abaxial (lower) portions of the mesophyll (Fig.10.4a; Balsamo and Uribe 1988). These fifth order veins are able to cross over higher order veins in order to reach new portions of the mesophyll, and provide water to this tissue. The evolution of 3D venation is believed to release the constraints on succulence, allowing plants to maintain adequate levels of water across the leaf, even in extremely thick leaves (Ogburn and Edwards 2013). It is intriguing to consider how the multiple independent origins of 3D venation across phylogenetically diverse lineages of plants might have contributed to the convergent evolution of CAM (Griffiths 2013).

B. Hydraulic Traits

For plants growing in environments where evaporative demands are high, excessive negative pressures within the xylem can cause the sap to change from liquid to gas in a process called cavitation (Lens et al. 2013a). Cavitation results in the formation of air emboli that often occur at the pits between xylem vessels, thus breaking the column of water in the xylem and resulting in hydraulic failure (Christman et al. 2012; Lens et al. 2013b). Given the hot and water-limiting habitats where CAM plants are competitive, it might seem intuitive that these species should be able to withstand highly negative pressures in their xylem vessels. However, the facultative CAM species *Clusia uvitana* was found to be less tolerant than sympatric C_3 species to highly negative pressures in the xylem vessels, as indicated by measurements of $\Psi 50$, the xylem pressure (negative water potential) at which a 50% loss in hydraulic conductivity can occur (Lüttge and Duarte 2007). However, as described above (Sect. 4.1) and elsewhere, CAM is a trait that evolved as a mechanism for drought avoidance (Griffiths 2013; Borland et al. 2015). Thus, the xylem vessels of CAM species are probably rarely exposed to pressures as negative as those experienced by C_3 species. Consequently it can be hypothesized that evolution has not driven the development of vasculature that is resistant to cavitation in CAM plants. Anecdotal support for this hypothesis comes from documented reports of low lignin content in leaves/stems of CAM species such as *Agave* and *Opuntia* (Cushman et al. 2015) which could be related to a low tolerance to xylem cavitation. Low lignin content is known to increase a plant's susceptibility to cavitation by affecting the permeability or thickness of vessel pits, the location where most xylem embolisms begin to form (Awad et al. 2012). Thus, the drought avoiding strategy of CAM may have reduced the requirement for lignification of the xylem.

The evolutionary relationship between CAM and the vasculature of plants may extend beyond these findings. One hypothesis is that CAM species are less likely to evolve secondary woodiness (i.e., evolve woodiness from an herbaceous ancestor). Evidence is emerging to suggest that the evolution of even small amounts of woodiness may increase tolerance to highly negative xylem pressures, either because strong vessels are resistant to cavitation, or because thicker vessels are less likely to form microfractures that nucleate embolisms (Lens et al. 2012, 2013b). Since the evolution of woodiness is believed to increase a plant's ability to withstand the negative pressures associated with high transpiration rates, it might be hypothesized that CAM lineages, which are known to have low transpiration rates, will rarely evolve from an herbaceous to woody growth habit. Furthermore, evidence from time calibrated phylogenies sug-

gests that aridity has driven the adaptive radiations of succulent CAM lineages as well as lineages that have recently evolved secondary woodiness in the last 9 million years (Arakaki et al. 2011; Lens et al. 2013a). It is intriguing to speculate that these adaptations are alternative solutions to tolerating drought, which may be mutually exclusive; i.e., CAM lineages avoid drought by changing the leaf to reduce transpiration whereas secondary woody lineages change the xylem structure to tolerate drought.

VI. Stomatal Traits in CAM Plants

A. Stomatal Patterning

In general, more succulent species show lower stomatal densities than less succulent species (Sayed 1998; Lüttge 2008b). This holds true for the photosynthetic organs of CAM species, which typically have low stomatal densities and subsequent low conductance to water vapor (Barrera-Zambrano et al. 2014; Males and Griffiths 2017). Such stomatal patterning concurs with the high water-storage capacity, low external surface area:volume ratio, and high water-use efficiencies for leaves and cladodes of CAM species (Osmond 1978; Nobel 1988). In stem succulent CAM species where ribs are obvious, stomata tend to be located at the base between ribs and in many cases the stomata are sunken. In leaf succulent CAM species, an amphistomatic (stomata on both upper and lower leaf surfaces) or hypostamatic (stomata only on the lower leaf surface) location of stomata appears to be related to leaf thickness. For instance, thick-leaved species of the Agavaceae, Crassulaceae and Aizoaceace, such as *Agave tequilana*, *Kalanchoe fedtschenkoi* and *Mesembryanthemum crystallinum,* respectively, are amphistomatic (Moreira et al. 2012; Monja-Mio et al. 2015). In contrast, the relatively thinner leaved *Clusias* are hypostomatic, regardless of the propensity for CAM (Barrera-Zambrano et al. 2014). Amphistomaty is considered an evolutionary adaptation to increase maximum leaf CO_2 conductance by the reduction of its diffusion pathway to the mesophyll, which is advantageous in thicker leaves (Mott et al. 1982), while hypostomaty is considered primarily an adaptive trait to avoid water loss (de Faria et al. 2012).

A negative correlation between stomatal density and size seems to hold for many C_3 species, where plants with lower stomatal densities show a greater mean stomatal size, and smaller stomata are found in leaves with higher stomatal densities (Doheny-Adams et al. 2012; Lawson and Blatt 2014). Such a relationship is attributed to spatial limits in the placing of stomata on the leaf surface that constrains the maximum size and density of stomata (Beaulieu et al. 2008; Franks et al. 2009). In a comparative study of stomatal patterning across nine species of *Clusia* that possess varying capacities for CAM, it was found that stomata were present in lower densities in the thicker-leaved CAM-performing species (Barrera-Zambrano et al. 2014). However, the stomatal pore areas tended to be larger in CAM *Clusias* compared to C_3 *Clusias,* which supports the spatial limitation view described above for C_3 species (Barrera-Zambrano et al. 2014).

As well as affecting the rate of transpiration, the size of the stomata exert a strong influence on the speed with which stomata open and close (Hetherington and Woodward 2003). Smaller stomata may have faster response times when opening and closing compared with larger stomata due to their high membrane surface area to volume ratio. This means that smaller guard cells require less water movement, relative to their size, to inflate or deflate, and affect the pore-facing membrane. It has been suggested that smaller stomata are better at improving water use efficiency due to their more rapid response to changes in environmental conditions such as humidity (Hetherington and Woodward 2003). Therefore, it is reasonable to predict

that CAM species, which are often subject to fluctuating levels of water availability, might have many small stomata. A comparative study of *Clusia* species with different photosynthetic physiologies, however, did not find this to be true. In fact, *Clusia* species that undertake a greater amount of night-time photosynthesis tend to have fewer, large stomata (Barrera-Zambrano et al. 2014). This is believed to be because the production of more, smaller stomata for a given leaf area may incur additional metabolic costs due to higher rates of guard cell respiration (Srivastava et al. 1995; Franks et al. 2009). These extra costs could be compensated for with high CO_2 assimilation rates, but only if environmental resources such as water and light are not limiting (Franks et al. 2009). Given that CAM-performing species of *Clusia* commonly inhabit water-limited environments the possession of larger stomata in lower densities might be the most appropriate strategy in terms of resource use. Furthermore, the positive correlation found within *Clusia* in terms of leaf thickness, mesophyll cell size and guard cell size may be a consequence of common genetic control of cell sizes (Beaulieu et al. 2008).

B. Physiological Implications of Stomatal Patterning

In C_3 plants, it has been proposed that size correlations between different cell types in the leaf (e.g., guard cells, epidermal cells, mesophyll and xylem) provide a highly efficient match between potential maximum water loss (determined by stomatal conductance) and the leaf vascular system's capacity to replace that water (which is determined by vein density; Brodribb et al. 2013). Ultimately, the anatomical potential for diffusive exchange across leaves may be calculated as a function of stomatal density and pore area (i.e., anatomical G_{smax}; (Lawson et al. 1998). Calculations of anatomical G_{smax} across nine *Clusia* species showed no clear trend between photosynthetic mode or the potential for water loss (Barrera-Zambrano et al. 2014). Similarly, a comparison of two facultative CAM species of *Clusia*, *C. minor* and *C. pratensis*, showed comparable anatomical potential for stomatal water loss compared to a related constitutive C_3 species (*C. tocuchensis*; Fig. 10.6). Comparing G_{smax} for these three *Clusia* species with that of *Kalanchoë fedtschenkoi* indicated a significantly reduced anatomical potential for stomatal water loss in the thicker leaved constitutive CAM *Kalanchoë* (Fig. 10.6d). However, in all three species, the calculated G_{smax} was at least 10-fold higher than the measured stomatal conductance (data not shown). Thus, endogenous control over stomatal conductance appears to be more important than stomatal patterning in determining the potential for water loss across different CAM species (Barrera-Zambrano et al. 2014).

In CAM plants, the diel process of malate turnover results in profound shifts in the leaf internal partial pressure of CO_2 (pCO_2), which in turn is believed to underpin the CAM-defining daytime closure and opening of stomata (Cockburn et al. 1979; Wyka et al. 2005). However, other stimuli/regulators are known to influence stomatal conductance in CAM plants either independently or in conjunction with the endogenous CAM cycle of malate turnover. Stomata are subject to regulation by light intensity, light quality, osmolyte concentration, humidity, temperature and the circadian clock, (von Caemmerer and Griffiths 2009; Males and Griffiths 2017). Recent data has demonstrated temporal reprogramming of the expression of several genes associated with various signal transduction mechanisms that regulate stomatal movement in the constitutive CAM species *Agave americana* relative to C_3 *Arabidopsis* (Abraham et al. 2016). These reprogrammed genes included the CO_2-sensing *HIGH LEAF TEMPERATURE 1* together with several redox-related genes; genes demonstrated to play important roles in abscisic acid signaling in *Arabidopsis thaliana*, and potassium, calcium and chloride channels known to

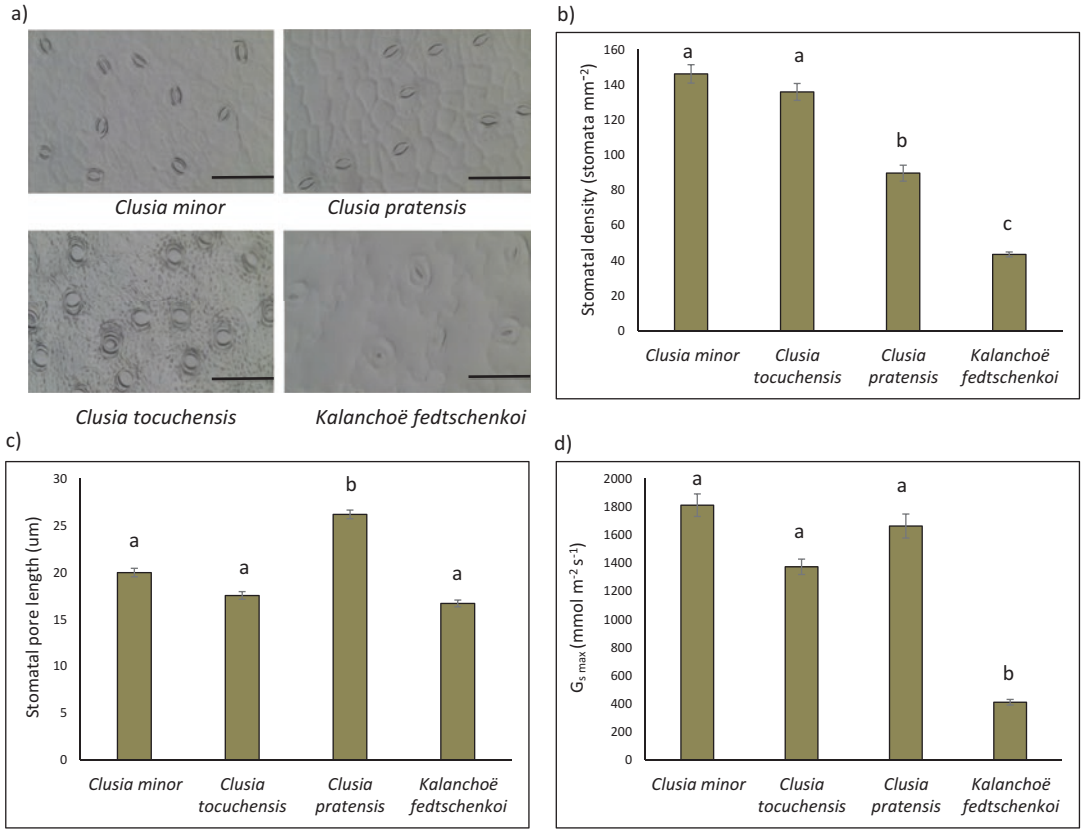

Fig. 10.6. Stomatal patterning characteristics in plants with different modes of photosynthesis. (**a**) Stomatal imprints were obtained using clear nail varnish applied to the lower surface of the leaf for 2 facultative CAM species, *Clusia minor* and *C. pratensis*, a constitutive C_3 species, *C. tocuchensis*, and the constitutive CAM species *Kalanchoe fedtschenkoi*. The scale bar is 100 μm, (**b**) stomatal densities for the 4 species (note stomata are located only on the abaxial leaf surface in *Clusia* and are amphistomatic in *Kalanchoë*), (**c**) stomatal pore length for the 4 species and (**d**) the calculated anatomical G_{smax} for all 4 species. All measurements are shown as the mean of 60 measurements taken from 4 biological replicates ± standard error. Statistical analysis of data was performed using ANOVA and for each graph, different letters above the bars indicate significant difference where $p < 0.05$

be key players in determining stomatal movement. Many of these shifts in transcript abundance were confirmed at the level of protein abundance (Abraham et al. 2016), suggesting a concerted re-programming of the temporal regulation of key components in the core signalling mechanism responsible for inverse stomatal activity in CAM plants. The complex and dynamic nature of diel stomatal regulation which underpins the 4 phases of CAM remains to be fully resolved (Males and Griffiths 2017).

VII. Engineering Anatomical Traits That Are Conducive to CAM

Major research efforts are underway to harness the inherently high WUE of CAM by engineering this pathway into existing food, feed, and bioenergy crops (Borland et al. 2014; Yang et al. 2015). The engineering of CAM into non-CAM crops offers the potential to sustain plant productivity in the hotter and drier climates that are predicted over the next 10–20 years (Dai 2013; Cook et al.

2014). This grand challenge will require further elucidation of the genomic features and regulatory mechanisms that underpin CAM in order to achieve the day/night separation of carboxylation processes catalysed via PPC and Rubisco as well as a temporal reprogramming of stomatal conductance (Yang et al. 2015; Abraham et al. 2016). In addition, leaf anatomical traits will be an important determinant of the mode and level of CAM that is engineered in a non-CAM species. As discussed above (Sect. 3.1), strong constitutive CAM requires adequate vacuolar storage capacity for malate, a trait that is associated with more succulent cells. Engineering succulent cells in a host species for bioengineered CAM could result in less intercellular air space (IAS) between mesophyll cells and a reduction in the length of mesophyll exposed to intercellular air spaces (L_{mes}/area), traits that reduce internal conductance to CO_2. Thus, succulence presents a 'trade-off' between the optimal leaf anatomy for CAM and the internal structure ideal for C_3 photosynthesis. For many productive non-CAM crops, the mode of engineered CAM used to improve WUE should not compromise productivity when water is in plentiful supply. Thus, the option to engage in CAM for only limited periods of time when water is in low supply (i.e., exploit CAM to maintain viability during periods of drought) would appear to be the best model configuration for engineering CAM in many crop species (Borland et al. 2015; Yang et al. 2015).

Studying the functional leaf anatomy of facultative CAM plants, where CAM can be reversibly induced in response to water limitation, should provide valuable pointers towards the optimal leaf anatomy that would accommodate the bioengineering of inducible CAM without incurring detrimental consequences for direct C_3-mediated photosynthesis. Across the different photosynthetic types of the dicotyledonous genus *Clusia*, it has been suggested that the relatively well-aerated spongy mesophyll of the facultative species helps to optimize direct C_3-mediated CO_2 fixation, whereas the enlarged and densely packed palisade mesophyll cells accommodate the potential for C_4 carboxylation and nocturnal storage of organic acids (Barrera-Zambrano et al. 2014). Thus, in principle, differentiated leaves that contain distinct layers of palisade and spongy mesophyll present further options for accommodating both direct Rubisco-mediated daytime uptake of atmospheric CO_2 and the nocturnal uptake of CO_2 that defines CAM. A differential distribution of PPC and Rubisco proteins between palisade and spongy mesophyll cells (with relatively more PPC localized to palisade versus spongy mesophyll cells) could further enhance the efficacy of engineered CAM within leaves made up of these cell layers (Barrera-Zambrano et al. 2014; Sect. 3.3 above). Selecting genotypes with increased levels of ploidy as potential hosts for bioengineered CAM should, in principle, provide increased cell size and biomass productivity due to the positive correlation that exists between ploidy and cell size (De Veylder et al. 2011). In addition to finding species with enlarged cells across the whole leaf, species with a well-developed and tightly packed palisade mesophyll layer should enhance the level of engineered CAM (Barrera-Zambrano et al. 2014). Enhanced development of palisade mesophyll tissue is commonly found in thicker-leaved C_3 species and is hypothesized to improve the harvesting of light, thereby helping to offset the increased investment of biomass in thicker leaves (Smith and Hughes 2009). Overexpression of CBF/DREB transcription factors has been shown to result in thicker leaves with more chlorophyll and higher rates of photosynthesis (Savitch et al. 2005) and this could be one possible strategy for genetically modifying the leaf anatomy of a C_3 host for optimal operation of engineered CAM.

In terms of which stomatal patterning traits might be preferred for bioengineered CAM,

similar values for G_{smax} (anatomical stomatal conductance) that exist between *Clusia* species with different modes of photosynthesis imply that endogenous control over stomatal conductance will be the key factor determining potential water loss from engineered CAM plants (Barrera-Zambrano et al. 2014; Fig. 10.6d). It is generally believed that stomatal conductance in CAM plants is regulated via the substantial diel changes in leaf internal partial pressure of CO_2 (pCO_2) that result from the day/night turnover of malate (Borland et al. 2014; Males and Griffiths 2017). Genotypic variation in stomatal responsiveness to pCO_2 has been reported within the genus *Populus* (AM Borland, unpublished observation), fast-growing bioenergy trees that have been targeted for CAM bioenegineering (Borland et al. 2014, 2015). Thus, it can be argued that an appropriate C_3 host for bioengineered CAM will possess stomata that open in response to low C_i (internal CO_2 concentration) at night and close completely in response to high C_i during the day.

Further detailed and comparative analyses of physiological and morphological characteristics across other lineages that contain C_3, CAM, and intermediate C_3/CAM species are needed to highlight the anatomical traits that are vital for nocturnal CO_2 uptake. A recent study that investigated biochemical, physiological and anatomical traits for the hybrid offspring of C_3 and CAM parents belonging to the genus *Yucca* (Asparagaceae) indicated that leaf anatomical traits seem to be segregating (i.e., show phenotypic variation) among individuals of the hybrid (Heyduk et al. 2016a, b). The *Yucca* hybrid system shows future promise for elucidating the genetic architecture of morphological and CAM related traits within a monocotyledonous genus. In turn, this system should help inform the bioengineering of CAM into economically important non-CAM monocots as a means of improving crop WUE.

VIII. Conclusions

Despite the convergence of CAM across taxonomically diverse groups of plants, common anatomical traits are found in the photosynthetic organs which have a profound influence on physiological strategies for photosynthetic CO_2 assimilation and water-use. Future research should seek to apply advances in the study of leaf and stem hydraulics alongside improved phylogenies and knowledge of geographical distribution in order to aid our understanding of environmental and physiological constraints that have shaped the evolution of CAM. As more CAM genomes become publically available, it should also be possible to identify key regulatory factors involved in the induction and development of leaf and/or stem succulence within the context of photosynthetic pathway divergence. Collectively, such approaches will be crucial for supporting the successful exploitation of engineered versions of constitutive or facultative CAM as a means of improving the water-use efficiency of non-CAM crops grown for food, feed, fibre and bioenergy.

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Chapter 11

Trade-offs and Synergies in the Structural and Functional Characteristics of Leaves Photosynthesizing in Aquatic Environments

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Summary

Aquatic plants, comprising different divisions of embryophytes, derive from terrestrial ancestors. They have evolved to live in water, both fresh and salty, an environment that presents unique challenges and opportunities for photosynthesis and growth. These include, compared to air, a low water stress, a greater density, and attenuation of light, and a more variable supply of inorganic carbon, both in concentration and chemical species, but overall a lower carbon availability, and the opportunity to take up nutrients from the water. The leaves of many aquatic plants are linear, dissected, whorled, or cylindrical with a large volume of air spaces. They tend to have a high specific leaf area, thin cuticles, and usually lack functional stomata. Exploiting the availability of chemicals in their environment, freshwater macrophytes may incorporate silica in their cell wall, while seagrasses contain sulphated polysaccharides, similar to those of marine macroalgae; both groups have low lignin content. This altered cell wall composition produces plants that are more flexible and therefore more resistant to hydraulic forces (mechanical stress arising from water movement). Aquatic plants may have enhanced light harvesting complexes conferring shade adaptation, but also have mechanisms to cope with high light. Aquatic plants have evolved numerous strategies to overcome potential carbon-limitation in water. These include growing in micro-environments where CO_2 is high, producing leaves and roots that exploit CO_2 from the air or sediment and operating concentrating mechanisms that increase CO_2 (CCM) around the primary carboxylating enzyme, ribulose-1,5-bisphosphate carboxylase-oxygenase. These comprise C_4 metabolism, crassulacean acid metabolism, and the ability to exploit the often high concentrations of HCO_3^-, and ~50% of freshwater macrophytes and ~85% of seagrasses have one or more CCM. Many of these adaptations involve trade-offs between conflicting constraints and opportunities while others represent 'synergies' that help to maximize the productivity of this important group of plants.

I. Introduction

Aquatic embryophytes (bryophytes, lycophytes, monilophytes, and angiosperms) are a heterogeneous group of plants with many common features. They have arisen as a result of convergent evolution from terrestrial ancestors to adapt to life in marine and fresh waters. In the ocean, seagrasses cover an area of about 3.10^{11} m^2 (Duarte et al. 2005) and have an average net productivity rate that ranges from 500 to 1200 g C m^{-2} $year^{-1}$ (van der Heijden and Kamenos 2015). This corresponds to a net global productivity of about 0.4 Pg C $year^{-1}$, which is about 1% of marine primary productivity. Nevertheless, seagrass beds help to support coastal fisheries and are important habitats for fish, birds, invertebrates, and marine mammals, play an important role in nutrient cycling (Waycott et al. 2009) and keep waterbone pathogens in check (Lamb et al. 2017). They are also globally important for organic carbon sequestration, contributing about 30–40 Tg C $year^{-1}$, or nearly 20% of global oceanic carbon burial (Duarte et al. 2005).

In fresh waters, macrophytes are important primary producers, particularly in shallow lakes and rivers. They can also be regarded as 'ecological engineers' producing a structurally complex environment that is very different from the open water and an important habitat for numerous organisms including invertebrates and fish (Chambers et al. 2008; Kovalenko et al. 2012). They also help to prevent turbid, phytoplankton-dominated systems as part of the alternative stable

states that can exist in shallow lakes (Scheffer et al. 1993). Their presence in shallow lakes increases food-chain length (Ziegler et al. 2015) probably through their contribution to productivity, environmental variability, and their provision of a refuge for higher trophic levels. In some shallow environments, such as the Amazonian floodplains, macrophytes can contribute a large portion of the net primary productivity of the system (Silva et al. 2013).

Macrophytes comprise a range of life forms in a gradient ranging from emergent plants with leaves that photosynthesize in air (not considered in this chapter), through species with leaves floating on the water surface but rooted in the sediment, free-floating plants with leaves on the water surface, to completely submerged plants with leaves photosynthesizing in water, either free-floating in the water column or, more commonly, rooted in the sediment. The latter group can be broadly divided into two types: (i) rosette plants (often called isoetids) with leaves that are short, stiff, and often cylindrical and (ii) tall caulescent shoots (often called elodeids) with leaves with a range of morphologies (Sect. 3). Seagrasses typically belong to the caulescent, elodeid group of submerged plants. The life form system of Wiegleb (1991) gives a more detailed breakdown of hydrophyte growth forms. While most aquatic plants are rhizophytes with roots penetrating the sediment, some, such as the bryophytes, are haptophytes, attached to, but not penetrating hard surfaces.

In this chapter, we compare air and water as environments for photosynthesis and growth and describe how the structure and function of aquatic leaves allows them to thrive in aquatic environments. We deal primarily with submerged embryophytes, freshwater macrophytes, and seagrasses, but make occasional reference to macrophyte and microphyte algae.

II. Adaptation of Aquatic Plants to the Environmental Challenges and Opportunities in Water

A. Evolution of Aquatic Embryophytes

All aquatic embryophytes evolved from terrestrial ancestors that in turn evolved from aquatic green algae, probably within the Zygnematophyceae (Wickett et al. 2014), and therefore contain evolutionary traces of adaptations to life in both air and water. For example, terrestrial bryophytes inherited features from their algal ancestors, such as desiccation tolerance and lignin-like cell wall polymers, that allowed them to colonize land (Graham et al. 2014) while present day macrophytes possess visible features of their terrestrial past such as cuticles and stomata (functional and non-functional), xylem (Sculthorpe 1967) and leaves themselves.

Aquatic angiosperms are found in 17% of angiosperm families, comprising over 6000 species (Cook 1990; den Hartog and Kuo 2006), but represent less than 2% of all angiosperms (Les et al. 1997). Freshwater angiosperms have arisen at least 100 times (Les and Tippery 2013) and are represented predominantly by monocotyledons (Liliopsida) rather than dicotyledons (Magnoliopsida) (Les and Schneider 1995). In fresh waters, the cline between terrestrial, wetland, and submerged environments, blurred by episodic and seasonally variable water levels, produces a gradient that probably facilitated movement of plants between land and water. Indeed, there is evidence for progressive evolution from emergent to floating-leaved then to submerged leaves in aquatic lineages (Du and Wang 2014). Fossil and phylogenetic data show that invasion of the aquatic habitat occured very early in the evolution of the angiosperms and that they existed in the Cretaceous 120 million years ago (Friis et al. 2001; Gomez et al. 2015; Les 2015).

In coastal and marine waters, there are 72 species of seagrasses within 13 genera and five families if euryhaline genera such as *Ruppia* are included. All belong to the monocotyledon, largely freshwater order Alismatales (Les et al. 1997; den Hartog and Kuo 2006); Short et al. 2007; Papenbrock 2012; Les and Tippery 2013) and so probably evolved from freshwater ancestors. Seagrasses are believed to have evolved relatively recently, around 64–72 million years ago (Olsen et al. 2016) or possibly only 16–41 million years ago (Chen et al. 2012). The apparent evolutionary barrier to colonization of marine systems by embryophytes, indicated by low species diversity, is not clear. It does not appear to be linked to salinity *per se* since seagrasses have a range of mechanisms to deal with this factor (Touchette 2007). It has been suggested to be caused by difficulties of efficient pollination and potentially to the lack of co-evolution with insects, a group that is also limited in its success in marine environments (Van der Hage 1996). It could also result from competition with large macroalgae that were already present in marine systems in contrast to fresh waters where macroalgae, essentially members of the family Characeae, are relatively small and occupy a different niche, or simply result from a physical barrier caused by the hydraulic forces.

B. *Comparison of Air and Water as Environments for Photosynthesis and Growth*

The fluids of air and water provide contrasting environments for the photosynthesis, growth, and survival of photoautotrophs. These properties (Maberly and Spence 1989) set contrasting ecological challenges and opportunities (Table 11.1) that have resulted in different evolutionary solutions to maximize fitness in aquatic and terrestrial plants (Maberly 2014).

The most obvious distinction between air and water is water availability itself. In fresh water, the water potential is, by definition, 0 MPa and in seawater about −2.5 MPa. In air, it can range from 0 at 100% humidity to as low as −200 MPa in very dry air, setting extreme challenges for leaf water balance. Despite the many structural, morphological, and physiological attributes of terrestrial plants, water availability is still a major factor controlling plant productivity on land (Hsu et al. 2012). A second environmental difference is the greater density of water, compared to air, that reduces the need for investment in structural material, but increases the drag on a plant in flowing water. For a given velocity, the drag and lift forces on an object in water are about 29-times greater than in air (Denny 1993). Thus, plants in rivers with a relatively low water velocity of 1 m s^{-1} experience the same forces as plants in air with a wind speed of 100 km h^{-1}. Plants, in littoral regions where waves are breaking, or in fast flowing water such as in rapids, often inhabited in sub-tropical and tropical areas by freshwater macrophytes of the family Podostemaceae (Koi et al. 2015), will experience much greater forces.

A third difference is the generally low levels of underwater light. This results from reflection at the air-water interface, but mainly by the much greater, and spectrally-selective, attenuation of light in water compared to air (Table 11.1). This is caused by light absorption by water molecules themselves, plus the ability of water to dissolve substances, such as coloured dissolved organic matter ((Kirk 2011). Furthermore, because of the density of water, particles including phytoplankton, sediment, and non-living organic material can be kept in suspension, scattering light and increasing attenuation. Finally, growth of epiphytes on aquatic leaves because of the absence of desiccation and presence of dissolved nutrients, can reduce light availability even further (Sand-Jensen 1977). As a consequence, light controls macrophyte depth limits (Krause-Jensen and Sand-Jensen 1998), macrophyte productivity at the whole population level

Table 11.1 Challenges, opportunities, and responses for plant leaves in the environments (Env) of air (A) and water (W)

Feature	Env	Attribute	Environmental opportunity	Response to opportunity	Environmental challenge	Response to challenge
Water availability	A	Low	–	–	Risk of desiccation and restriction of productivity	Transpiration. Cuticle, stomata, sub-stomatal chambers, C_4, CAM
	W	High	No risk of desiccation	High productivity; epidermal chloroplasts, thin leaves	Competition with algae	Allelochemicals
Density	A	Low	Low aerodynamic drag	Tall plants possible	Structural support required	Lignin and secondary thickening
	W	High	Support of plant by water	Low investment in structural components	High hydrodynamic drag	Flexible leaves and shoots
Light availability	A	High	High incident light in unshaded habitats	High productivity in absence of water stress	Possibility of photoinhibition and UVB damage	Hairs, cuticle, epidermal pigments, xanthophyll cycle, antioxidants
	W	Low	Less likely to be photoinhibited or photodamaged	Low investment in photoprotection mechanisms	Restriction of photosynthesis and colonization depth	Epidermal chloroplasts
Temperature variability	A	High	Possible promotion of diurnal photosynthesis and restriction of nocturnal respiration	–	Temperature stress, elevated photorespiration at high temperature	Stomatal control of cooling, C_4, CAM
	W	Low	Low risk of temperature stress	–	–	–
Gas diffusion rate	A	High	Rapid gas exchange between leaf and air	High productivity in absence of water stress	–	–
	W	Low	Generation of micro-habitats with high CO_2	Exploitation of particular habitats	Restriction of photosynthesis by CO_2 and O_2 storage	CCMs: HCO_3^- use, C_4, CAM

(continued)

Table 11.1 (continued)

Feature	Env	Attribute	Environmental opportunity	Response to opportunity	Environmental challenge	Response to challenge
O_2 availability	A	High	No restriction of aerobic respiration	–	Photorespiration risk	C_4, CAM
	W	Low	Potential reduced photorespiration	–	Reduced O_2 supply to below-ground organs	Lacunal connection shoot to root, pressurised ventilation
Inorganic carbon availability	A	High	Relatively constant, not strongly limiting	–	–	–
	W	Low	High CO_2 in some habitats/ sites & HCO_3^- present	Exploitation of particular habitats; use of HCO_3^-	Highly variable and can be depleted to near zero	CCMs: HCO_3^- use, C_4, CAM
Nutrient solubility	A	Low	–	–	Supply largely from soil water	Vessels to supply nutrients to leaf taken up by roots
	W	High	Nutrients available to leaves and roots	Shoot and root uptake	Competition with algae	Allelochemicals

(Sand-Jensen et al. 2007), and can also control the overall productivity of some freshwater systems (Karlsson et al. 2009). Although aquatic environments are essentially shade environments, wave focussing at the water surface can produce bursts of high light with a duration of milliseconds (Schubert et al. 2014). These differ from sunflecks, well-known in terrestrial vegetation (Pearcy 1990), in producing levels of surface light that can be five-times greater than surface light and consequently have the potential to cause photoinhibition and photodamage.

A fourth difference is the heat capacity of water that is around 3500-times greater than that of air on a volume basis (Maberly and Spence 1989). This tends to reduce the magnitude of diel temperature variation in water compared to air. Also, because in fresh water, ice is slightly less dense than liquid water at 4 °C, it forms at the surface in winter, thereby protecting freshwater plants from freezing in all but the shallowest or extremely cold environments.

A fifth difference relates to the availability of inorganic carbon, a key requirement for photosynthesis (Table 11.1). The solubility of CO_2 in water decreases with temperature, but at 15 °C, the molar concentrations in air and water are similar. In contrast, oxygen is about 30-times less soluble in water than in air and this might suggest that aquatic photosynthesis would be favored over that in air because of reduced photorespiration. Furthermore, CO_2 is the only form of inorganic carbon in air, while in water it is supplemented by bicarbonate (HCO_3^-) and carbonate (CO_3^{2-}). Bicarbonate and carbonate are produced by dissolution of limestone and weathering of silicates (Pagani et al. 2009) and hence their concentration is catchment-dependent and highly variable among fresh waters (Talling 1985), but relatively constant in the oceans. The different forms of inorganic carbon are interconnected by equilibria controlled largely by pH. In fresh water, HCO_3^- is the dominant form of inorganic carbon at pH values between the two carbonate dissociation constants at about 6.4 and 10.4. In seawater, the equivalent dissociation constants are about pH 6.0 and 9.1 and the concentration of HCO_3^- is around 2 mmol L^{-1}, which is about 140-times greater than the concentration of CO_2 at air-equilibrium.

However, these apparent photosynthetic advantages of inorganic carbon in water are not always realized because of other characteristics that reduce inorganic carbon availability. First, although atmospheric concentrations of CO_2 have varied substantially over geological time (Pearson and Palmer 2000), they are relatively stable over decades with current seasonal ranges of the order of 2% of the annual mean and annual growth of less than 1% (Thoning et al. 1989). In contrast, concentrations of CO_2 are highly variable in productive aquatic systems where rates of biological transformation between organic and inorganic carbon can greatly exceed rates of re-supply from the atmosphere, input from rivers, and entrainment from depth. Consequently, inland waters can be both substantially under- or over-saturated with CO_2 relative to the atmosphere at different times of year (Cole et al. 1994; Maberly 1996).

Another major constraint on the supply of CO_2 to photosynthesizing leaves in water is the approximately 10,000 lower rate of diffusion of CO_2 compared to that in air, caused by the greater density of water (Raven 1970). Consequently, a transport limitation is imposed on leaf photosynthesis (Black et al. 1981) causing the $K_{½}$ for CO_2 uptake in water to be between 100 to 200 μmol L^{-1}, roughly six- to eleven-times air-equilibrium concentrations (Maberly and Madsen 1998).

The high solubility of many ions and compounds in water provides an opportunity for nutrients, such as nitrogen and phosphorus, to be taken up by shoots as well as roots. This also produces a challenge because nutrients in the water column are a resource for planktonic and attached algae that may

compete with the plants for these and other resources (Table 11.1). High solubility also allows allelochemicals and chemical signalling to produce an aquatic "smellscape" that affects interactions between different types of organism, potentially altering the competitive balance between plants and algae directly or indirectly via food-web interactions (van Donk and van de Bund 2002; Gross 2003).

III. Response of Leaf Morphology, Structure, and Composition to Aquatic Environments

Section 2 above describes some of the environmental opportunities and challenges faced by aquatic plants (outlined in Table 11.1) and the consequent biological responses are outlined below in Sects. 3 and 4.

A. Leaf Morphology

Aquatic leaves vary hugely in area (Pierce et al. 2012) from the very small leaves of free-floating lemnids with a leaf area of <1 mm^2 as in *Wolfia arrhiza*, through leaves of typical area of around 300 mm^2, to the very large floating leaves of water lilies of over 44,000 mm^2 for *Nuphar alba* and exceptionally nearly 5 10^6 mm^2 in the giant water lily *Victoria amazonica*. The morphology of aquatic leaves is less variable than those of terrestrial plants. A number of different leaf forms are widespread as discussed in classic texts such as those of Arber (1920) and Sculthorpe (1967). For example, in freshwater macrophytes, the 'isoetid' leaf shape is common, comprising short, stubby, cylindrical leaves with a large lacunal volume found in the eponymous lycophyte *Isoetes* (Fig. 11.1a) and also in dicotyledons such as *Littorella*, *Lobelia*, and *Subularia* and monocotyledons such as *Eleocharis*. Dissected whorled leaves are found in many genera of dicotyledon macrophytes, including *Myriophyllum* (Fig. 11.1b), *Limnophila*, and *Ceratophyllum* while dissected leaves are also found in *Cabomb*a and *Ranunculus* (*Batrachium*). Whorled, but mainly entire, leaves are present in monocotyledon genera such as *Egeria* (Fig. 11.1c), *Elodea, Hydrilla*, and *Lagarosiphon* and also in dicotyledon genera such as *Hippuris*. Rarely, large entire leaves are produced as in the monocotyledon *Ottelia* (Fig. 11.1d) and the submerged leaves of the dicotyledon *Nuphar lutea*. Within the large monocotyledon genus *Potamogeton*, there is a diversity of form including larger entire leaves such as in *P. lucens* (Fig. 11.1e), small linear leaves as in *P. maackianus* (Fig. 11.1f), and filiform leaves as in *P. pectinatus* (now *Stukenia pectinata*). Linear strap-like leaves are common, including those of the monocotyledons *Vallisneria* (Fig. 11.1g) and *Sparganium*. For the eudicots at least, small variations in a common development program can produce large changes in morphology (Runions et al. 2017). In seagrasses, all of which are monocotyledons, there is an even lower diversity of leaf-form with linear strap-like leaves being widespread, e.g., in *Posidonia*, *Thalassia* (Fig. 11.1h), and *Zostera*, although some genera contain species with linear leaves, e.g., *Amphibolis* or ovate leaves as in species of *Halophila* (Fig. 11.1i; (Kuo and den Hartog 2006).

Thus, there is a high degree of convergent evolution in the leaf form of aquatic embryophytes, implying common responses to environmental pressures. This is supported by the different leaf morphologies commonly seen in amphibious freshwater plants with terrestrial-like floating or emergent leaves produced in air and typical aquatic leaves produced underwater (Arber 1920; Sculthorpe 1967; Maberly and Spence 1989).

B. Leaf Structure

One of the necessary developments in the invasion of land by aquatic plants was the evolution of stomata, which occurred about

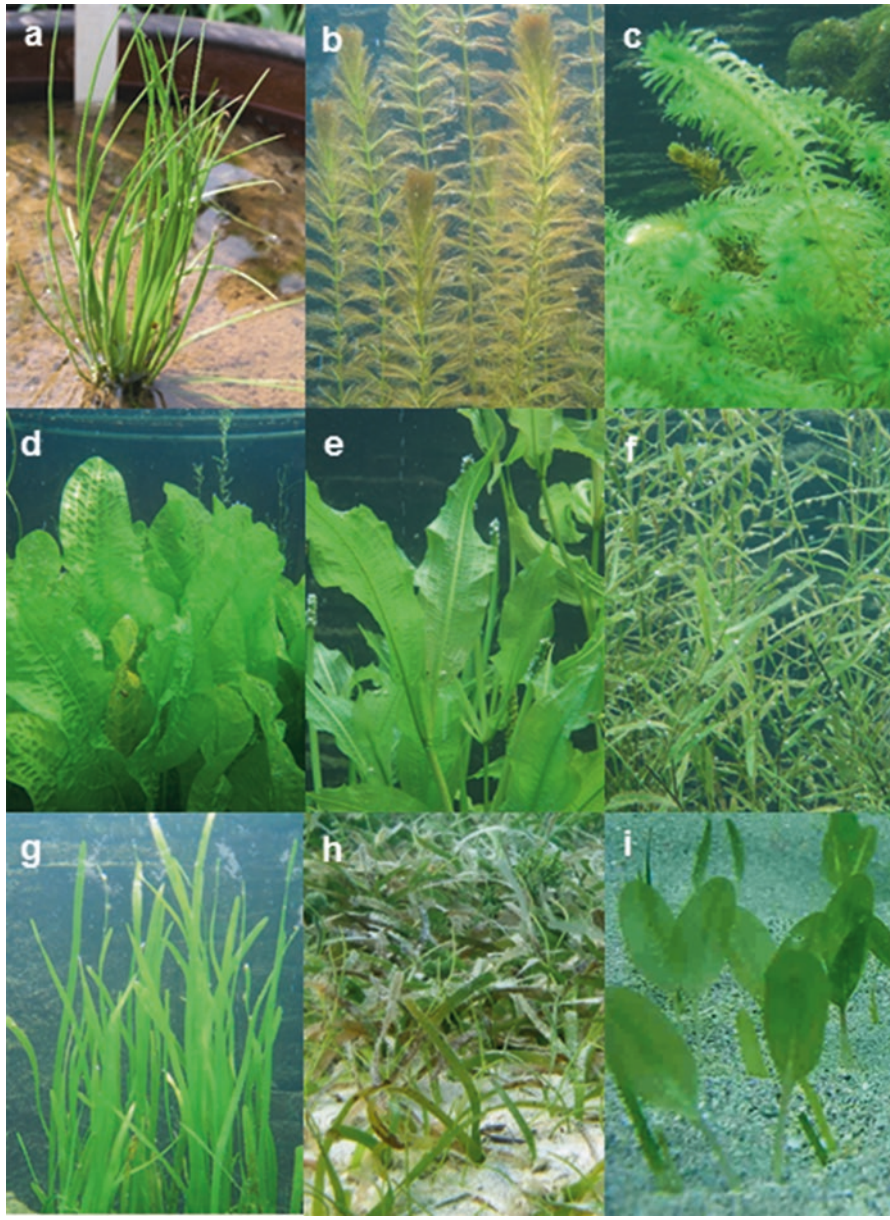

Fig. 11.1. Leaf form in freshwater macrophytes. (**a**) *Isoetes sinensis* (leaves in air), (**b**) *Myriophyllum verticillatum*, (**c**) *Egeria densa*, (**d**) *Ottelia acuminata*, (**e**) *Potamogeton lucens*, (**f**) *Potamogeton maackianus*, (**g**) *Vallisneria spiralis*, (**h**) *Syringodium filiforme* and *Thalassia testudinum*, and (**i**) *Halophila ovalis*. Photograph (**a**) was taken by Qing-Feng Wang, (**h**) by Ole Pedersen, and (**i**) by Marion Cambridge; the remainder were taken by the authors at the Botanical Garden of the Chinese Academy of Sciences in Wuhan

400 million years ago (Edwards et al. 1998; Ruszala et al. 2011). Since stomata control gas exchange including water loss, they are obsolete in aquatic environments. Indeed, most submerged leaves do not possess stomata, or, where they do exist, they are nonfunctional (Sculthorpe 1967). However, leaves of aquatic plants that float at the surface of the water, such as water lilies (e.g., *Nymphaea violacea*), have stomata at the

upper surface (Rudall and Knowles 2013). Stomata are also only present on the upper surface of the floating leaves of the aquatic pteridophyte, *Marsilea*, while stomata are present on both adaxial and abaxial surfaces of its aerial leaves (Lin and Yang 1999). In the seagrass *Zostera marina,* the genome of which has been sequenced (Olsen et al. 2016), the stomatal genes appear to have been lost. However, in another seagrass, *Thalassia testudinum,* paracytic stomata were observed in plants grown under 'stressed' conditions (Benzecry 2013). These contrasting data indicate that the loss of stomata is not universal in the seagrasses and there could be phylogenetic differences since *Zostera* belongs to the Zosteraceae while *Thalassia* is a member of the Hydrocharitaceae.

The lack of, or very low, water stress allows the cuticle to be very thin in many freshwater plants and seagrasses, typically about 0.1 μm (Frost-Christensen et al. 2003; Kuo and den Hartog 2006). It also allows chloroplasts to occur in epidermal cells (Fig. 11.2). This structure is typical of most aquatic plants, some of which have thin leaves with laminae of only two or three cell layers (Fig. 11.2g, h). Both laminar (Fig. 11.2c, d) and cylindrical leaves (Fig. 11.2e, f) can have large lacunal volumes although, unlike the sub-stomatal cavities of terrestrial leaves, these are not directly connected to the external environment. Floating leaves, in contrast, are thick exceeding 0.5 mm with many cells and a dorsi-ventral mesophyll structure similar to terrestrial leaves (Fig. 11.2a) or a homogenous mesophyll structure (Ronzhina and P'Yankov 2001). Within the seagrasses, small species such as *Halophila* (Figs. 11.1 and 11.2) and *Halodule* tend to have thin leaves while larger plants such as *Thalassia* and *Posidonia* tend to have thicker leaves (Papenbrock 2012) Figs. 11.1 and 11.2) presumably because they have to withstand greater hydraulic forces.

Leaf thickness in freshwater macrophytes and seagrasses has been compared to terrestrial plants by Enriquez et al. (1996). The median thickness of these aquatic leaves was about 130 μm while the median thickness of terrestrial herbs was 240 μm. Linked to this, the thickness of leaves of submerged freshwater macrophytes and seagrasses is much less than that of leaves that photosynthesize in air (Fig. 11.3). Since there is a negative relationship between leaf thickness and specific leaf area (m^2 of projected leaf area per kg dry mass; (Vile et al. 2005), specific leaf area tends to be greater in submerged freshwater macrophytes than in terrestrial leaves. An extensive meta-analysis of leaf mass per area, the converse of specific leaf area, was undertaken by Poorter et al. (Poorter et al. 2009). They showed that aquatic leaves, particularly those of freshwater macrophytes, had a much lower leaf mass per unit area than terrestrial leaves (Fig. 11.4). Similar differences can be seen in comparisons of aquatic, floating, and terrestrial leaves (Klančnik et al. 2014). Some of the ecological and physiological consequences of this are discussed in Sect. 4. Leaf construction costs, the amount of energy required to produce a unit weight or area of leaf (Williams et al. 1987), varies with the structure of aquatic leaves and their ecological habitat (Ronzhina and Ivanov 2014). Construction cost was lower in submerged compared to floating leaved rooted hydrophytes and lower still in free-floating leaves. Leaves with large cells had lower construction costs than leaves with more, but smaller, cells (Ronzhina and Ivanov 2014).

Riparian plants close to the air-water interface essentially photosynthesize in air but are frequently inundated and so have to survive, and preferably photosynthesize, during periods of flooding. Some species have hydrophobic leaves that trap an air film around them when submerged (Colmer and Pedersen 2008). This has been dubbed a 'plant plastron' by Raven (2008) by analogy with some aquatic insects. The plastron can

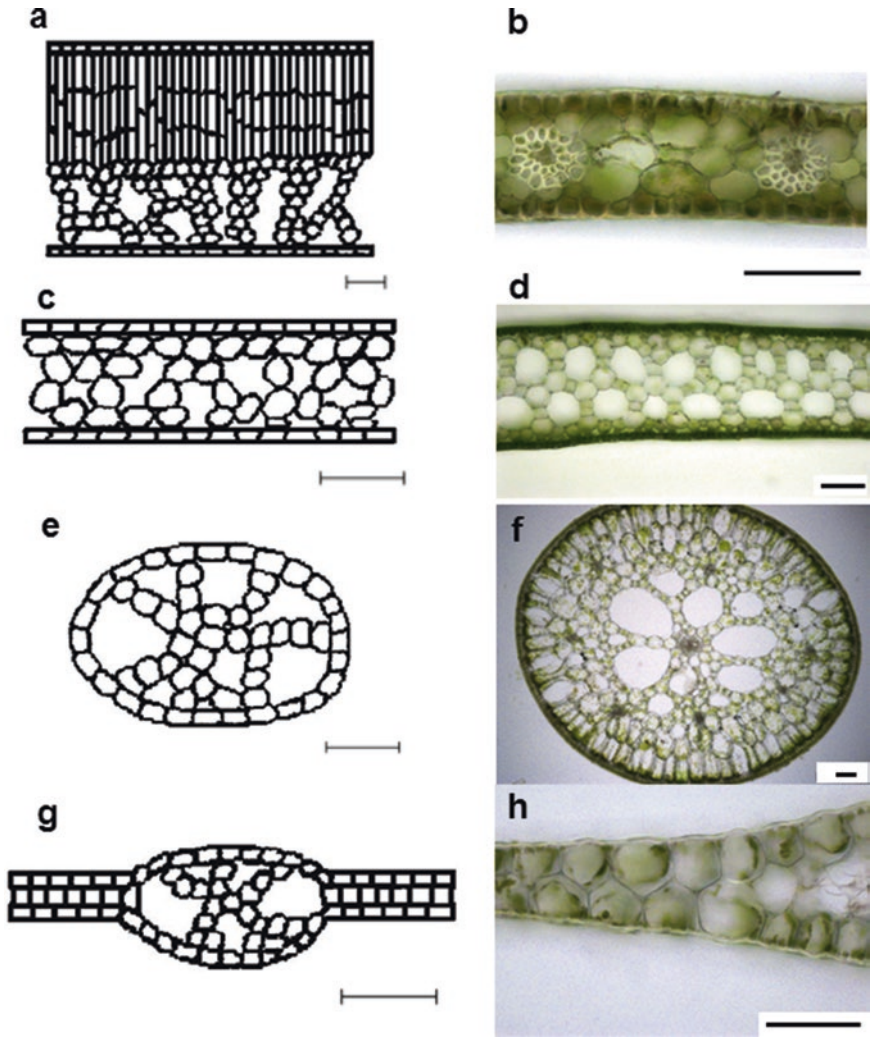

Fig. 11.2. Cross-sections of leaves of freshwater macrophytes (left-hand column) and seagrasses (right-hand column). (**a**) *Nuphar lutea* floating leaf, (**b**) *Amphibolis antarctica*, (**c**) *N. lutea* submerged leaf, (**d**) *Posidonia australis*, (**e**) *Ranunculus trichophyllous subsp. eradicatus*, (**f**) *Syringodium isoetifolium*, (**g**) *Potamogeton compressus*, and (**h**) *Halophila ovalis*. (Panels **a**, **c**, **e** and **g** reproduced with permission from Ronzhina and P'Yankov (2001). Panels **b**, **d**, **f** and **h** courtesy of Lukasz Kotula. Scale bar 100 μm)

increase the rate of CO_2 uptake by leaves in water by up to sixfold and the rate of oxygen influx to the leaf, helping to support respiration in the dark (Verboven et al. 2014; Voesenek and Bailey-Serres 2015).

The low solubility of oxygen in water, coupled with a 10,000-fold lower rate of diffusion, leads to an oxygen supply rate that is 300,000 times lower in water than air (Verberk et al. 2011). This, along with high rates of organic carbon breakdown in some environments such as the sediment, can lead to oxygen concentrations that are at or close to zero. This can prevent aerobic respiration of roots and rhizomes causing toxic products of glycolysis and fermentation, such as ethanol, to form. It can also produce redox shifts in heavy metal ions causing them to become more toxic (Crawford 1992).

The extensive lacunae of aquatic leaves, which are often continuous between shoots and roots, facilitate the transport of oxygen

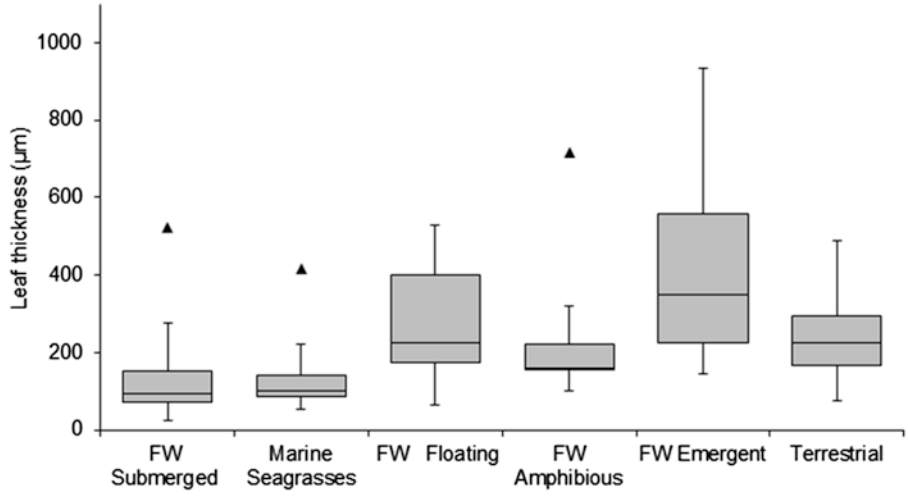

Fig. 11.3. Leaf thickness for leaves from different habitats calculated following Vile et al. (2005) for data from Pierce et al. (2012). Box and whisker plots show the lower and upper quartiles shaded in grey and the median as the horizontal line. The length of the whiskers is 1.5 times the interquartile range. The triangles represent the extreme outliers. Data for freshwater submerged (n = 33), freshwater floating (n = 19), freshwater secondary (n = 9), and terrestrial leaves (n = 506) were derived from (Pierce et al. 2012), data for marine seagrasses (n = 9) were from (Borum et al. 2015), and data from freshwater emergent plants (n = 6) were from (Colmer and Pedersen 2008). *FW* freshwater

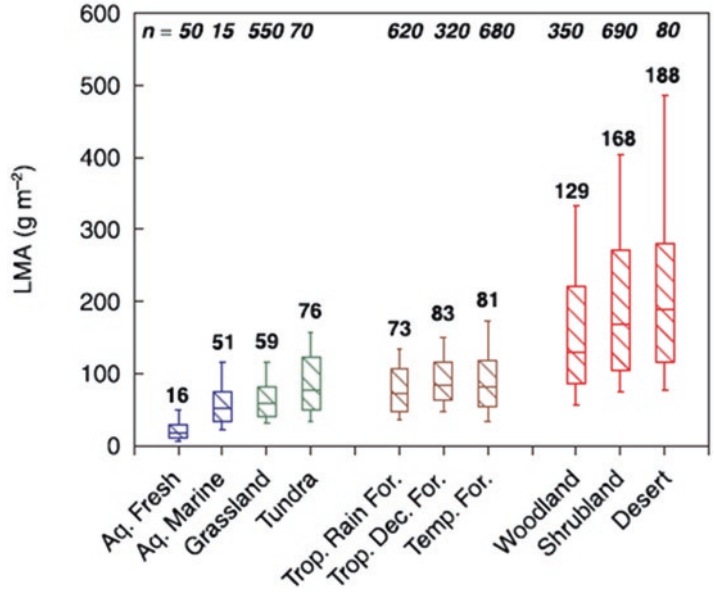

Fig. 11.4. Leaf mass per unit area (LMA, the inverse of specific leaf area) for different types of aquatic and terrestrial leaves. The bottom and top part of the box indicate the 25th and 75th percentile, respectively, the two whiskers the 10th and the 90th percentile, and the horizontal line within the box the median value. The median value is also printed above the box plots. The total number of species present in each functional group or habitat is indicated at the top of the figure. *Aq.* Aquatic, *Trop.* Tropical, *Dec* Deciduous, *For.* Forest, *Temp.* Temperate. (Reproduced with permission from Poorter et al. 2009)

into the sediment (Sand-Jensen et al. 1982; Soana and Bartoli 2013). In aquatic plants with leaves in the air, pressurized ventilation can occur (Dacey 1980, 1981; Grosse et al. 1991). This arises from thermo-osmosis driven by a temperature difference between the ambient environment and a leaf, which forces gas molecules to diffuse into the warmer compartment. The process requires pores that are small enough to generate Knudsen diffusion, around 1 μm, which in the case of *Nuphar lutea* occurs in young, newly emerging floating leaves within a monolayer of cells that separates the palisade and spongy parenchyma (Schroder et al. 1986). The flow, into the young leaves and out via the old leaves, at rates of up to five litres per hour, can supply substantial amounts of oxygen to the roots and rhizomes, helping them to reduce damage in flooded sediments. However, the oxic conditions in the sediment around the roots produced by diffusion or ventilation may decrease the availability of phosphorus through changes in redox state (Wigand et al. 1997) see Sect. 3.3.1).

C. Leaf Composition

Leaf composition is influenced by the growth environment of the plant. Unlike most terrestrial leaves where essential mineral resources derive largely from uptake from the soil, aquatic leaves can access nutrients from the sediment and directly from the water.

1. Nitrogen and Phosphorus

Many early studies focused on the extent to which water or the sediment supplied the nitrogen and phosphorus requirements of aquatic plants (Denny 1980; Barko et al. 1991). Differences in nutrient availability in the sediment porewater and overlying water have a major effect on the contribution of roots and leaves to nutrient uptake. Thus, in conditions typical of nutrient-rich Danish streams, Madsen and Cedergreen (2002) showed that the water could completely satisfy the nutrient requirement of macrophytes since experimental removal of the roots, or enriching the water with additional nutrients, had no effect on the relative growth rate of the four species of plant tested.

Early work established critical average yield-limiting elemental composition for freshwater macrophytes of 0.13 and 1.3 as a percentage of dry mass for phosphorus and nitrogen, respectively (Gerloff and Krombholz 1966). These values represent the lower limits of content (yield-limiting), while rate-limiting contents for phosphorus are about 1.6- and 3-times greater for photosynthesis and growth respectively (Colman et al. 1987) and presumably similar for nitrogen. A survey of nutrient contents of 344 plant samples from 65 rivers and lakes in NE Scotland was undertaken by Demars and Edwards (2007). They found that only a few samples were close to being yield-limited, but many were potentially rate-limited, especially by phosphorus availability. There was a large taxon-specific variation in nutrient content: aquatic bryophytes (lacking roots) had lower tissue contents of nitrogen and particularly phosphorus than vascular plants. This suggests that, in addition to anchoring plants in the sediment, roots may play an important role in supplying nutrients to the plant when concentrations in the water are low. Furthermore, macrophytes can store excess phosphorus and levels exceeding 1% of dry mass have been recorded (Thiébaut 2008). These reserves can then prevent or reduce phosphorus limitation when demand exceeds supply. Overall, the flexibility afforded by alternative nutrient supply, from sediment or water, may be one reason why nutrient limitation appears not to be a major factor controlling the growth of freshwater plants.

2. Cell Walls

Cellulose, hemi-cellulose, and pectin are major components of angiosperm cell walls, as is lignin in terrestrial plants. Lignin is a complex phenolic polymer that increases mechanical strength and reduces the permeability of the vessel walls that transport water from the roots to the leaves. The content of lignin varies greatly in angiosperms: grasses contain 5–10% on a dry mass basis and some tropical hardwoods contain more than 40%; an average lignin content of 20% has been estimated for modern land plants (Novaes et al. 2010). Some aquatic species have a similar lignin content to modern land plants, however in many it is lower, although highly variable among species, ranging between 0.3% and 20% for aquatic species and between 2% and 14% for wetland species (Schoelynck et al. 2010). For example, the lignin content in submerged *Berula erecta* is only 0.8–1.4% while in *Nuphar lutea* it is about 1.2% in submerged leaves and 2% in floating leaves (derived from Fig. 11.1 in (Grasset et al. 2015)).

The cell walls of seagrass leaves and shoots also have low lignin contents (Espineira et al. 2011) and contain sulphated polysaccharides (anionic polymers), like marine macroalgae, but unlike freshwater and terrestrial plants (Aquino et al. 2005; Silva et al. 2012). In seagrasses, sulphated polysaccharides are made up of galactose units (Papenbrock 2012). The carbohydrate sulfotransferases encoding genes appear to be an ancient eukaryotic feature (Collen et al. 2013) that was lost in land plants, possibly because sulphate concentrations in soils are generally much lower than in the ocean, but regained in seagrasses (Olsen et al. 2016). In marine macroalgae, sulphated polysaccharides confer rheological properties such as flexibility (Kloareg and Quatrano 1988) and presumably perform a similar function in seagrasses.

In fresh waters, leaves have direct access to minerals in water and have a higher mineral content than terrestrial leaves (Ronzhina et al. 2009). The silica concentration in fresh water can be high (on average 200 μM in rivers; (Meybeck 1979)) and some freshwater macrophytes use silica in their cell walls since this produces some rigidity at a 10- to 20-fold lower energy cost than the production of lignin or cellulose (Raven 1983). The biogenic silica content varies between 7 and 28 mg g^{-1} dry matter for true aquatic species and between 2 and 14 mg g^{-1} dry matter for wetland species (Schoelynck et al. 2010). Across aquatic and wetland species, there is an antagonist relationship between biogenic silica, lignin, and cellulose content. Interestingly, the freshwater macrophyte *Egeria densa* acclimated for 3 weeks to higher hydrodynamic stress (exposure to 0.5 m s^{-1} water flow compared to low water movement) showed 'thigmomorphogenetic' responses. The composition of silica and lignin in leaves and stems increased at the higher flow, causing a greater resistance to breaking and a greater tensile strength, although flexibility was unaltered (Schoelynck et al. 2015).

There is a varied response of aquatic plants to the mechanical stress experienced in their environment (Schutten et al. 2004; Bornette and Puijalon 2011; Miler et al. 2012). Two broad strategies have been identified to minimize the risk of shoot breakage, 'avoidance' that minimizes the forces experienced by a plant and 'tolerance' that maximizes resistance to breakage (Puijalon et al. 2011). In a survey of 30 species, Puijalon et al. (2011) found an inverse correlation between the two strategies, implying that they are alternatives such that each involves a cost as well as a benefit. For example, 'avoidance' strategies may incur a cost resulting from self-shading (see Sect. 5), while 'tolerance' strategies require a greater resource allocation to structural compounds.

3. Storage Compounds

Starch is a polymer of glucose and a primary product of photosynthesis that serves as a storage compound supporting metabolism

and growth when carbon and energy demands outstrip supply (Fondy and Geiger 1982; Weise et al. 2011). Its metabolism is highly influenced by key environmental factors such as day length, temperature, and nutrient availability (Zeeman et al. 2004). In land plants, starch content is extremely variable and ranges between 1 (e.g., in the fern *Polypodium punctatum*) and 40% (e.g., in the lycophyte *Selaginella rupestris*; (Sharkey et al. 2004). The starch content of freshwater plants is generally between 0.3% and 2.5% (Steinbachova-Vojtıskova et al. 2006; Cao et al. 2011; Grasset et al. 2015; Yang and Liu 2015). In seagrasses, starch contents as high as 14% have been reported (Touchette and Burkholder 2000) representing around 65% of the total non-structural carbohydrate content. For a given species, starch content varies with nutrient content. For example, in *B. erecta* and *N. lutea,* starch content decreased as the habitat nutrient content increased, presumably as starch can accumulate when biomass is limited by availability of nutrients such as nitrogen and phosphorus (Grasset et al. 2015). Ronzhina et al. (2009) found a slightly, but significantly, greater average non-structural polysaccharide (starch and fructosans) content in aquatic compared to terrestrial leaves, possibly caused by the greater lignin and structural polysaccharide content of terrestrial leaves.

4. Regulation of Leaf Form and Structure

Amphibious aquatic plants that live in habitats with fluctuating water levels have to survive and preferably thrive in air and water. Some species produce leaves of similar overall morphology (homophyllous) while others produce leaves of very different appearance in the two environments (heterophyllous; (Maberly and Spence 1989). Leaves of low stature plants, such as the isoetid *Littorella uniflora*, only differ slightly in morphology when growing in air or water. In a study of this species growing on the shores of a reservoir with seasonal fluctuating water levels, Robe and Griffiths (1998) showed that leaves produced in air were nearly twofold longer and 1.6-fold thinner than leaves produced underwater. Submerged leaves had a few stomata that appeared to be non-functional and terrestrial leaves had a tenfold greater stomatal density and 3.7-fold lower lacunal volume.

In heterophyllous amphibious plants, leaves produced in air are often similar to terrestrial leaves with thick cuticles, functional stomata, and sub-stomatal cavities, while leaves produced in water are typically aquatic in morphology and structure. A range of different environmental factors are known to trigger the switch between aquatic and terrestrial leaves (Maberly and Spence 1989; Wells and Pigliucci 2000). Low versus high temperature (Johnson 1967), short versus long daylength (Kane and Albert 1982), high versus low concentration of CO_2 (Bristow 1969), and high versus low water potential (Deschamp and Cooke 1983) favor the production of submerged versus aerial leaves and vice versa. Phytochrome, affected by the ratio of red to far-red light, is involved in the production of aerial leaves in *Hippuris vulgaris.* Aerial leaves are produced by a low red:far-red ratio in the field and the laboratory even when underwater (Bodkin et al. 1980). This mechanism of sensing the environment is not present in the seagrass *Z. marina*, which has lost the genes coding for phytochrome production (Olsen et al. 2016), but appears to be present in the brackish water seagrass *Ruppia maritima* that responds to different red:far-red ratios (Rose and Durako 1994).

Some or all of these responses to environmental cues are probably mediated by hormones. Gibberellic acid often promotes the formation of submerged leaves, while abscisic acid, frequently produced in response to water stress, often promotes the formation of aerial leaves (Maberly and Spence 1989; Wells and Pigliucci 2000). Another plant hormone, ethylene, is involved in shoot elongation, a flooding response in

some species including *Callitriche platycarpa*, *Ranunculus sceleratus*, and *Rumex palustris* (Jackson 1985). *Potamogeton pectinatus* (*Stukenia pectinata*) lacks the ability to produce ethylene, but responds to exogenously supplied ethylene (Summers and Jackson 1998). The seagrass *Z. marina*, in contrast, appears to lack ethylene responsive genes (Golicz et al. 2015; Olsen et al. 2016) but, apart from tidal fluctuations, seagrasses do not experience the same seasonal and episodic changes in water level as freshwater and riparian macrophytes.

IV. Resource Acquisition and Responses to Aquatic Environments

A. Light Acquisition

The aquatic environment is basically a shade environment because of a reflection loss at the air-water interface and the absorption of light by water and dissolved and suspended material, which can be exacerbated further by growth of epiphytes on leaf surfaces (Sand-Jensen 1977; Sect. 2.2). The rooting depth limit of freshwater macrophytes is controlled by light and varies between 2.2% of surface light in bryophytes, 5% in charophytes and caulescent (elodeid) angiosperms, 12.9% in *Isoetes*, and 16.3% in rosette (isoetid) angiosperms (Middelboe and Markager 1997). The differences among groups are partly caused by the proportional extent of roots with a large respiratory burden in isoetids and the ability of some species, such as caulescent angiosperms, to elongate their shoots at low light and therefore grow into shallower water with higher light levels. Seagrasses only have a depth limit of 16 to 18% of surface light (Lee et al. 2007), although in the generally more transparent marine waters, it has been suggested that this could be caused by the effects of pressure, rather than low light, at depth (Beer and Waisel 1982).

Enriquez (2005) studied the light absorption efficiency in *Thalassia testudinum* and other seagrasses. As expected, leaf light absorption efficiency decreased with pigment content per unit area as a consequence of the package effect. The lower pigment light absorption efficiency of *T. testudinum* compared to the more typical terrestrial leaf of *Mentha aquatica* might be explained by the absence of palisade cells and spongy mesophyll cells in seagrasses. However, the thin, flat leaves of tropical seagrasses were more efficient than *M. aquatica,* perhaps because of greater scattering within the leaf that helps to offset the package effect.

Individual submerged freshwater and marine macrophytes are generally shade-adapted with median compensation points for net photosynthesis (I_c) at a photon irradiance of about 16 μmol m^{-2} s^{-1} and the onset of light-saturation (I_k) at about 130 μmol m^{-2} s^{-1} (Binzer et al. 2006). In a compilation of data from temperate and tropical seagrasses, the median values of I_c and I_k were 30 and 116 μmol m^{-2} s^{-1} respectively (Lee et al. 2007). Freshwater macrophytes from deeper, low-light environments are more shade-adapted than those from shallower water (Spence and Chrystal 1970a, b). The genome of the seagrass *Z. marina* contains ten genes for LHCB1, one of three proteins that form the trimers of the photosystem II light-harvesting complex. The number of genes for LHCB1 in *Z. marina* is greater than in *Spirodela polyrhiza* and *Arabidopsis thaliana* (Olsen et al. 2016), consistent with enhancing performance at low light. Although individual leaves may be saturated by low levels of light, an individual leaf within a dense stand of plants maybe shaded by other leaves and therefore receive very low light so that the stand may not be fully light-saturated even at maximum light levels (Binzer et al. 2006). Individual leaves or shoots, however, may experience high light and photoinhibition may therefore occur that, although photoprotective, (Adams et al. 2013), will reduce net photosynthesis. When

H. verticillata, grown at moderate light, was exposed to full sunlight for 15 minutes, photosynthesis was photoinhibited by about 50% at an inorganic carbon concentration of 0.6 mmol L^{-1} but was not inhibited at 2 mmol L^{-1} because carbon fixation provided a greater sink for the light energy absorbed (White et al. 1996). When three species of freshwater macrophyte, grown at low light, were exposed to light from 1.4-fold to 14-fold higher, a photoprotective response was triggered that down-regulated their light harvesting machinery (decreased chlorophyll content) and the photosynthetic electron transport chain (Hussner et al. 2010).

Enzymes and molecules that scavenge reactive oxygen species (ROS) are also involved in photoprotection. The amphibious plant *Lobelia cardinalis* relies on the xanthophyll cycle for the pre-emptive dissipation of excitation energy as heat preventing ROS formation (Nielsen and Nielsen 2006). In contrast, another amphibious species, *Nesaea crassicaulis,* when grown in water appeared to lack the xanthophyll cycle but had very high levels of anthocyanin that is a powerful antioxidant and acts as sunscreens and quencher of free radicals a (Nielsen and Nielsen 2006).

Ultraviolet radiation at the water surface can be substantial but it is rapidly attenuated with depth, especially in water with high concentrations of coloured dissolved organic carbon (Morris et al. 1995). However, no significant differences were found in the content of UV-B screening pigments (flavonoids) in floating versus submerged leaves of the amphibious macrophytes *Ranunculus trichophyllum* and *Potamogeton alpinus* (Germ et al. 2002). The authors concluded that, in these species, levels of screening pigments were saturating and sufficient to prevent damage at current or elevated (17%) levels of UV-B. In contrast, differences in UV-absorbing compounds were found on a unit area basis between submerged and floating or emerged leaves of *Sagittaria sagittifolia* and *Ranunculus lingua* (Klančnik et al. 2014). In the seagrass *Z. marina*, in contrast to the LHCB1 genes, a gene involved in UV-B-sensing and triggering photo-protective responses, UVR8 (Rizzini et al. 2011), appears to have been lost (Olsen et al. 2016), although associated genes such as COP1 are present. Olsen et al. (2016) hypothesized that the loss of UVR8 is linked to the attenuation of UV radiation in aquatic environments since UVR8 is present in another Alismatales that floats on the water surface, *Spirodela*. More sequenced genomes are required from a range of different aquatic plants to test this and other hypotheses.

B. Carbon Acquisition

The potential problems of acquiring inorganic carbon in aquatic environments are outlined in Sect. 2.2. Aquatic embryophytes exhibit a large range of anatomical, morphological, biochemical, physiological, and ecological carbon acquisition strategies to minimize this constraint (Klavsen et al. 2011; Maberly and Gontero 2017). This diversity of strategies reflects the various costs and benefits involved in acquiring inorganic carbon in different aquatic environments and contrasts with terrestrial plants where there are only three carbon dioxide acquisition strategies, all based on carboxylation enzymes as described below.

1. C_3 Metabolism

Terrestrial and aquatic leaves assimilate CO_2 by a common pathway known as the reductive pentose phosphate pathway, the Calvin-Benson-Bassham cycle or the C_3 cycle. This pathway uses the products of the light reactions of photosynthesis, ATP and NADPH, to produce carbon skeletons that lead to the production of sucrose and starch and involves 13 reactions catalyzed by 11 enzymes. In the first step, ribulose-1,5-bisphosphate carboxylase-oxygenase (Rubisco) carboxylates ribulose-1,5-bisphosphate (RuBP) with CO_2 to produce two molecules of phosphogly-

ceric acid (PGA), each with three carbon atoms. Rubisco can also oxygenate RuBP producing one molecule of PGA and one molecule of phosphoglycolate leading to photorespiration when the concentration of O_2 at its active site is high relative to CO_2 (Bowes et al. 1971; Bowes and Ogren 1972). The terrestrial evolutionary history of aquatic embryophytes means that the properties of their Rubisco enzyme are likely to be more similar to that of their direct land plant ancestors than to the microalgal and macroalgal photoautotrophs (Tabita et al. 2008) with which they grow and compete. Similarly, carboxylation in plants is unlikely to involve other enzymes (e.g., use of carbon monoxide dehydrogenase/Acetyl-CoA synthase; (Tabita et al. 2008; Berg 2011; Raven et al. 2012; Hadj-Saïd et al. 2015; Kroth 2015), in contrast to archaea and eubacteria. In order to be fully active, Rubisco must be activated by a non-substrate CO_2, or carbamylated at the ϵ-amino group of a specific lysine residue at position 201 (numbered from the plant spinach enzyme) with the divalent cation Mg^{2+} (Lorimer and Miziorko 1980). Although the regulatory properties of Rubisco activase have not been examined in aquatic organisms, the regulation of this activase is probably similar to that employed by terrestrial shade plants (Gontero and Salvucci 2014).

While the properties of Rubisco in aquatic plants are likely to be similar to their terrestrial forebears, the levels of activity tend to be lower reflecting the lower resource availability in water than in air. A survey of 21 freshwater plants found a median Rubisco activity of about 150 μmol h^{-1} mg^{-1} Chla (range of 12 to 464 μmol h^{-1} mg^{-1} Chla; (Beer et al. 1991). Rubisco activity was lower in submersed than in emergent freshwater leaves (Beer et al. 1991; Fig. 11.5), in agreement with previous observations (Farmer et al. 1986). Moreover, Rubisco activity from seagrasses was similar to that of submersed freshwater species (Beer et al. 1991). From compiled data for C_3 land plants, the average activity of Rubisco from 11 species was higher with a median of about 220 μmol h^{-1} mg^{-1} Chla (ranging from 80 to 622 μmol h^{-1} mg^{-1} Chla; Fig. 11.5).

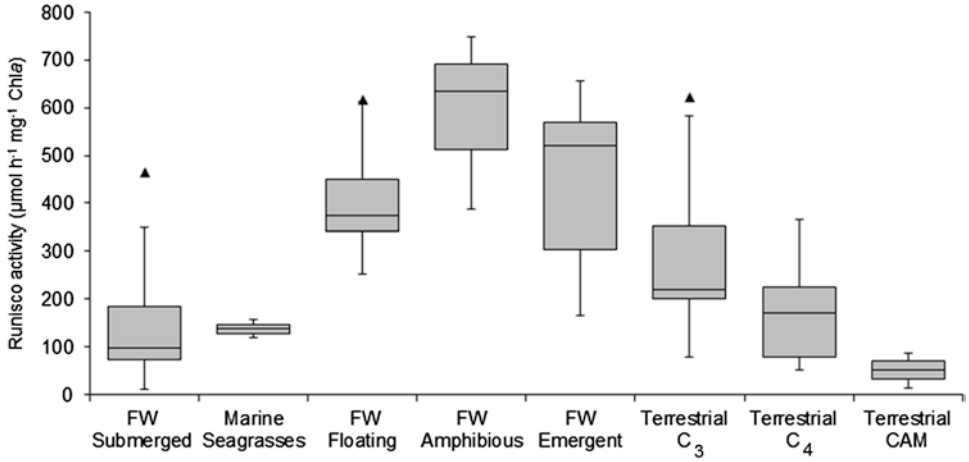

Fig. 11.5. Activity of Rubisco per unit chlorophyll *a* in different plant types. Box and whisker plots show the lower and upper quartiles shaded in grey and the median as the horizontal line. The length of the whiskers is 1.5 times the interquartile range. The maximum outlier is shown by a closed triangle. (Data for aquatic plants from Beer et al. (1991) and terrestrial plants predominantly from Vu et al. (1984). FW = freshwater)

2. Avoidance Strategies

Avoidance strategies (*sensu* Klavsen et al. (2011) are employed by plants that live and grow in microhabitats with locally high CO_2 concentrations, such as above the sediment surface close to sites where decomposition of organic matter produces CO_2, and hence avoid problems caused by low CO_2 availability. For example, the low-growing aquatic moss *Fontinalis antipyretica* is restricted to CO_2 as a carbon source (Bain and Proctor 1980) yet grows in a lake, Esthwaite Water UK, where surface concentrations of CO_2 are close to zero in the summer. It survives by growing just above the sediment surface where concentrations of CO_2 are 2- to 10-times air-equilibrium (Maberly 1985a, b). About 20% of freshwater macrophytes lack a CO_2-concentrating mechanism (CCM) but, based on plant stature, grow close enough to the sediment to have the possibility of benefitting from a high-CO_2 microenvironment (Fig. 11.6).

3. Exploitation Strategies

Exploitation strategies (*sensu* Klavsen et al. (2011)) involve morphological or anatomical features that give access to higher concentrations or higher availability of CO_2. Examples include floating leaves that exploit the more constant and available concentrations of CO_2 in the atmosphere. These can make a major contribution to carbon uptake of amphibious plants (Janauer and Englmaier 1986; Prins and Deguia 1986; Madsen and Breinholt 1995), but they may not be able to prevent overall carbon limitation, so restricting some species to sites with high concentrations of CO_2 (Nielsen and Borum 2008). The high concentrations of CO_2 in the sediment can be exploited by short isoetids, because extensive lacunae in roots and leaves form a continuous pathway for CO_2 to diffuse from the sediment to the leaves. This was first shown by Wium-Andersen (1971) for *Lobelia dortmanna* and can account for a large, but variable proportion of carbon uptake in many isoetids (Raven et al. 1988; Madsen et al. 2002). The rate of diffusion restricts the length of leaf over which this process can occur. Accordingly, Bagger and Madsen (2004) found that leaves were longer in populations of *L. uniflora* growing in sediment with high concentrations of CO_2. The thick cuticle of some isoetids, especially *Lobelia dortmanna*, reduces exchange of CO_2 with the water, but acts to trap CO_2 within the leaf. In *Isoetes australis*, achlorophyllous leaf bases in the sediment, comprising 34% of shoot surface area, are additional important points of CO_2 entry to the photosynthesizing leaves (Pedersen et al. 2011b). Overall, about 30% of the tested species have access to the atmosphere via floating or emergent leaves but only about 3% have access via lacunae to CO_2 within the sediment (Fig. 11.6).

4. Amelioration Strategies

C_4 Metabolism

Amelioration strategies involve additional, energy-requiring processes. Some land plants, mainly those living in high light, hot, dry and/or saline environments, have evolved adaptations in which CO_2 is first fixed by a supplementary pathway, namely crassulacean acid metabolism (CAM) or C_4 photosynthesis. These CCMs elevate the CO_2 concentration around Rubisco, thereby suppressing photorespiration (Giordano et al. 2005; Raven et al. 2011, 2012; Meyer and Griffiths 2013).

In both pathways, the first carboxylation step is achieved by the oxygen-insensitive enzyme phosphoenol pyruvate carboxylase (PEPC) that catalyzes carboxylation of phosphoenol pyruvate with HCO_3^- to yield a four-carbon molecule, oxaloacetate, that is subsequently converted to malate (or aspartate). Malate (or aspartate) is then decarboxylated to produce CO_2 near Rubisco by one of three enzymes: NADP malic enzyme

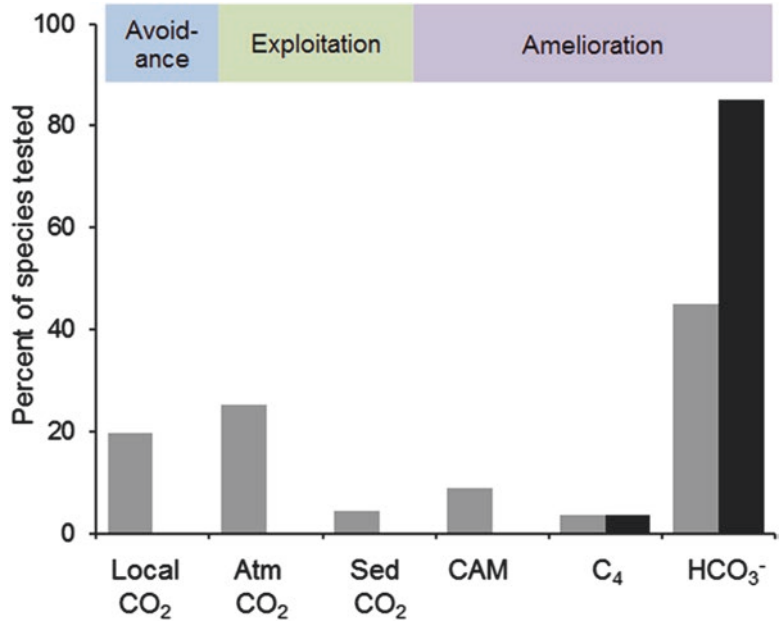

Fig. 11.6. Carbon acquisition strategies in freshwater macrophytes (grey histograms) and seagrasses (black histograms); data from (Maberly and Madsen 2002; Koch et al. 2013; Borum et al. 2015). Local = leaves that have access to elevated levels of CO_2 in their immediate vicinity; Atm = species with access to CO_2 in the atmosphere; Sed = species that receive the bulk of CO_2 from sediments via anatomical conduits and from their own respiratory CO_2; CAM = species that utilize crassulacean acid metabolism to acquire CO_2 during the night for subsequent use in light-driven chloroplast-localized photosynthesis during the day; C_4 = plants that utilize C_4 photosynthesis to acquire CO_2 more efficiently during the day; and HCO_3^- = plants that utilize bicarbonate as a source of carbon for photosynthesis

(ME), NAD-ME, or PEP carboxykinase (PEPCK). In terrestrial plants, C_4 metabolism has long been associated with a radial leaf anatomy (Kranz anatomy), involving two types of cells, to separate physically synthesis of the four carbon acids from decarboxylation in order to prevent futile cycling. Fixation of CO_2 by PEPC occurs in mesophyll cells that surround bundle sheath cells where decarboxylation occurs and where Rubisco is present (Gutierre et al. 1974; Hatch 1987; Edwards et al. 2001). Detailed phylogenetic and carbon isotope studies have resolved C_3, C_4 and C_3–C_4 intermediate relationships within many taxonomic groups and showed that the C_4 pathway of photosynthesis has evolved many times, suggesting that compartmentation is an intrinsic feature maximizing the efficiency of the process (Sage 2004; Sage et al. 2012). Interestingly, about a decade ago, variants of the C_4 pathway within a single cell were discovered in terrestrial plants growing in salty, semi-desert regions, including *Bienertia cycloptera* and *Borszczowia aralocaspica* (family Chenopodiaceae) (Voznesenskaya et al. 2001; Voznesenskaya et al. 2002; Edwards et al. 2004) and more recently *Bienertia sinuspersici* (Offermann et al. 2011). These results show that Kranz anatomy is not essential for C_4 metabolism.

In aquatic systems, a number of 'amelioration strategies' involving CCMs exist to counteract the limitations in inorganic carbon availability (Raven 1970; Maberly and Madsen 1998, 2002; Giordano et al. 2005; Klavsen et al. 2011). It is likely that C_4 photosynthesis arose in aquatic systems, in response to limitations in dissolved CO_2, before its advent in terrestrial ones, based on

PEPCK in the green macroalga *Udotea flabellum* (Reiskind and Bowes 1991), and more controversially, and geologically later, in some marine diatoms putatively based on PEPC (Roberts et al. 2007; Reinfelder 2011; Clement et al. 2016).

Like the single-cell C_4 metabolism in some terrestrial plants, aquatic C_4 metabolism takes place in a single cell. The best characterized freshwater C_4 plant is the submerged aquatic monocotyledon *Hydrilla verticillata* (Hydrocharitaceae); (Holaday and Bowes 1980; Salvucci and Bowes 1981; Bowes et al. 2002). C_4 metabolism in *H. verticillata* is facultative, being induced by various environmental conditions that reduce the availability of CO_2 and promote photorespiration (Reiskind et al. 1997). In *H. verticillata*, physical separation of PEP carboxylation and decarboxylation is achieved within a single cell since PEPC is confined to the cytosol and Rubisco to the chloroplast.

The regulatory patterns and properties of the enzymes involved in C_4 metabolism have been studied in detail in *H. verticillata*. When C_4 metabolism is induced in *H. verticillata*, the activities of PEPC, pyruvate phosphate dikinase (PPDK), NADP–ME, and asparagine and alanine aminotransferases are elevated, while Rubisco activity remains constant (Magnin et al. 1997). One out of the three PEPC isoforms was expressed uniquely in C_4 leaves and transcript levels for this isoform increased (Rao et al. 2002). Genes for two isoforms of PPDK, as well as genes that encode a transporter, an aminotransferase, and two chaperonins, were also up-regulated (Rao et al. 2006). The activity of NADP-ME increased tenfold in C_4 plants (Magnin et al. 1997) and a specific chloroplast isoform (HVME1) was induced. This isoform had specific and unusual regulatory properties that are likely to facilitate C_4 metabolism in this species (Estavillo et al. 2007). Furthermore, the catalytic efficiency of recombinant HVME1 was twofold higher than that of rice but lower than the plastid forms of maize, and the K_m for malate was higher than that for the maize enzyme but fourfold lower than that found in rice. Bowes and co-workers concluded that NADP-ME is the decarboxylating enzyme in *H. verticillata* (Holaday and Bowes 1980). Although both NAD- and NADP-ME increased under limiting CO_2 conditions (Table 4 in Salvucci and Bowes 1981), 95% of the total NAD-ME activity was found in the mitochondrial fraction. Therefore, if NAD-ME was the decarboxylating enzyme, CO_2 would be released in the cytosol causing a futile cycle since PEPC is also present within this compartment. In contrast, the NADP-ME isoform, HVME1, is specifically induced in the chloroplast, the site where CO_2 is concentrated (Reiskind et al. 1997) and it was therefore concluded that it was the main decarboxylating enzyme in this species.

Egeria densa, another species from the Hydrocharitaceae, also exhibits a NADP-ME C_4 syndrome, under high light and high temperature, that can also be triggered by exogenous application of the plant stress hormone abscisic acid (Casati et al. 2000). The kinetic properties of the purified NADP-ME that was induced were similar to those of terrestrial C_3 plants. The partially purified PEPC synthesized during the induction of C_4 photosynthesis had a K_m value (*ca* 8 μM) for HCO_3^- that is lower than those reported in the literature for other PEPC enzymes (Casati et al. 2000). PEPC isoforms are not only induced in leaves (Casati et al. 2000), but also in stems in *E. densa* under low CO_2 conditions (Gu et al. 2015). Similar results were obtained for PPDK and NADP-ME, possibly indicating that the stem could act as a photosynthetic organ as has been described in some terrestrial plants such as tobacco and celery (Hibberd and Quick 2002) and several species of trees (Berveiller and Damesin 2008). As is often the case for an enzyme catalyzing the first step of a pathway, PEPC is finely regulated: phosphorylation and feedback inhibition by malate are well established in terrestrial plants (Chollet et al. 1996) and possibly also occur in aquatic sys-

tems (Casati et al. 2000; Lara et al. 2002; Gu et al. 2015). Recently, two further Hydrocharitaceae, *Ottelia alismoides* and *O. acuminata,* were also shown to exhibit the C_4 syndrome in the absence of Kranz anatomy (Zhang et al. 2014; Shao et al. 2017). When grown at low CO_2, the activity of PEPC and NAD-ME was higher than when grown at high CO_2. If NAD-ME is the decarboxylating enzyme in these species it will be the first reported case for an aquatic plant. Four carbon acids accumulate in other freshwater macrophytes, such as *Elodea canadensis* (Degroote and Kennedy 1977), but there is currently no evidence for turnover and transfer of CO_2 from these metabolites into the C_3 pathway.

In seagrasses, the capacity to perform C_4 photosynthesis seems limited to a few species. Evidence is strongest for *Cymodocea nodosa* (Beer et al. 1980), but *Halophila stipulacea* and *Thalassia testudinum* may have a facultative C_4 metabolism induced by low CO_2 concentrations (Koch et al. 2013; Larkum et al. 2017); further investigation is required to test this possibility. If confirmed, this would be another example of C_4 photosynthesis in aquatic plants lacking Kranz anatomy since there is no indication of Kranz anatomy in any seagrasses (e.g., Fig. 11.2). Presently, about 4% of tested freshwater macrophytes and seagrasses show the capacity for C_4 metabolism (Fig. 11.6).

CAM

The other supplementary pathway in terrestrial plants, CAM, is very similar to C_4 photosynthesis, but PEPC and Rubisco are temporally regulated, the former being active at night and the latter during the day. At night, the four-carbon compound malic acid is stored in vacuoles and decarboxylated during the following day. The features of CAM are therefore: net uptake of CO_2 and accumulation of malic acid during the night, diel change in acidity and starch content of the cells, opposite to one another, a high PEPC activity during the night, and a high PEPC:Rubisco activity ratio (Osmond 1984; Winter et al. 2015).

For decades, CAM was regarded as a water conserving measure in plants from environments with intermittent water availability. The intriguing discovery by J. Keeley that this metabolism was also present in an aquatic plant (*Isoetes howellii*; (Keeley 1981) was first rejected but later confirmed (Keeley 2014). In these plants, there is net CO_2 uptake accompanied by malic acid accumulation during the night (Keeley et al. 1983; Keeley and Busch 1984). CAM has subsequently been found in all other species of *Isoetis* and other submerged macrophytes including *L. uniflora* (Madsen 1987a) and *Crassula helmsii* (Newman and Raven 1995; Klavsen and Maberly 2009). Other freshwater species show some evidence for diurnal acid fluctuations that may be related to CAM (Webb et al. 1988) including *Vallisneria spiralis* (Keeley 1998) and *V. spinulosa*, *Nechamandra alternifolia*, and *E. densa* (Yin et al. 2017). Recently, it was shown that *O. alismoides* (Zhang et al. 2014) and *Deinostema violaceum* (a member of the Plantaginaceae, like *L. uniflora*; (Yin et al. 2017) are able to operate CAM facultatively. CAM has been found in about 9% of tested aquatic species, which is comparable to the percentages estimated for terrestrial plants (Silvera et al. 2010).

Crassulacean acid metabolism is under environmental control in aquatic plants. Typically CAM is up-regulated when leaves are in water and down-regulated when leaves are in air in *Isoetes howellii* and *L. uniflora* (Keeley and Busch 1984; Aulio 1986; Robe and Griffiths 2000); the opposite to expectations for a water-conserving measure and to what occurs in some terrestrial CAM plants (Cushman and Borland 2002). After about 3 days emersion, CAM activity in *L. uniflora* aquatic leaves decreased by 70% and new terrestrial leaves had no CAM activity and exhibited high Rubisco activity (Robe and Griffiths 2000). Recently, using a range of

approaches, including titratable acidity measurements, mRNA levels, and activity measurements of C_3 and C_4 enzymes, it was shown that *Isoetes sinensis* possesses a stronger CAM activity under submerged than under terrestrial conditions (Yang and Liu 2015), although, unlike other species of *Isoetes*, it appeared not to be completely down-regulated in leaves grown in air (Yin et al. 2017). CAM is also down-regulated when *L. uniflora* is grown at low light (43 μmol photon m^{-2} s^{-1} of photosynthetically active radiation) versus high light (450 μmol photon m^{-2} s^{-1}) and CAM activity is reduced when this species is grown at high concentrations of CO_2 (Madsen 1987b). Activity can also change seasonally (Klavsen et al. 2011; Klavsen and Madsen 2012).

CAM in aquatic ecosystems is correctly considered to be a carbon-conserving strategy that increases net inorganic carbon acquisition. For instance, many species with CAM live in soft-water lakes or in shallow seasonal pools of moderate alkalinity where the availability of CO_2 is low (Sondergaard and Sand-Jensen 1979; Keeley et al. 1983; Madsen 1985). Aquatic CAM is beneficial in this habitat because, unlike the situation for many terrestrial CAM plants, uptake of external CO_2 is not suppressed in the light allowing CO_2 to take place over 24 hours and to exploit nocturnal concentrations of CO_2 that are often elevated relative to those during day. A major carbon-conserving advantage of aquatic CAM is its ability to reduce respiratory loss of carbon by converting CO_2 to malate. For example, in *C. helmsii*, CAM activity was equivalent to 74% of night-time respiration in spring and over 200% in summer (Klavsen and Maberly 2009). CAM also has a beneficial effect by increasing rates of photosynthesis and suppressing photorespiration in *Isoetes australis* (Pedersen et al. 2011a).

About 98% of the malic acid that is decarboxylated during the day to produce endogenous CO_2 is re-assimilated in *Littorella* and *Isoetes*. This high efficiency is likely to be related to internal storage in the extensive lacunal system of the leaves, their relatively thick cuticles, and the resistance of the surrounding liquid medium. In these species, the maximum CO_2 concentration in the lacunal air ranged from 0.8 to 1.5%, which is comparable to the values for terrestrial plants (Madsen 1987a). Last, it has been suggested that CAM could function as a nitrogen conserving mechanism because an increased concentration of CO_2 at the active site of Rubisco makes the enzyme more efficient (Sage and Kubien 2003). However, for *L. uniflora* at least, this hypothesis could not be confirmed (Baattrup-Pedersen and Madsen 1999).

There is no suggestion so far that any seagrasses operate a CAM system (Koch et al. 2013). Some accumulation of ^{14}C label in four carbon acids has been detected in *Thalassia hemprichii*, *Thalassodendron ciliatum*, and *Halophila stipulacea* that did not, however, appear to be linked to CAM metabolism (Beer et al. 1980). Nonetheless, more research is warranted to test the possibility that a form of CAM is in operation in some species.

Bicarbonate Use

Bicarbonate is the most abundant form of inorganic carbon in seawater and many fresh waters (Sect. 2.2) (Maberly and Gontero 2017). However, some form of active transport is required to acquire HCO_3^- from the surrounding aqueous medium because the plasmalemma is impermeable to HCO_3^- and the aquatic leaf has a negative internal membrane potential (Denny and Weeks 1970) creating a large electrochemical gradient opposing passive HCO_3^- entry. A widespread mechanism of HCO_3^- acquisition in freshwater macrophytes is the generation of low and high pH at the abaxial and adaxial leaf surfaces respectively (the so-called polar leaf). This mechanism has similarities to that associated with the well-studied banding on the giant cells of some freshwater green

Fig. 11.7. Marl on the upper surface of a *Potamogeton lucens* leaf, indicated by the arrow, growing among *Hydrilla verticillata* and *Myriophyllum spicatum*. Photograph taken by the authors

macroalgae (charophytes) *Chara* and *Nitella* (Lucas and Smith 1973). The formation of marl (calcite) on the upper surface of aquatic leaves of species within certain genera, e.g., *Elodea*, *Egeria*, *Hydrilla* and *Potamogeton* (Fig. 11.7), has been known for decades and studied as a mechanism of carbon uptake by scientists such as Arens, Ruttner, and Steemann-Nielsen in the middle of the twentieth century (see review by Prins (1989). Extensive studies by Prins and co-workers (1980, 1982; Prins and Elzenga 1989) have elucidated the various mechanisms involved. The cells of the lower epidermis often, but not always, have extensively folded plasma membranes with the appearance of transfer cells. The proposed mechanism involves active extrusion of protons at the lower epidermis, aided by the larger plasma membrane area, generating a local reduction of pH (down to pH 4). This converts HCO_3^- into CO_2 within the boundary layer that can then diffuse into the leaf. In order to maintain electrochemical balance, cations are transported through the apoplast from the lower to the upper side of the leaf and a net export of OH^- occurs at the upper epidermis generating high pH values (pH 10 to 11) causing HCO_3^- to be converted to carbonate and calcite to be precipitated the marl seen in (Fig. 11.7). The precipitation of carbonate also leads to the generation of CO_2 close to the cell surface in *Chara corallina* that may be taken up (McConnaughey 1991) and a similar process may occur at the upper leaf surface. This is not the only mechanism involved in HCO_3^- utilization since some species within genera such as *Ranunculus* or *Myriophyllum* are effective users of HCO_3^- (Maberly and Spence 1983) but do not possess the physical leaf features described above, as is also true for *Vallisneria spiralis* (Prins et al. 1980). However, there might still be spatial horizontal patterns of high and low pH that act in a similar way, analogous to the banding in some species of *Chara*. Alternatively, or in addition, HCO_3^--H^+ co-transport might be involved in HCO_3^- uptake, but more work is needed to elucidate the mechanisms involved that are likely to vary among species.

Of the species of freshwater macrophyte tested, about 45% have the ability to use HCO_3^- (Maberly and Madsen 2002; Fig. 11.6). Less extensive work has been performed on HCO_3^- use in seagrasses compared to freshwater macrophytes. Compiled data (Carr and Axelsson 2008; Koch et al. 2013; Borum et al. 2015) suggest that about 85% of the 27 species that have been studied are able to use HCO_3^- (Fig. 11.6). This is greater than the 57% found in the freshwater macrophytes tested, but the marine habitat has a higher HCO_3^- concentration than many fresh waters, so the ecological advantage may be greater in the oceans. Detailed studies of the mechanism of HCO_3^- use in seagrasses have been performed using inhibitors by a number of workers. For example, in *Z. marina* and *Ruppia cirrhosa*, HCO_3^- use mainly involves HCO_3^- dehydration in external acid zones, catalyzed by periplasmic carbonic anhydrase, followed by diffusion into the leaf (Hellblom et al. 2001; Hellblom and Axelsson 2003). In addition, non-catalyzed

HCO_3^- dehydration or direct uptake of HCO_3^- has also been suggested to be involved. In a study of nine seagrass species (Borum et al. 2015), seven were definitely able to use HCO_3^- and of these, five were suggested to operate a mechanism involving external acidification. In the two additional species, internal conversion of HCO_3^- to CO_2 was inferred. External carbonic anhydrase played a role in four of the species with the ability to use HCO_3^- (see Table 3 in Borum et al. 2015). Fewer studies of this nature have been undertaken on freshwater macrophytes.

The more constant concentration of CO_2 in the oceans and the closer coupling of the aqueous CO_2 concentration with the atmospheric CO_2 content makes it possible to assess whether seagrass photosynthesis is currently saturated by inorganic carbon and likely to be increased by changing atmospheric CO_2 content. Borum et al. (2015) found that the rate of net photosynthesis in eight of the nine species they tested, the exception being *Zostera polychlamys*, was limited at pre-industrial concentrations of CO_2 (280 ppm). This suggests that relief of carbon-limitation in future elevated CO_2 environments might alter the competitive interactions among the species in non-light-limited situations. Experiments to determine carbon limitation in stream macrophytes showed that *in situ* rates of photosynthesis were 35% of CO_2-saturated rates for a species unable to use HCO_3^- and 60% for a species able to use HCO_3^- despite the stream having a high CO_2 concentration of about 220 μmol L^{-1} (Madsen and Maberly 1991). A similar type of assessment, but based on growth carried out in two contrasting Danish lakes with lower CO_2 concentrations than the stream, showed that a species restricted to using CO_2 was unable to grow while a species able to use HCO_3^- grew slowly but the rate was stimulated by about 50% when supplied with higher CO_2 concentrations (Vadstrup and Madsen 1995). These experiments, and others like them, show that freshwater macrophytes without the ability to use HCO_3^- can be severely limited by carbon availability at typical environmental concentrations of CO_2 and that although HCO_3^- helps to reduce this it may not remove carbon-limitation completely.

V. Trade-Offs, Synergies, and Future Prospects

The contrasting environmental challenges and opportunities of air and water as environments for photosynthesis and growth have been described in the text and summarised in Table 11.1. These represent responses to a single environmental factor while in reality a leaf has to respond simultaneously to multiple environmental conditions. These may require a trade-off, where one response is optimised for one environmental factor but is less beneficial for another or a synergy where a particular feature has more than one benefit. Consequently, different characteristics are beneficial in different environments, although this is complicated by environmental variability that may alter the cost-benefit balance of a trade-off, and by acclimation at a range of time-scales that will help to optimize fitness by altering morphology and physiology. It is this interaction among environmental conditions, characteristics, trade-offs, and synergies that control fitness and the ecological distribution of species. In this penultimate section, three examples of different possible types of trade-offs, and three examples of synergies, are discussed.

A. Trade-Offs

Structural or physiological characteristics typically have energy or opportunity costs as well as fitness benefits. As a result, they confer an advantage under some, but not all, environments. As a first example, the flexible nature of many aquatic plants reduces the mechanical stress they experience in flowing

water and thus reduces the risk of shoots breaking or being uprooted. However, the reconfiguration of shoots can have negative consequences by increasing self-shading and therefore reducing light availability to the plant (Sand-Jensen 1998).

A second example concerns the morphology of many aquatic leaves. Madsen (1991) showed that entire, as opposed to dissected, leaves were more common in oligotrophic compared to eutrophic lakes and more common in streams and marine systems than in lakes. This was attributed to trade-offs between minimizing boundary-layer thickness, and hence maximising rate of material exchange, and preventing mechanical damage at high flow. In seagrasses, the general pattern of thin leaves in some small species and thicker leaves in larger species is probably linked, at least in part, to a similar trade-off.

A third example relates to the use of HCO_3^- in aquatic plants. This has costs and benefits that find different balances in different environments. The most obvious cost relates to the extra energy required to produce and operate the machinery required for HCO_3^- use (Raven and Lucas 1985; Raven et al. 2008). The relevance of this cost in the 'real world' is supported by the lack of HCO_3^- use in red macroalgae growing in low light environments in the ocean (Maberly 1990) and in bryophytes (Bain and Proctor 1980) that are frequently found at the depth-limit in lakes (Middelboe and Markager 1997). If energy was the only cost, one would expect HCO_3^- to be ubiquitous in optically shallow water, but this is not the case as was observed (Maberly et al. 2015) in a transect down a fairly shallow river fed by a spring with very high HCO_3^- concentrations (>4 mmol L^{-1}). Concentrations of CO_2 were very high close to the source, and the species found there were restricted to those that use CO_2. A few kilometers downstream, where concentrations of CO_2 were much lower but the concentration of HCO_3^- was virtually the same, all the species tested were able to use HCO_3^- (Maberly et al. 2015). A similar response also occurs within a species, *Elodea canadensis*, grown at high light and very high concentrations of CO_2 (2200 μM), down-regulated its ability to use HCO_3^- (Sand-Jensen and Gordon 1986). An additional cost of using HCO_3^- relates to the affinity of using CO_2 (Maberly and Madsen 1998). At a given limiting concentration of CO_2, the CO_2-dependent rate of photosynthesis is around twofold higher in species restricted to CO_2 than in ones also able to use HCO_3^-. This difference appears to be related to a lower internal permeability in species able to use HCO_3^- (Madsen and Maberly 2003). This is teleologically consistent with, although not yet proved to be linked to, the prevention of futile cycling in species able to use HCO_3^-: a low permeability reduces the efflux of inorganic carbon actively taken up by the leaf. It is also possible that species limited to the use of CO_2 have a lower leaf construction cost than those species that also use HCO_3^-. There are virtually no data on this, but in Table 11.1 in Ronzhina and Ivanov (2014), the single submerged species tested that lacks the ability to use HCO_3^-, *Utricularia vulgaris*, had the lowest area-based construction cost. Clearly, this is worthy of further study.

B. Synergies

Some structural and physiological features have a synergistic benefit for more than one environmental challenge. For example, the lack of water stress allows chloroplasts to be present in the epidermis of the leaves of many submerged plants (e.g., Fig. 11.2). This is likely to be beneficial both for the supply of light and carbon although it has been noted (Black et al. 1981) that the large external transport resistance around the leaves of freshwater macrophytes means that internal diffusion resistances are relatively small in comparison.

In a second example, flexible shoots reduce the risk of breakage but also reduce water flow rate within the plant stand and so promote sedimentation. Sediment within a patch has been found to be 4.1 cm above the river bed on average (Sand-Jensen and Pedersen 2008) and since this comprises fine organic sediment, it has a high carbon, nitrogen, and phosphorus content. This may make an important contribution to nutrient availability to plants in streams and rivers that are not already eutrophic (Sand-Jensen 1998). However, in rivers with sufficient nutrients in the water, high organic carbon content in the sediment can cause a negative trade-off by limiting oxygen supply to the roots (Moller and Sand-Jensen 2011) and, in some species at least, reducing the strength of anchorage provided by the roots, increasing the risk that the whole plant might be washed out of the system (Sand-Jensen and Moller 2014).

In a third example, floating or emergent leaves give access to the much more reliable supply of CO_2 in the atmosphere. This is a common feature in many emergent amphibious plants and makes them among the most productive plants in the world (Westlake 1975). Moreover, access to the atmosphere also helps to minimize the common problem of restricted oxygen supply to roots and rhizomes by providing a pathway for atmospheric oxygen to reach roots and rhizomes, especially in species with 'forced ventilation'(Dacey 1980). The transpiration stream may also promote the transport of nutrients from root to shoot at a rate greater than that possible by acropetal transport (Pedersen 1993).

C. *Future Prospects*

As is hopefully apparent from this chapter, there is a large and growing body of information about how aquatic leaves respond to the challenges and opportunities presented by their environment and hence maximize their fitness. There are also some clear knowledge gaps, some of which are highlighted below. Unlike most terrestrial plants (but the C_4/CAM *Portulaca oleracea* is an exception; (Koch and Kennedy 1980)), some aquatic plants can operate more than one CCM or carbon acquisition strategy. There are some apparent, but presently unexplained, associations between the different CCMs in freshwater macrophytes and seagrasses. All species known to have the ability to perform C_4 photosynthesis are also able to use HCO_3^-. While it is true that HCO_3^- is the substrate for PEPC, the link between these two facts is not necessarily straightforward and direct. One can speculate that the association could be linked to the low concentrations of CO_2 and high concentrations of O_2 that can occur in productive beds where C_4 plants may be found. While both processes act as a CCM, the C_4 pre-fixation by PEPC may help to reduce oxygen sensitivity but HCO_3^- provides the continued supply of carbon. In contrast, species known to have the ability to perform CAM are largely restricted to CO_2. It is possible to speculate that species with the ability to undergo CAM tend to rely on a relatively thick cuticle to trap CO_2 produced by decarboxylation of malate within the plant. This might be an effective strategy in species such as the isoetids that can obtain carbon from the sediment where it will also serve to increase internal concentrations of CO_2, but will be detrimental to plants that rely on HCO_3^- in the water. One interesting exception is *O. alismoides* that appears to operate three CCMs, HCO_3^- use, C_4 photosynthesis, and, facultatively, CAM (Zhang et al. 2014; Shao et al. 2017). If these mechanisms are all confirmed to be present, it will raise interesting questions about how these different processes are regulated and interact.

Unlike terrestrial plants, including *A. thaliana*, few aquatic plants have been sequenced, the recently published sequence of *Z. marina* (Olsen et al. 2016) being an exception as are the genomes of two species

of two floating freshwater plants, *Lemna minor* (Van Hoeck et al. 2015) and *Spirodela polyrhiza* (Wang et al. 2014). Genomic sequencing and analysis of more aquatic species and their comparison with terrestrial species will lead to novel phylogenetic and functional insights. Along with analyses of proteomes, transcriptomes and metabolomes, it will dramatically increase our understanding of how aquatic plants respond to their environment at the level of their morphology, physiology, and biochemistry.

VI. Conclusions

The challenges and opportunities faced by aquatic plants photosynthesising in water are very different to those of their terrestrial ancestors photosynthesising in air. This has led to distinctive differences in the morphology, structure and resource acquisition processes of aquatic compared to terrestrial leaves. The contrasting properties of air and water, and the biological characteristics that they trigger, can be useful to highlight fundamental processes. For example, the interrelationships between CCMs discussed above are also relevant to discussions about the evolution of terrestrial C_4 photosynthesis and CAM (Sage 2002). In another example, the discovery of CAM in aquatic plants (Keeley 1981) and the subsequent realization that its function was linked to carbon-conservation, rather than water-conservation, has also illuminated the carbon-conserving function of CAM in terrestrial plants. Within the field of food security, the possibility of introducing genes for C_4 photosynthesis into C_3 crops such as rice (Leegood 2013) has relied in part on the extensive work on the best-characterized plant with single-cell photosynthesis, *Hydrilla verticillata* (Bowes 2011). Aquatic leaves are also part of the global spectrum of leaf and shoot types. In a recent analysis, Díaz et al. (2016) showed that aquatic leaves lie on one of the major axes that they identified. They fell within the group of ephemeral, small plants with low investment in vegetative structures along with small terrestrial ruderals such as *Arabidopsis thaliana*. Recent work that related the size of terrestrial leaves (Wright et al. 2017) to leaf-energy budgets and water-loss could be confronted with data from aquatic leaves where other challenges are likely to be dominant as a result of the physical properties of water.

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Chapter 12

Leaf Photosynthesis of Upland and Lowland Crops Grown under Moisture-Rich Conditions

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Summary

Decreasing soil water potential decreases leaf water potential, causing stomatal closure to reduce water loss and restricting CO_2 uptake. Water stress decreases photosynthesis under intense transpiration even with sufficient soil moisture. Root resistance to water transport (the reciprocal of hydraulic conductance) then controls leaf water potential and photosynthesis.

Stomatal closure occurs earlier in rice (*Oryza sativa*) than in other crop plants. This high sensitivity significantly decreases midday and afternoon photosynthesis even in irrigated fields. The reduction is particularly remarkable in varieties with low hydraulic conductance. Their maximum early-morning stomatal conductance and photosynthetic rates, when the vapor-pressure deficit is small, are small due to reduced leaf water potential. In contrast, stomatal conductance and photosynthetic rates in varieties with high hydraulic conductance are higher in the morning and remain high at midday and in the afternoon. Varieties with high root surface area have high whole-plant hydraulic conductance. High root capacity to transport cytokinins to the shoot can keep leaf nitrogen content high and preserve high photosynthetic rates during senescence. Researchers have detected quantitative trait loci (QTLs) that increase hydraulic conductance and leaf nitrogen content by increasing root surface area. A near-isogenic line carrying such QTLs showed greater photosynthesis, and an introgression line with two QTLs for increased hydraulic conductance had higher stomatal conductance and photosynthetic rates than lines carrying only one QTL. This suggests that photosynthesis can be improved by marker-assisted selection for these QTLs.

Photosynthesis of upland crops is also affected by root system development, which depends on soil moisture. In Japan's monsoon climate, summer crops in rainfed fields show decreased growth during the hotter, drier late summer. Since upland crops develop vigorous shoots with poorly developed roots during the rainy season, they suffer from water stress in late summer, even when soil moisture is available. In soybean (*Glycine max*), this greatly reduces midday photosynthesis and photosynthesis during reproductive growth (when leaf senescence occurs), thereby reducing yield. Winter crops also experience soil moisture fluctuations: mid-March to April is a wet period, approximately 1 month before flowering, when winter grain crops grow rapidly, significantly affecting root and shoot growth. In wheat (*Triticum estivum*), a wet spring can inhibit root growth before flowering, greatly reducing photosynthesis by advancing senescence during ripening, thereby reducing grain yield. Because roots absorb water and nitrogen and synthesize cytokinins, improving drainage will promote vigorous root growth and increase photosynthesis of upland crops.

I. Introduction

A. Plant Responses to Water Stress

Plants have evolved sophisticated mechanisms to balance water consumption and uptake under conditions of intense transpiration and in soils with deficient moisture. These mechanisms include long-term regulation of root and shoot development, short-term adjustment of hydraulic conductance, short-term adjustments of leaf water potential (Ψ_{leaf}) and turgor, and short-term stomatal control of gas exchange (Kramer 1983; Hummel et al. 2010; Christmann et al. 2013; Simonin et al. 2015). Soil water deficits cause large morphological and physiological changes, including reduced expansion of leaves, promotion of root growth (Hsiao 1973; Hsiao and Xu 2000; Saidi et al. 2010), and accumulation of osmotica (Hsiao 1973; Morgan 1992). These changes help plants reduce water stress and continue photosynthesis to support growth and reproduction.

The rate of C_3 photosynthesis is controlled by CO_2 diffusion and biochemical processes

(Sage et al. 2017). The rate of CO_2 diffusion from atmosphere into chloroplasts depends on stomatal resistance (reciprocal of stomatal conductance), which controls the diffusion of CO_2 into the intercellular spaces, and mesophyll resistance (reciprocal of mesophyll conductance; g_m), which controls CO_2 flux from the intercellular spaces to the site of carboxylation in the chloroplast stroma (Terashima et al. 2011). The biochemical process is controlled by the capacity of ribulose 1,5-bisphospate carboxylase/oxygenase (Rubisco) to consume ribulose 1,5-bisphospate (RuBP) and the capacity to regenerate RuBP (Farquhar et al. 1980).

As the soil water potential (Ψ_{soil}) around the roots decreases, so too does Ψ_{leaf}, and leaf stomata therefore close to reduce water loss, thereby restricting the entry of CO_2 and reducing photosynthesis, a phenomenon called "stomatal limitation" (Hirasawa et al. 1989; Chaves 1991; Quick et al. 1992; Kramer and Boyer 1995). Further reduction of Ψ_{leaf} inhibits the biochemical processes of photosynthesis in the chloroplast, a phenomenon called "non-stomatal limitation". The differential impacts that soil water deficits have on photosynthesis and shoot growth result in increased availability of carbon to support root growth and osmotic adjustment so that the plant can continue taking up both water and carbon dioxide (Hummel et al. 2010). In plants growing in a drying soil, hydraulic signals decrease stomatal conductance and leaf expansion without any reduction in Ψ_{leaf} (Zhang and Davies 1990; Davies and Zhang 1991; Buckley 2005; Christmann et al. 2007, 2013; Pintin et al. 2013). Soil water deficits also accelerate senescence, resulting in a decrease in leaf area and photosynthesis (Nooden 1988; Sade et al. 2018).

However, even when growing under moisture-rich conditions, plants may show physiological responses that are similar to responses seen under moisture-deficient conditions. Under intense midday and afternoon transpiration, water will be lost faster than it can be replaced by the roots; as a result, Ψ_{leaf} decreases, leading to stomatal closure, which in turn leads to decreased stomatal conductance and photosynthetic rates. The Ψ_{leaf} reduction depends on the water uptake and transport characteristics of plants as well as on the intensity of transpiration, as explained in the next section.

B. Water Uptake and Transport by Plants

Liquid water moves from soil to leaf in the soil-plant-atmosphere continuum (SPAC; Kramer 1983), where the driving force for water transport is the difference in water potential between the root-surface soil and the leaf ($\Psi_{soil} - \Psi_{leaf}$) and the resistance to water transport (the reciprocal of conductance) of the roots (root resistance, R_{root}), stem (stem resistance, R_{stem}), and leaves (leaf resistance, R_{leaf}) is arranged in series in a plant, as shown in Eq. (12.1):

$$R_{plant} = R_{root} + R_{stem} + R_{leaf} = \left(\Psi_{soil} - \Psi_{leaf}\right) / F \tag{12.1}$$

where R_{plant} is the whole-plant resistance to water transport from root surface to leaf, and F is the water flux across the SPAC. For plants in soil with sufficient moisture, the equation can be simplified as Eq. (12.2) because Ψ_{leaf} should be far lower (more negative) than Ψ_{soil}:

$$R_{plant} = -\Psi_{leaf} / F \tag{12.2}$$

Water moves from the leaf into the atmosphere as vapor, and the driving force for water transport is the difference in the vapor pressure (which is proportional to the vapor concentration) between the leaf and the atmosphere ($e_{leaf} - e_{air}$) (Kramer 1983).

Based on Eq. (12.1), Ψ_{leaf} decreases when Ψ_{soil} decreases at constant F (Slatyer 1967). Ψ_{leaf} also decreases when F increases, even with sufficient soil moisture. The vapor-pressure difference between leaf and air,

which increases with increasing leaf temperature and with decreasing atmospheric vapor pressure, affects F. R_{plant} also affects Ψ_{leaf}: the greater the resistance, the more negative the Ψ_{leaf} that is required to cause water to flow.

Water passes through the epidermis and cortex of the roots, following a radial path, and enters the xylem of the root's stele. It then moves through the xylem to the leaves following an axial path through the stem. Although R_{root} is not always the greatest resistance within the liquid part of the SPAC (Newman 1966; Tinklin and Wheatherley 1966; Boyer 1971; Neumann et al. 1974; Black 1979; Meyer and Ritchie 1980; Hirasawa et al. 1992a; Sack and Holbrook 2006; Koizumi et al. 2007), R_{root} is the most variable component of R_{plant}; for example, it increases greatly with increasing root age (Hirasawa et al. 1992a; Hubbard et al. 2001) and responds strongly to growth conditions (Hirasawa et al. 1992b; Tomar and Ghildyal 1975), because water follows a symplastic path through the root by crossing living membranes along its radial path (Steudle and Peterson 1998; Steudle 2000). Resistance to water transport is usually far smaller in the axial path (towards the leaves) than the radial path; once xylem cells have matured, resistance to water transport in the axial path does not vary except when a xylem embolism occurs (Steudle 2000; Tyree and Zimmermann 2002). At a given water potential difference ($\Psi_{soil} - \Psi_{leaf}$) and a given water flux across the SPAC (F), the shoot's water potential is usually dominated by the drop in water potential across the root (Steudle 2000), especially for plants in a soil with sufficient moisture, as in the case of paddy fields.

In contrast with roots, the hydraulic properties of leaves have received little attention. Recently, they have been investigated intensively in trees, and researchers have clarified that R_{leaf} differs among species and between plants of a given species grown under different conditions (Sack and Holbrook 2006), and that it changes in response to changes in the transpiration rate in some trees and also an herbaceous plant (Simonin et al. 2015). However, it was demonstrated that whole-plant resistance remains constant irrespective of the transpiration rate when a plant transpires intensively in rice (*Oryza sativa*), maize (*Zea mays*), and soybean (*Glycine max*) (Fig. 12.1; Hirasawa and Ishihara 1991). Water movement from the leaf into the atmosphere within the vapor part of the SPAC is controlled by stomatal resistance. When Ψ_{leaf} decreases beyond a certain critical value, stomatal resistance increases and the photosynthetic rate decreases due to limitations on CO_2 uptake (Slatyer 1967; Hsiao 1973).

The water uptake capacity of the root system, which is referred to as the "whole-root hydraulic conductance" (L_{root}) can be written as:

$$L_{root} = L_p A_r \tag{12.3}$$

where L_p is the root hydraulic conductivity (hydraulic conductance per unit root surface area) and A_r is the root surface area. L_p and A_r are strongly affected by soil conditions and plant age (Kramer 1983; Kramer and Boyer 1995) and they also depend on plant species (Yoshida and Hasegawa 1982; Kramer 1983; Steudle 2000).

C. *Individual-Leaf Photosynthesis*

We can characterize individual-leaf photosynthesis in terms of (1) the maximum rate of photosynthesis in a young, fully expanded leaf measured under conditions of optimum temperature, a low atmospheric vapor-pressure deficit, ambient CO_2 concentration, and saturated light intensity (referred to hereafter as the "photosynthetic capacity"); (2) the degree of the midday and afternoon depression in photosynthesis; and (3) the reduction in the photosynthetic rate that occurs during senescence. Each of these factors can affect crop growth and yield. In this chapter, I discuss photosynthesis in crop plants with different productivity from the perspective of water

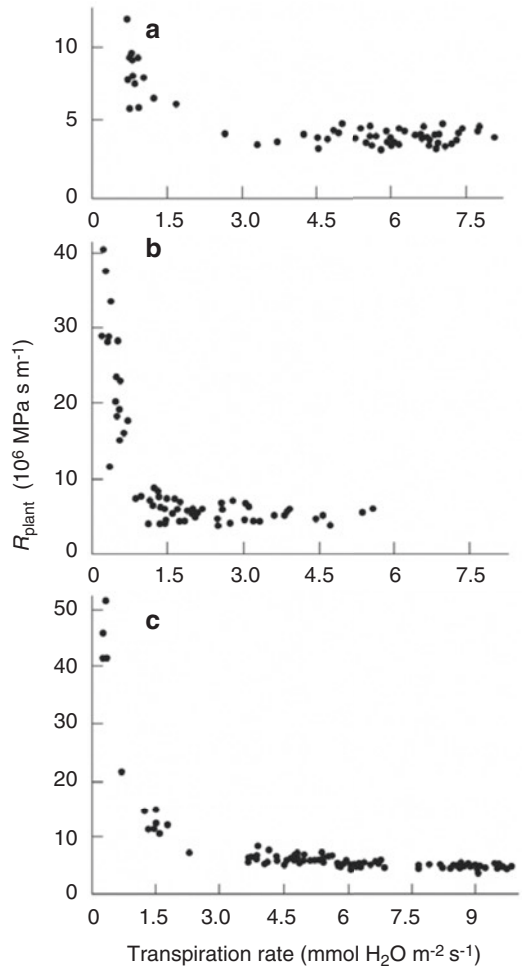

Fig. 12.1. Relationships between the transpiration rate and whole-plant resistance to water transport (R_{plant}) from the roots to the leaves in (**a**) paddy rice, (**b**) maize, and (**c**) soybean grown in wet soil. (Redrawn from Hirasawa and Ishihara 1991). In all three species, R_{plant} becomes constant when the transpiration rate exceeds 1.5–3 mmol H_2O m^{-2} s^{-1}. The resistance between the stem and the leaves is almost constant, irrespective of transpiration rate, compared with R_{plant} in rice. This means that changes in R_{plant} are mostly attributable to changes in root resistance

uptake, transport capacity, and other root functions under moisture-rich conditions.

II. Rice

A. *Characteristics of Rice-Water Relationships*

In paddy rice, the rate of photosynthesis reaches a maximum in the early morning; thereafter, it decreases in response to intense transpiration in the late morning and afternoon on clear days with strong solar radiation (Fig. 12.2a), even though water is not limiting because the rice is growing under flooded conditions (Ishihara and Saito 1987; Jiang et al. 1988b; Asanuma et al. 2008). Rice is a well-understood crop that shows this characteristic midday and afternoon depression in photosynthetic rate (Ishihara 1995). Stomatal conductance follows the same pattern of changes as photosynthesis (Fig. 12.2b), and the intercellular CO_2 concentration (C_i) decreases as CO_2 is consumed by photosynthesis (Fig. 12.2c). This indi-

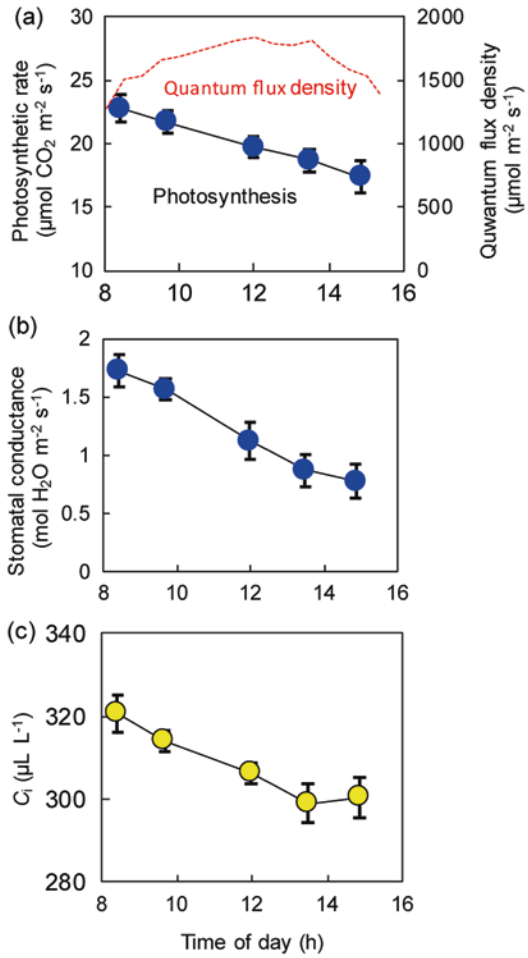

Fig. 12.2. Diurnal changes in the (**a**) rate of photosynthesis, (**b**) stomatal conductance, and (**c**) intercellular CO_2 concentration (C_i) of rice (mean values ± standard deviation, n = 4). (Redrawn from Asanuma et al. 2008)

cates that the reduction in the photosynthetic rate is caused mainly by the reduction in stomatal conductance, which reduces CO_2 uptake and therefore represents a stomatal limitation on photosynthesis (Hirasawa et al. 1989; Kramer and Boyer 1995). In addition, some non-stomatal limitations may contribute to the midday and afternoon reduction in photosynthesis (Ishihara and Saito 1987; Muraoka et al. 2000). Although root hydraulic conductivity is small (Steudle and Peterson 1998; Miyamoto et al. 2001), plant resistance to water transport from the roots to the leaves is not large in rice compared with other crops under the conditions of intense transpiration (Fig. 12.1). This is probably due to the larger root surface area in rice (Yoshida and Hasegawa 1982).

The reduction of Ψ_{leaf} from early morning to midday is usually only 0.1–0.2 MPa in paddy rice based on measurements with a thermocouple psychrometer (Koizumi et al. 2007). Stomatal conductance and the rate of photosynthesis start to decrease immediately after Ψ_{leaf} decreases, and they decrease to approximately 50% of the values that occur before Ψ_{leaf} decreases to −1 MPa (Fig. 12.3a; Hirasawa et al. 1988; Hirasawa 1999). In contrast, in upland crops except for upland rice (Fig. 12.3b, c), the rate of photosynthesis remains high until Ψ_{leaf} decreases to −1 MPa (Hirasawa 1999). Compared with these upland crops, the stomata of rice plants are very sensitive to reductions in Ψ_{leaf} (Hirasawa et al. 1988); this sensitivity causes a significant decrease in the rate of photosynthesis in response to a small reduction in Ψ_{leaf} in the afternoon on clear days, although a stomatal response to one or more signals might also be involved in the sensitivity (Christmann et al. 2013; Deans et al. 2017). Plants with low resistance to water transport could maintain a high rate of photosynthesis during the day because, even under intense transpiration, the reduction of Ψ_{leaf} is much slower in these plants (Fig. 12.4; Hirasawa et al. 1992b; Hubbard et al. 1999, 2001).

B. Photosynthetic Characteristics of High-Yielding Rice

Rice yield increased remarkably in the last half of the twentieth century. The increase in harvest index (the fraction of dry matter production that is allocated to the harvested parts) is far larger than the increase in biomass during the same period (Evans 1993); this indicates that the increase in yield was mainly due to improvement of the harvest index. Recently developed high-yielding rice varieties, released in the Philippines (Peng et al. 2000) and in Japan for manufacturing, bread production, livestock fodder, etc.

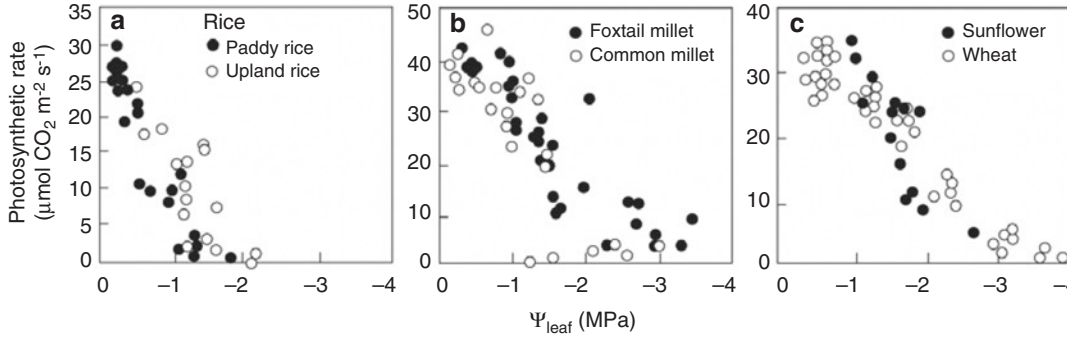

Fig. 12.3. Relationships between leaf water potential (Ψ_{leaf}) and the photosynthetic rate in (**a**) rice and (**b**, **c**) four upland crops: foxtail millet (*Setaria italica*), common millet (*Panicum miliaceum*), sunflower (*Helianthus annuus*), and wheat (*Triticum aestivum*). (From Hirasawa 1999)

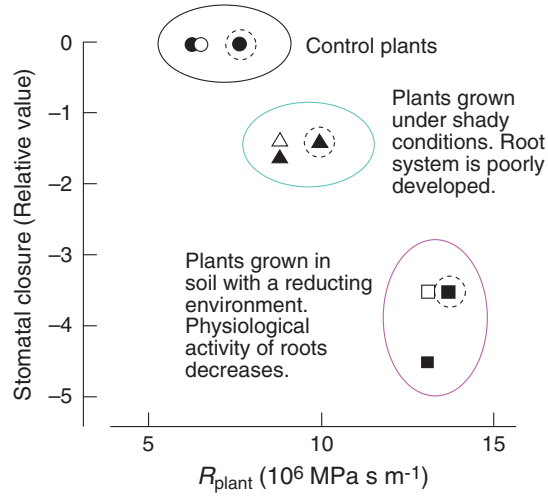

Fig. 12.4. Relationship between whole-plant resistance to water transport (R_{plant}) and the relative degree of midday stomatal closure in rice. (Redrawn from Hirasawa et al. 1992b). Midday stomatal closure is expressed as the difference in the stomatal aperture score measured at midday (from 11 am to 2 pm) on a clear day between control plants and treated plants. Negative values mean that the stomatal aperture was smaller in the treated plants than in the control plants. Changes in stomatal closure were measured on three different days, indicated by black symbols, white symbols and encircled symbols

(Ishihara 1996), yield a significantly heavier dry matter than conventional commercial rice cultivars and older cultivars; this indicates that the increase in dry matter production contributed to their increase in yield. In these cultivars, dry matter production increases significantly after heading (Jiang et al. 1988a; Peng et al. 2000; Asanuma et al. 2008; Taylaran et al. 2009). This, in turn, results in a higher harvest index in some cultivars. A high-yielding cultivar, 'Akenohoshi', derived from a cross between *japonica* and *indica* cultivars, produces grain yield approximately 20% higher than typical commercial *japonica* cultivars (Jiang et al. 1988a). High-yielding 'Habataki' and 'Takanari', derived from crosses between *indica* cultivars, produce grain yield approximately 25% and 40% higher, respectively, than commercial *japonica* cultivars (Asanuma et al. 2008; Taylaran et al. 2009) (Table 12.1). These high-yielding rice cultivars have superior individual-leaf photosynthesis.

Table 12.1 Photosynthetic characteristics of high-yielding rice cultivars compared with those of commercial rice cultivars[a]

Cultivar	Level of yield (brown rice[b], t ha^{-1})	Photosynthesisc capacity (flag leaf, μmol CO_2 m^{-2} s^{-1})	Photosynthetic rate at 1–2 pm (%)[c]	Leaf nitrogen content (flag leaf, %)[c]	Nitrogen accumulation in a plant (%)[c]	Whole-plant resistance to water transport (%)[c]	Root mass (%)[c]	References
Commercial *japonica* cultivars	5–6	22–25	100	100	100	100	100	
'Akenohoshi'	ca. 7	22–25	111	100	118	75	145	Jiang et al. (1988a, b) and Ookawa et al. (2004)
'Habataki'	7–7.5	28–31	106	126	134	50	245	Asanuma et al. (2008) and Adachi et al. (2011a, b)
'Takanari'	ca. 8	28–31	135	125	137	39	202	Taylaran et al. (2009, 2011)

[a]Measurements for all parameters except yield were done at the full heading to early ripening stages. For the commercial *japonica* cultivars, 'Koshihikari', 'Nipponbare', and 'Sasanishiki' were used as the standard of comparison
[b]Yield at the farm of the Tokyo University of Agriculture and Technology
[c]Value relative to that of commercial cultivars (set at 100%)

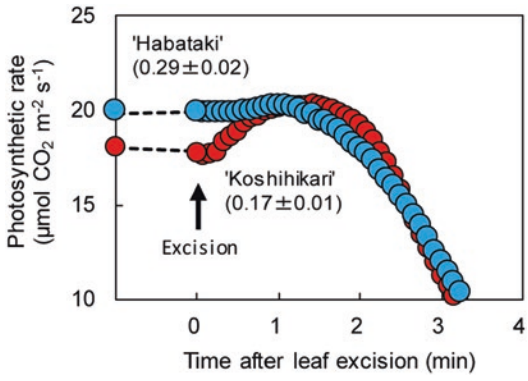

Fig. 12.5. Changes in rates of photosynthesis after excision of a leaf of 'Habataki' (blue circles) and 'Koshihikari' (red circles). (From Adachi et al. 2011b). Each leaf was excised at the base of the leaf blade after leaf gas exchange had reached a steady state. Values in brackets represent the hydraulic conductance (10^{-6} m MPa^{-1} s^{-1}) from the roots to the leaves, expressed per unit leaf area (mean values ± standard deviation, n = 3–5). The leaf–air vapor-pressure difference was approximately 1.5 kPa before leaf excision. Leaf nitrogen content was 1.24 ± 0.11 g m^{-2} for 'Habataki' and 1.31 ± 0.03 g m^{-2} for 'Koshihikari'. Leaf water potential (Ψ_{leaf}) measured with a pressure chamber was −0.32 ± 0.03 MPa for 'Habataki' and − 0.42 ± 0.04 MPa for 'Koshihikari'. This suggests that water stress was more severe in 'Koshihikari' than in 'Habataki' despite the small vapor-pressure difference and that stomatal conductance of 'Koshihikari' increased relative to that of 'Habataki' after xylem tension was released by leaf excision (Colour figure online)

1. *Photosynthetic Capacity*

Compared with the commercial *japonica* cultivars, 'Habataki' and 'Takanari' take up more nitrogen from the soil, have higher foliar nitrogen and Rubisco levels, and exhibit higher capacities for photosynthesis (Asanuma et al. 2008; Adachi et al. 2011a,b; Makino 2011; Taylaran et al. 2011) (Table 12.1). In addition, 'Habataki' and 'Takanari' have root surface areas approximately 2.5 and 2.0 times, respectively, the value for the *japonica* cultivars, and their resistance to water transport from roots to leaves is approximately 50% and 39%, respectively, of the value for the *japonica* cultivars (Asanuma et al. 2008; Adachi et al. 2011a,b; Taylaran et al. 2011). The far smaller R_{plant} keeps Ψ_{leaf} high in the morning, when a relatively low atmospheric vapor-pressure deficit is combined with saturated solar radiation for photosynthesis (Fig. 12.5). This is another reason for the higher photosynthetic capacity in these higher yielding cultivars (Adachi et al. 2011a,b; Taylaran et al. 2011) because rice stomata are very sensitive to the reduction of Ψ_{leaf} (Hirasawa et al. 1989). Compared with the *japonica* cultivars, R_{root} in 'Takanari' is approximately 50% smaller (C. Otsuka, S. Adachi, K. Yoshikawa, T. Ookawa and T. Hirasawa, unpublished, 2015). This makes R_{plant} of 'Takanari' far smaller than that of the *japonica* cultivars (Table 12.1).

2. *Midday and Afternoon Depression of Photosynthesis*

The midday and afternoon reduction in photosynthesis is caused by reduction of Ψ_{leaf} as well as increases in atmospheric vapor pressure deficit (Hirasawa et al. 1988, 1989; Koizumi et al. 2007). The degree of reduction in Ψ_{leaf} depends on the plant's resistance to water transport and on its transpiration rate. Compared to the typical commercial cultivars, 'Akenohoshi' has roots that are approximately 45% heavier and has an approximately 25% lower R_{plant} (Table 12.1). This lets the variety maintain a high photosynthetic rate from midday through the afternoon by maintaining high Ψ_{leaf} (Fig. 12.6). The smaller R_{root} can be attributed to the larger root mass. R_{root} is approximately 55% smaller in 'Akenohoshi' than in the *japonica* cultivars (T. Hirasawa, unpublished, 2016). This decreases R_{plant} of 'Akenohoshi'. These characteristics allow 'Akenohoshi', as well as the similar cultivars 'Habataki' and 'Takanari', to maintain a high rate of photosynthesis from midday through the afternoon, a period when the commercial *japonica* cultivars show reduced photosynthesis (Jiang et al. 1988b; Xu et al. 1997; Asanuma et al. 2008).

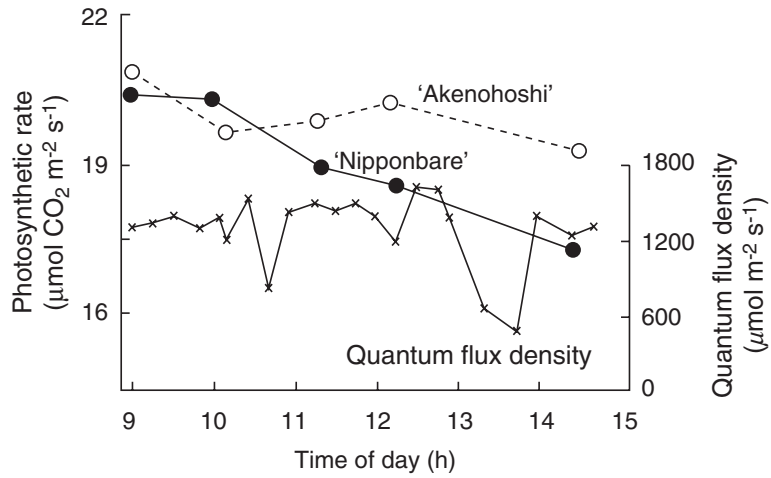

Fig. 12.6. Diurnal changes in the rate of photosynthesis in the high-yielding rice cultivar 'Akenohoshi' compared with commercial Japanese rice cultivar 'Nipponbare' growing in flooded paddy fields. (From Jiang et al. 1988b)

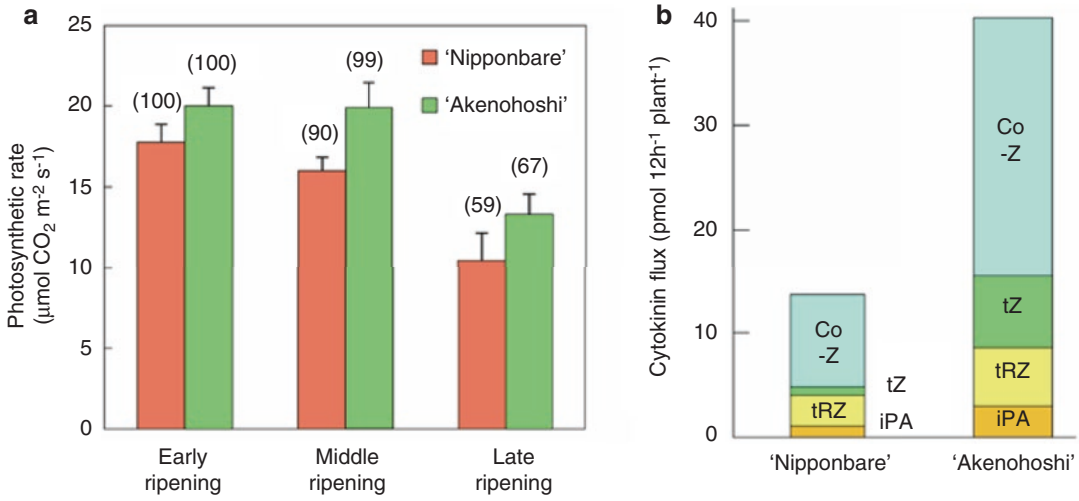

Fig. 12.7. (**a**) Changes in the rate of photosynthesis (mean values ± standard deviation, n = 3) in flag leaves of 'Nipponbare' and 'Akenohoshi'. (Redrawn from Jiang et al. 1988b) and (**b**) cytokinin flux from roots to shoots in 'Nipponbare' and 'Akenohoshi' 17 days after heading. (Redrawn from Soejima et al. 1992). Measurements were for rice growing in a paddy field. In (**a**), values in brackets are percentages relative to those at early ripening. In (**b**): tZ, *trans*-zeatin; tRZ, *trans*-ribosylzeatin; iPA, N^6-isopentenyladenosine; Co-Z, conjugated zeatin

3. *Depression of Photosynthesis During Senescence*

During ripening, when leaf senescence begins, 'Akenohoshi' shows a smaller reduction of the photosynthetic rate than for typical commercial *japonica* cultivars (Jiang et al. 1988b, 1999); Fig. 12.7a shows this difference in comparison with 'Nipponbare', a *japonica* cultivar. More nitrogen accumulates in 'Akenohoshi' than in *japonica* cultivars during ripening (Ookawa et al. 2003; Yamamoto et al. 2017). In addition, cytokinin transport from roots to shoot during ripening is greater in 'Akenohoshi' (Soejima et al. 1992) (Fig. 12.7b). Cytokinins increase

nitrogen transport to the leaves from other organs, and this increases the leaf nitrogen content (Ookawa et al. 2003). Nitrogen transport into a leaf enhances the expression of the Rubisco gene (Imai et al. 2008), thereby increasing the Rubisco content in 'Akenohoshi' leaves during ripening (Ookawa et al. 2004).

Roots are essential for absorbing water and minerals, providing anchorage, and synthesizing plant hormones and organic compounds (Kramer 1983; Waisel et al. 2002), and are therefore essential for plant growth. However, in contrast with our knowledge of the role that shoots play in photosynthesis, the actual role of roots in photosynthesis and plant production has not been quantified. The case studies of high-yielding rice cultivars discussed in this section suggest that root functions related to nitrogen accumulation, water uptake, and cytokinin transport enhance photosynthetic capacity, maintain high photosynthetic rates from midday through the afternoon, and maintain high photosynthetic rates during ripening. Root functions might therefore be a key to improving paddy rice grain yield. However, additional research will be required to quantify these contributions.

C. *Quantitative Trait Loci (QTLs) That Increase Photosynthesis, and Their Functions*

The rate of individual-leaf photosynthesis in rice plants is controlled by several physiological processes (Sage et al. 2017), and enhancement of photosynthetic rate might be possible if key characteristics related to these processes can be improved. Comparisons of cultivars with different photosynthetic rates will provide clues to potential target characteristics for improving photosynthesis. These characteristics are likely to be quantitative traits, so the loci of many important quantitative traits are currently being identified (Yamamoto et al. 2014). If loci for enhancing the photosynthetic rate can be identified, near-isogenic lines (NILs) can be developed to investigate details of how the loci function and their effects on the rate of photosynthesis. Now that sequencing of the whole rice genome is complete (International Rice Genome Sequencing Project 2005), various kinds of materials, including high-yielding cultivars, are being developed for genetic analysis (Ebitani et al. 2005; Ando et al. 2008; Takai et al. 2013, 2014; Yamamoto et al. 2016). The photosynthetic characteristics of previously identified high-yielding cultivars can be confirmed by detecting alleles in the high-yielding cultivars, and the genes responsible for enhancing photosynthetic rate can be introduced in breeding programs by means of marker-assisted selection to improve yield.

1. *Detecting QTLs That Increase the Rate of Photosynthesis by Using Rice Cultivars with a High Photosynthetic Rate*

By analyzing chromosome segment substitution lines derived from crosses between 'Koshihikari' (a commercial *japonica* cultivar) and 'Habataki' (a high-yielding *indica* cultivar), and between 'Sasanishiki' (a commercial *japonica* cultivar) and 'Habataki', Adachi et al. (2011a, 2014) identified four QTLs that enhanced the photosynthetic rate in a plant with the 'Koshihikari' genetic background. The QTLs were detected on chromosomes 4 (*qCAR4*), 5 (*qCAR5*), 8 (*qCAR8*), and 11 (*qCAR11*) (Fig. 12.8). Using NILs that carry a locus for enhancing the rate of photosynthesis, they discovered that a 'Habataki' allele of the locus on chromosome 4 increases leaf nitrogen content and plant hydraulic conductance from roots to leaves by increasing the root surface area, and 'Habataki' alleles of the loci on chromosomes 5 and 11 increase leaf nitrogen content (Adachi et al. 2011a). 'Habataki' has these physiological traits (Table 12.1), which confirms the QTL results. Although a 'Habataki' allele of the locus on chromosome 8 increases plant hydraulic conductance by increasing root hydraulic conductivity (Adachi et al. 2011b), 'Habataki' does not exhibit higher

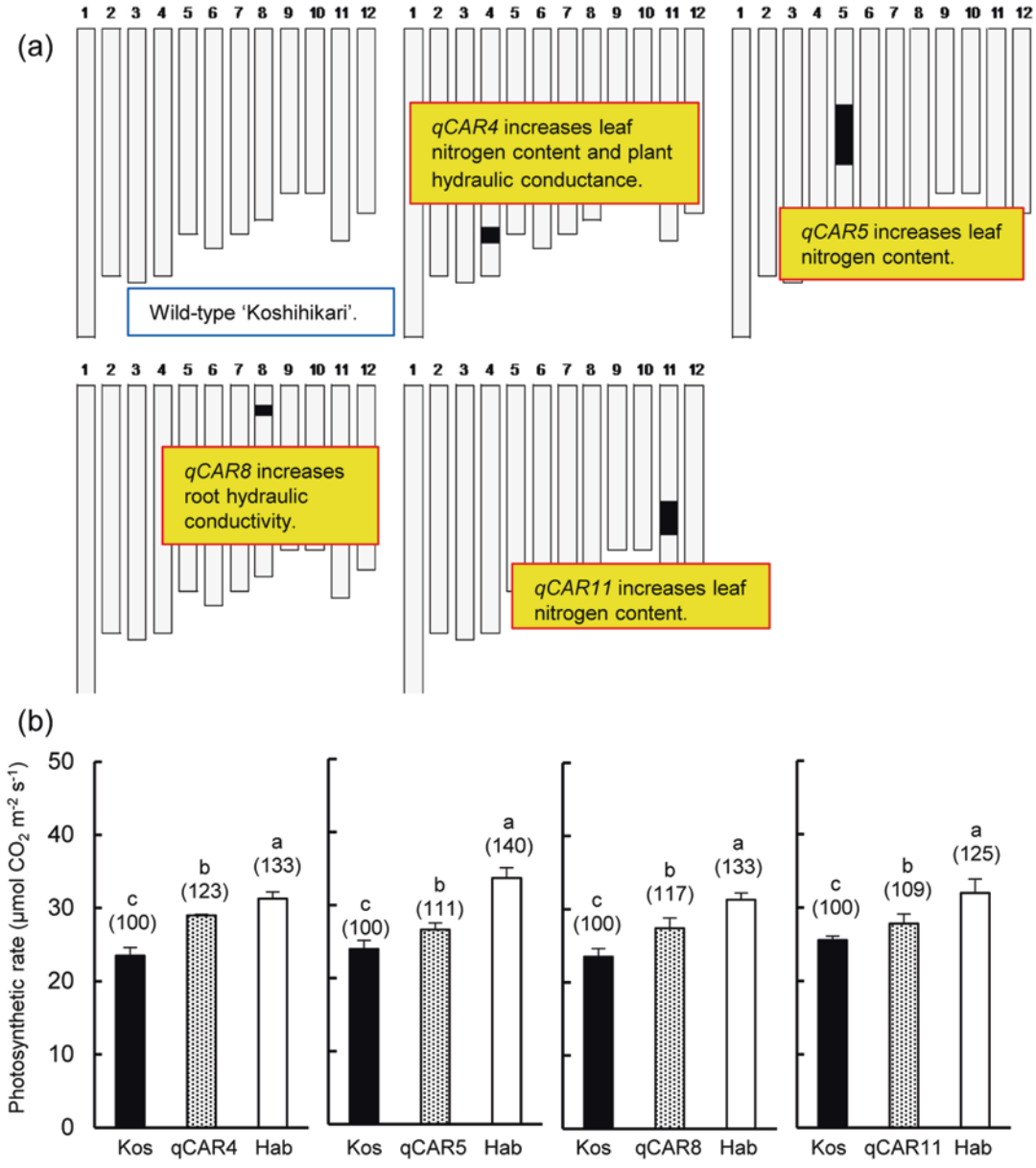

Fig. 12.8. (**a**) Estimated locations and functions of four QTLs, in which the alleles of 'Habataki' (a high-yielding *indica* cultivar) enhance the photosynthetic rate in the 'Koshihikari' genetic background. The QTLs are found on chromosomes 4 (*qCAR4*), 5 (*qCAR5*), 8 (*qCAR8*), and 11 (*qCAR11*). (**b**) The rates of photosynthesis (mean values ± standard deviation, n = 3–5) of near-isogenic lines (NILs) carrying one of the four QTLs. (From Adachi et al. 2014). In (**a**), black bars represent the 'Habataki' chromosome segment and white bars represent the 'Koshihikari' genetic background. In (**b**), Kos and Hab represent 'Koshihikari' and 'Habataki', respectively, and qCAR4, qCAR5, qCAR8, and qCAR11 represent NILs carrying *qCAR4*, *qCAR5*, *qCAR8,* and *qCAR11,* respectively. Values above the bars are the percentages relative to 'Koshihikari' (set to a value of 100%). Bars labeled with different letters differ significantly (Tukey's test, $P < 0.05$)

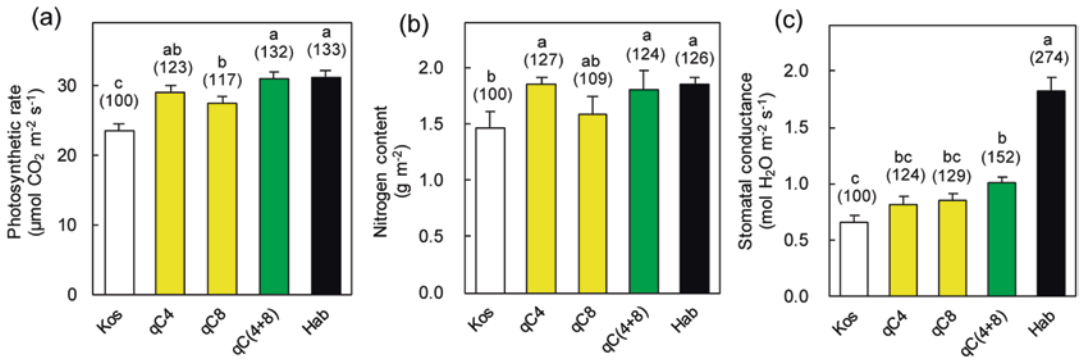

Fig. 12.9. Effects of introgression of QTLs for enhancing photosynthesis on the (**a**) rate of photosynthesis, (**b**) nitrogen content, and (**c**) stomatal conductance of a leaf (mean values ± standard deviation, n = 3–6). (Redrawn from Adachi et al. 2014). Bars labeled with different letters differ significantly (Tukey's test, $P < 0.05$). Abbreviations: NIL, near-isogenic line in the 'Koshihikari' genetic background; Kos, 'Koshihikari'; Hab, 'Habataki'; qC4, NIL(qCAR4) that carries QTL *qCAR4*; qC8, NIL(qCAR8) that carries QTL *qCAR8*; qC(4 + 8), NIL(qCAR4 + qCAR8) that carries QTLs *qCAR4* and *qCAR8*

root hydraulic conductivity; thus, this effect might arise from an interaction between a 'Habataki' allele and the 'Koshihikari' genetic background.

An allele of the high-yielding cultivar 'Akenohoshi' on chromosome 2 increases hydraulic conductance by increasing root surface area (Yamamoto et al. 2016) and an 'Akenohoshi' allele on chromosome 3 in the 'Koshihikari' genetic background maintains a high photosynthetic rate during ripening (Yamamoto et al. 2017). The 'Akenohoshi' allele on chromosome 3 increases nitrogen partitioning to the leaves and maintains high leaf chlorophyll and nitrogen contents during the ripening stage (Yamamoto et al. 2017).

2. *Introgression of QTLs to Enhance Photosynthesis*

In NILs that carry only one of the four QTLs described in the previous section, the photosynthetic rate is significantly smaller than that of 'Habataki' (Fig. 12.8b). The photosynthetic rate is improved by introgression of more than one of the QTLs into rice (Fig. 12.9a): it is higher in NIL(qCAR4 + qCAR8) plants that carry pyramided *qCAR4* and *qCAR8* than in NIL(qCAR4) and NIL(qCAR8) plants, which carry only *qCAR4* or *qCAR8*, respectively. The rate is equivalent to that of 'Habataki' despite having introgression of only two of the four QTLs (Fig. 12.8). The high rate of photosynthesis of NIL(qCAR4 + qCAR8) plants may be attributable to the high leaf nitrogen content (Fig. 12.9b) inherited from NIL(qCAR4) plants and to the larger hydraulic conductance inherited from NIL(qCAR8) plants and (due to the large root surface area) from NIL(qCAR4) plants that contribute to higher stomatal conductance (Fig. 12.9c).

Two rice lines (HP-a and HP-b) with extremely high rates of photosynthesis have been identified among backcross inbred lines derived from the *indica* cultivar 'Takanari' and the *japonica* cultivar 'Koshihikari' ('Kos hihikari'/'Takanari'//'Takanari') (Adachi et al. 2013). The rates of photosynthesis of the two lines at an ambient CO_2 concentration of 370 μmol mol^{-1} are 20% to 50% higher than those of the parental cultivars, and the difference is significant (Fig. 12.10a). Compared with 'Takanari', these lines have neither a higher Rubisco content nor higher Rubisco activity when the leaf nitrogen contents are similar, but they do have significantly higher g_m (Fig. 12.10b) due to the higher density and more highly developed

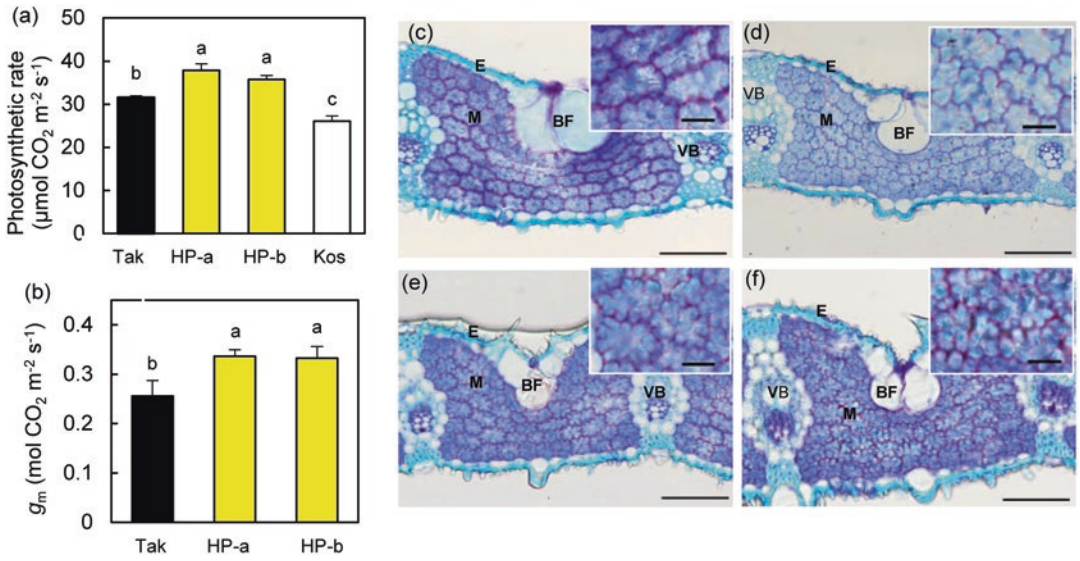

Fig. 12.10. (**a**) Photosynthetic rates (mean values ± standard deviation, n = 3–5), (**b**) mesophyll conductance (g_m, mean values ± standard deviation, n = 3–5), and (**c**-**f**) transverse sections of the flag leaf of (**c**) 'Takanari', (**d**) 'Koshihikari', (**e**) HP-a, and (**f**) HP-b. (From Adachi et al. 2013) Rice lines HP-a and HP-b were identified among backcross inbred lines derived from the *indica* cultivar 'Takanari' and *japonica* cultivar 'Koshihikari' ('Kosh ihikari'/'Takanari'//'Takanari'). Bars labeled with different letters differ significantly (Tukey's test, $P < 0.05$). Abbreviations: *Kos* Koshihikari, *Tak* Takanari, *BF* bulliform cell, *E* epidermis, *M* mesophyll cell, *VB* vascular bundle. Scale bars in (**c**–**f**) represent 50 μm; those in the insets represent 10 μm

lobes of their mesophyll cells (Fig. 12.10c–f). Since the lobes of mesophyll cells are better developed in 'Koshihikari' than in 'Takanari', HP-a and HP-b might have inherited this trait from 'Koshihikari' (Fig. 12.10c–f). Furthermore, the thicker mesophyll layer in these lines might have been inherited from 'Takanari' and the smaller mesophyll cells might have been inherited from 'Koshihikari'. HP-a and HP-b carry at least three to four 'Koshihikari' QTLs for enhancing photosynthetic rate in the 'Takanari' genetic background, and an introgression line carrying these four QTLs has a photosynthetic rate as high as that of HP-a (T. Ochiai, S. Adachi, T. Yamamoto, T. Ueda, T. Ookawa, and T. Hirasawa, unpublished, 2016). These anatomical traits of the mesophyll cells appear in HP-a and HP-b during the reproductive period (He et al. 2017).

A NIL that carries one or more QTLs for enhancing the photosynthetic rate can increase dry matter and grain production if the QTL does not shift heading time to an earlier date, the leaf area smaller, or the canopy extinction coefficient larger (T. Hirasawa, C. Ootsuka, M. Yamashita, S. Adachi, T. Yamamoto, T. Ueda, T. Ookawa, unpublished, 2015).

III. Upland Crops

A. Distribution of Seasonal Precipitation and Water Relations of Upland Crops

Growth of upland crops is strongly affected by soil moisture conditions. The midday reductions in Ψ_{leaf} and photosynthetic rate become pronounced as soil moisture decreases (Slatyer 1967; Tazaki et al. 1980; Tenhunen et al. 1987). It is well known that a plant's rooting depth, rooting density, and hydraulic conductance are important for maintaining high Ψ_{leaf} in the field as the soil dries (Loomis and Conner 1992). A midday

and afternoon reduction in photosynthetic rate has been observed in upland crops (Huck et al. 1983; Quick et al. 1992; Hirasawa and Hsiao 1999) as well as in trees (Pettigrew et al. 1990; Hubbard et al. 1999; Koyama and Takemoto 2014), even when soil moisture was sufficient. This is because the increased atmospheric vapor-pressure deficit that develops from midday through the afternoon decreases both Ψ_{leaf} and stomatal conductance. These reductions are pronounced in plants with decreased hydraulic conductance, as described in the previous section. Under conditions with sufficient soil moisture and moderately deficient soil moisture, R_{root} is both high and highly variable compared with other resistances in a plant (Steudle 2000). Soil moisture conditions affect shoot and root growth differently (Kramer and Boyer 1995), and moisture conditions in an upland soil can change significantly in response to seasonal precipitation and irrigation. Root system development can therefore change more in upland crops than in paddy rice.

Seasonal changes in precipitation, which affect soil moisture, differ among the global climatic zones, and these patterns can significantly affect plant growth. In regions where precipitation is distributed in relatively small amounts and uniformly throughout the year and in regions with low summer rainfall, soil moisture decreases gradually with crop growth during the summer, and crops adjust their growth in response to water stress. Although shoot growth is suppressed under conditions with gradually decreasing soil moisture, root growth is typically enhanced (Sharp and Davies 1979; Kramer and Boyer 1995; Saidi et al. 2010). On the other hand, under moisture-rich conditions due to sufficient precipitation, plant growth adapts to the moisture-rich conditions and plants develop larger shoots and poorly developed roots. Plant productivity and water-stress tolerance might therefore differ between plants grown under moisture-deficient conditions and plants grown under moisture-rich conditions during the vegetative phase.

Japan is located in the Asian monsoon climate region. The annual mean precipitation in Japan is far greater than the annual mean evaporation. Crop plants in Japan seldom suffer from the severe water stress that is frequently experienced by plants in and around the world's arid and semi-arid regions. Nevertheless, the growth of crop plants in Japan is influenced by fluctuations in soil moisture conditions. Figure 12.11 shows the average 5-day precipitation throughout the year in Tokyo. During the growing seasons of summer and winter upland crops, the differences in temperature and precipitation, and their seasonal changes, are pronounced.

Summer crops receive high precipitation from the middle of June to the middle of July. This rainy season is called *baiu* or *tsuyu* in Japanese. Atmospheric moisture conditions change greatly and rapidly in mid-summer after *baiu*. Incident solar radiation and air temperature increase and evaporation greatly exceeds precipitation in midsummer (Hirasawa et al. 1994). *Baiu* coincides with vigorous vegetative growth of summer crops before flowering.

Winter wheat (*Triticum aestivum*) is grown from fall to the next summer in Japan. The internodes of the plants start to elongate and to grow rapidly in mid-March to mid-April, or later in some northern regions. The period from mid-March to April occurs during the wet period known as *natanezuyu* (which means the "rainy season for growing rapeseed"), when precipitation is high and the atmospheric vapor-pressure deficit is small (Nakamura et al. 2003). Wheat plants grow rapidly and vigorously for approximately 1 month before flowering under these wet conditions. After the wet period, the decrease in precipitation is not large, but conditions become somewhat drier because of the higher level of solar radiation, higher temperatures, and larger vapor-pressure deficit until early June.

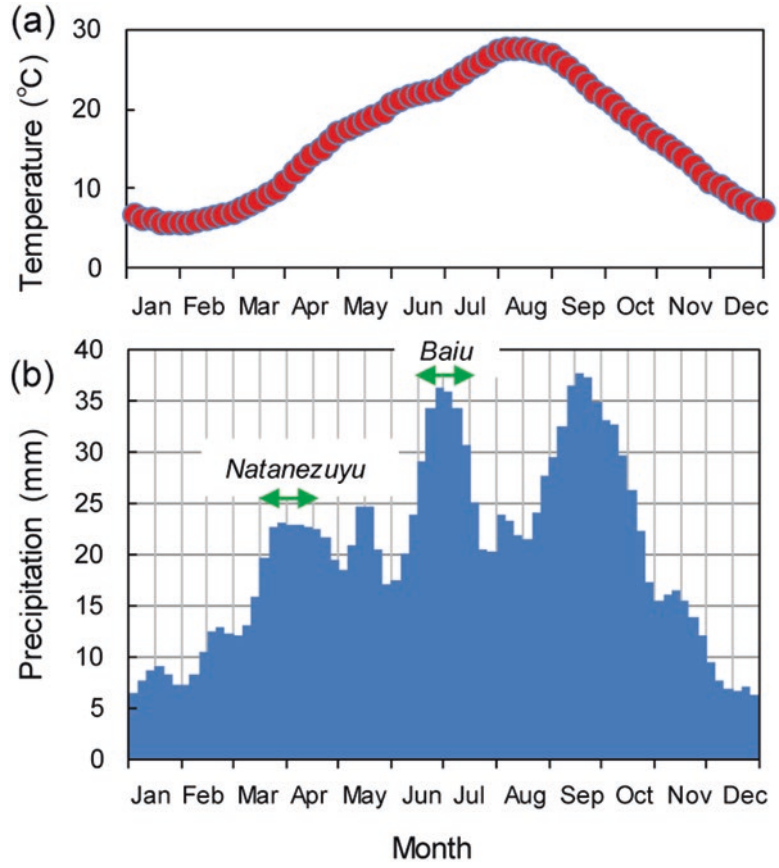

Fig. 12.11. Mean (**a**) temperature and (**b**) precipitation of every 5 days in Tokyo from 1971 to 2000. (Source: National Astronomical Observatory of Japan 2000) Seasons: *baiu* represents the rainy season from the middle of June to the middle of July; *natanezuyu* represents the wet period from mid-March to April

Investigations using soybean (Hirasawa et al. 1994, 1998) and wheat (Nakamura et al. 2003; Nakagami et al. 2004) show that high precipitation before flowering strongly affects photosynthesis and grain filling, as described in sections B and C.

B. Growth Response of Soybeans

The effects of seasonal moisture conditions on soybean growth and photosynthesis (Fig. 12.12) were studied by Hirasawa et al. (1998). After soybean seedlings were established in the field, some plants were grown under deficient soil moisture by withholding irrigation (the D plot) and others were grown under conditions of sufficient soil moisture provided by irrigation two or three times per week (the W plot) during the vegetative stage. Both plots were covered with a rain-out shelter when it rained. At the flowering stage, plants in the D plot were irrigated until the soil moisture reached levels similar to those in the W plot. Thereafter, plants in both plots were grown with additional irrigation whenever soil moisture decreased below a target level.

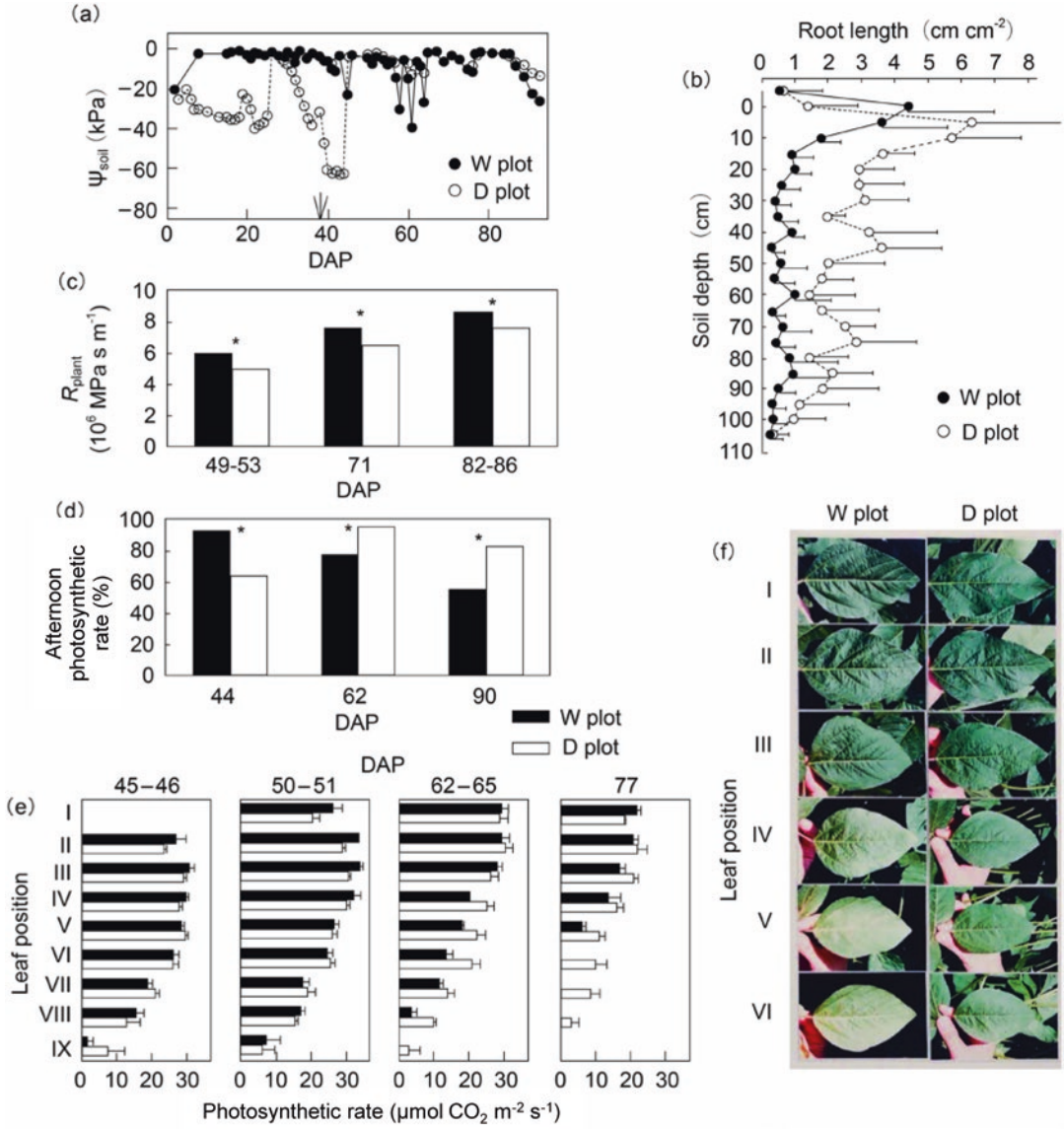

Fig. 12.12. (**a**) Changes in soil water potential (Ψ_{soil}) at a depth of 30 cm from the soil surface, (**b**) vertical root distribution of soybean (*Glycine max* (L.) Merr. cv. 'Enrei') at the time of flowering measured using a minirhizotron root observation tube system (mean values ± standard deviation, n = 5), (**c**) resistance to water transport from the roots to the leaves (R_{plant}), (**d**) afternoon photosynthetic rate (% of the maximum rate in the morning), (**e**) morning photosynthetic rate of leaves at different positions (lower numbers are closer to the flag leaf) on the stem (mean values ± standard error, n = 3–4), and (**f**) photographs of leaves at different positions on the stem approximately 70 days after planting (DAP) in the W plot and the D plot. (Redrawn from Hirasawa et al. 1994, 1998). Soybean plants were planted at the end of June and grown at a density of 16.7 plants m^{-2} in the farm of the Tokyo University of Agriculture and Technology. The soil in the field was classified as a loam or clay loam to a depth of 34 cm below the surface, a light clay from 34 to 44 cm, a sandy loam from 44 to 71 cm, and a light clay from 71 to 120 cm. Fertilizer was applied as a basal dressing at a rate of 30, 44, and 83 kg ha^{-1} for N, P, and K, respectively. Both plots were covered with a rainout shelter when it rained. Plants in the D plot were grown under conditions of deficient soil moisture by withholding irrigation water before flowering. Plants in the W plot were grown with adequate soil moisture by irrigation with approximately 30 mm provided per 3 days during the study period to reflect the average recorded precipitation for the study region. At flowering (45 DAP), the plants in the D plot were irrigated sufficiently until soil moisture became similar to that in the W plot. Thereafter, plants in both plots were grown under natural rainfall conditions and irrigated when soil moisture decreased below a target level. The arrow in (**a**) indicates the date when flowering started. In (**c**) and (**d**), * represents a significant difference between the W and D plots (Student's t-test, $P < 0.05$)

1. Growth and Photosynthesis of Plants in the Irrigated and Water-Deficient Plots Before Flowering

Ψ_{soil} in the W plot remained higher than −10 kPa during the vegetative phase. In the D plot, it decreased gradually to −40 kPa by 25 days after planting (DAP) and finally reached −60 kPa at 40 DAP (Fig. 12.12a). The midday water potential of the leaves decreased significantly more in plants in the D plot. The afternoon reduction in stomatal conductance, and the corresponding reduction in the photosynthetic rate, was also significantly larger in plants in the D plot at 44 DAP (Fig. 12.12d). The stem length and leaf area index (LAI) were smaller in plants in the D plot before and during flowering. On the other hand, roots developed more robustly in the plants in the D plot before flowering (Fig. 12.12b), and the larger root system was retained after flowering until the early ripening (grain-filling) stage.

2. Growth and Photosynthesis of Plants in Irrigated and Water-Deficient Plots After Flowering

R_{plant} increased with increasing time after planting, and was always significantly lower after flowering in the D plot than in the W plot (Fig. 12.12c). Ψ_{leaf} in the afternoon of a clear day after flowering decreased significantly more in plants in the W plot even though both groups of plants were now growing in soil with sufficient moisture. The afternoon depression of the rate of photosynthesis (Fig. 12.12d) and of stomatal conductance were significantly smaller in plants in the D plot. Although the sensitivity of stomatal conductance and photosynthesis to the reduction of Ψ_{leaf} was somewhat greater in this study than in a previous report (Boyer 1970), the significantly higher resistance to water transport in plants in the W plot probably led to the larger afternoon depression in photosynthesis and stomatal conductance through the larger reduction in Ψ_{leaf} caused by this higher resistance.

The photosynthetic rate decreased markedly from 50 DAP onwards in the leaves of the lower and middle positions on the stem and from 62 DAP onwards in the upper leaves as a result of senescence (Fig. 12.12e). The decrease was larger in the leaves at the lower and middle positions on the stem in plants in the W plot. The difference in leaf senescence can be clearly observed visually (Fig. 12.12f).

Seed yield was significantly larger in plants in the D plot (Table 12.2). This might be attributable to the higher photosynthesis during grain-filling in the D plot. In the yield components, the number of fully ripened seeds and the single-seed weight tended to be larger in the D plot, although the difference between plots was not significant.

The plants of the D and W plots were grown under deficient soil moisture conditions during grain-filling by withholding irrigation in the field with a rain-out shelter in another season (Hirasawa et al. 1994). Plants in the D plot also produced more dry matter and a higher seed yield than in the W plot because of (1) their better-developed root system in the deep soil layers, (2) their higher capacity to absorb a large amount of soil water from these deep soil layers, (3) the maintenance of a higher Ψ_{leaf} and, therefore, a higher photosynthetic rate in the afternoon, and (4) a delay in the decrease in the photosynthetic rate due to senescence (Hirasawa et al. 1994).

Similar phenomena have been observed in other upland crops. Ψ_{leaf} decreased below the value at which the photosynthetic rate started to decrease and a reduction of photosynthesis occurred from midday through the afternoon even in continuously irrigated maize (Hirasawa and Hsiao 1999). However, a concommitantly conducted study found only a small midday reduction in the photosynthetic

Table 12.2 Yield and yield components in soybean plants[a] (from Hirasawa et al. 1998)

Plot	No.of pods (m^{-2})	Pod-flower set ratio (%)	No.of seeds (m^{-2})	Single seed weight (mg)[b]	Yield per unit area (g m^{-2})	Stem weight (g m^{-2})
W	663[a]	55.5[b]	1078[a]	271[a]	304[b]	121[a]
D	701[a]	64.5[a]	1199[a]	282[a]	342[a]	117[a]

[a]Plants in the D plot were grown under conditions of deficient soil moisture by withholding irrigation water before flowering. Plants in the W plot were grown with adequate soil moisture by irrigation with approximately 30 mm provided per 3 days during the study period to reflect the average recorded precipitation for the study region. At flowering, the plants in the D plot were irrigated sufficiently until soil moisture became similar to that in the W plot. Thereafter, plants in both plots were grown under natural rainfall conditions and irrigated when soil moisture decreased below a target level. Means followed by the same letter in the same column are not significantly different (Student's t-test, $P < 0.05$)
[b]Seed weight at 15% moisture

rate in maize that was grown without irrigation until a few days before the measurements (Hirasawa and Hsiao 1999). The midday through afternoon reduction in photosynthetic rate was remarkable in sugar beet (*Beta vulgaris*) grown in a highly compacted soil where it had a smaller root-length density than beet grown in less compacted soil (K. Komatsu, T. Hirasawa, S. Hayashi, H. Ito, unpublished, 2003).

C. Growth Response of Wheat

Nakamura et al. (2003) and Nakagami et al. (2004) performed the same experiments for wheat as those described for soybean in the previous section. Wheat plants were grown under natural rainfall conditions until the middle of March. Some plants were then grown under conditions with deficient soil moisture by withholding irrigation water for 1 month until mid-April (the D plot). Other plants were grown with adequate soil moisture by providing irrigation every 3 days during the same period (the W plot) to reflect the average recorded precipitation. At the flowering stage, plants in the D plot were irrigated sufficiently until soil moisture became similar to that in the W plot. Thereafter, plants in both plots were grown under irrigated conditions to reflect the average recorded precipitation. Both plots were covered with a rain-out shelter when it rained. For comparison, wheat plants were also grown under natural rainfall conditions during the entire growth period in an adjacent field (the C plot).

1. Growth of Plants in Irrigated and Water-Deficient Plots Before Flowering

Ψ_{soil} remained higher than −10 kPa during the vegetative phase in the W and C plots (Fig. 12.13a). In the D plot, Ψ_{soil} decreased gradually and reached −50 kPa by mid-April. Midday Ψ_{leaf} decreased to approximately −0.7 MPa in plants in the D plot, but remained near −0.4 MPa in plants in the W plot. Roots tended to develop better in the deeper soil layers at the flowering stage, and root length in the deeper soil layers remained larger at the early ripening stage (Fig. 12.13b) in plants in the D plot.

2. Growth and Photosynthesis of Plants in Irrigated and Water-Deficient Plots After Flowering

Plants in the D plot accumulated more nitrogen and showed greater cytokinin activity in xylem exudates at the flowering stage (Fig. 12.13e). These characteristics of plants in the D plot can be attributed to the delayed leaf senescence resulting from maintaining a high leaf nitrogen content and thereby maintaining high photosynthetic rates until the

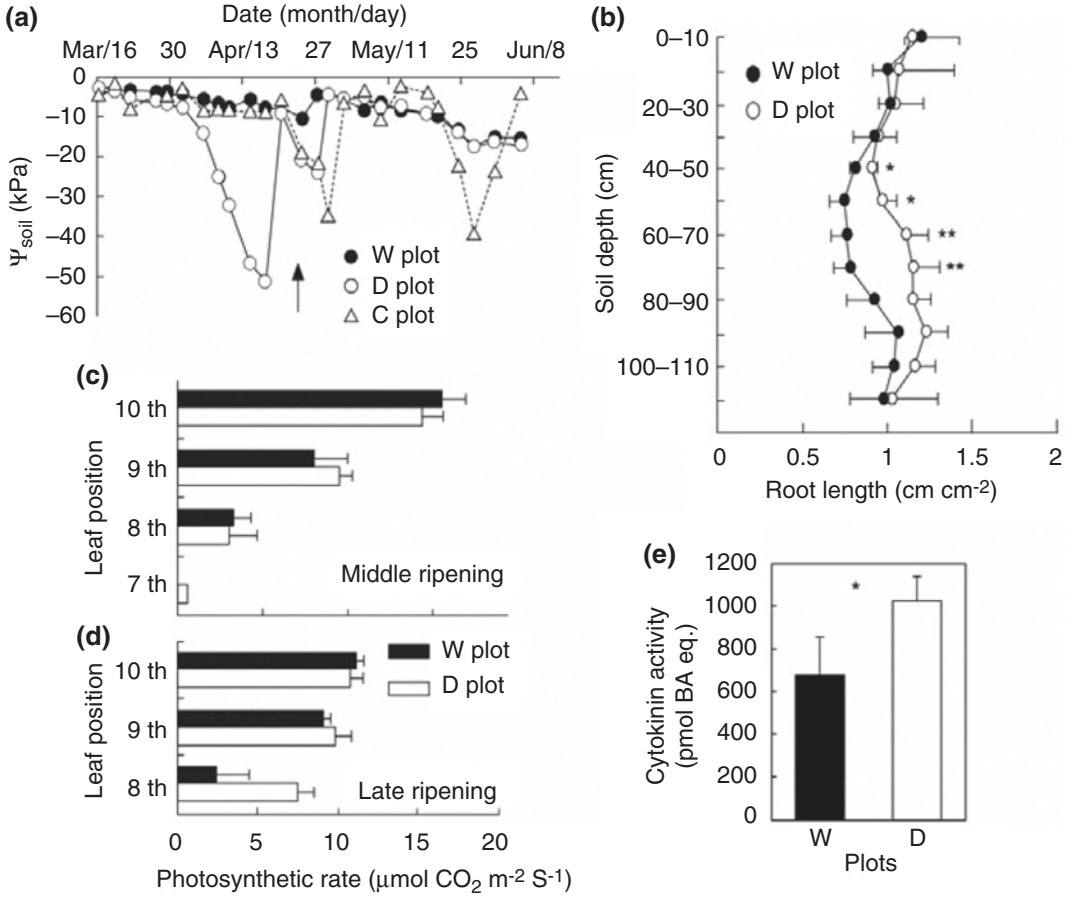

Fig. 12.13. (**a**) Changes in soil water potential (Ψ_{soil}) at a depth of 30 cm from the soil surface, (**b**) vertical root distribution of wheat (*Triticum aestivum* L. cv. 'Bandowase') at the early ripening stage measured using a minirhizotron root observation tube system (mean values ± standard deviation, n = 4), (**c**, **d**) the morning photosynthetic rate of leaves (mean values ± standard deviation, n = 3) at different positions on the stem at the (**c**) middle ripening and (**d**) late ripening stages, (**e**) cytokinin activities of xylem exudates (mean values ± standard deviation, n = 3–4) at the flowering stage in the W and D plots. (Redrawn from Nakamura et al. 2003 and Nakagami et al. 2004). Wheat plants were sown at a rate of 60–80 kg ha^{-1} with a between-row distance of 32.5 cm in mid-November at the farm of the Tokyo University of Agriculture and Technology. The soil in the field was classified as a loam or clay loam to a depth of 34 cm below the surface, a light clay from 34 to 44 cm, a sandy loam from 44 to 71 cm, and a light clay from 71 to 120 cm. Fertilizer was applied as a basal dressing at rates of 100, 44 and 83 kg ha^{-1} for N, P and K, respectively. Plants in the W and D plots were grown under natural rainfall conditions until the middle of March. Thereafter, both plots were covered with a rainout shelter when it rained. Plants of the D plot were grown under conditions with deficient soil moisture by withholding irrigation water from mid-March to mid-April. Plants in the W plot were grown with adequate soil moisture by providing irrigation at 10–12 mm per 3 days during the study period to reflect the average recorded precipitation. The plants in the D plot were irrigated sufficiently from mid- to late April so that the soil moisture became similar to that in the W plot. Thereafter, plants in both plots were irrigated to reflect the average recorded precipitation. White triangles in (a) represent plants grown under natural rainfall conditions during the entire growth period in an adjacent field with the same soil conditions as those in the moisture-controlled field (the C plot). The arrow in (**a**) indicates the date of heading (spike emergence). In (**b**) and (**e**), * and ** represent significant differences between the W and D plots (Student's t-test, $P < 0.05$ and $P < 0.01$, respectively). BA in (**e**) is N^6-benzylaminopurine

Table 12.3 Dry weight of aboveground plant parts at harvest and grain yield in wheat grown under different soil moisture conditions[1)] (from Nakamura et al. 2003)

Year	Plot	Dry weight (g m^{-2})	Grain yield (g m^{-2})[2)]
1994	W	1845[b]	627[b]
	D	2158[a]	735[a]
	C	1730[b]	648[a,b]
1995	W	1308[b]	518[b]
	D	1649[a]	724[a]
	C	1253[b]	589[a,b]

[1)]Treatments: Plants in the W and D plots were grown under natural rainfall conditions until the middle of March. Plants in the W plot were grown with adequate soil moisture by providing irrigation at 10–12 mm per 3 days during the study period to reflect the average recorded precipitation. Plants of the D plot were grown under conditions with deficient soil moisture by withholding irrigation water from mid-March to mid-April and then irrigated sufficiently from mid-to late April such that the soil moisture became similar to that in the W plot. Plants in the C plot were grown under natural rainfall conditions during the entire growth period in an adjacent field with the same soil conditions as those in the moisture-controlled field. Values of a parameter in a given year labeled with the same letters did not differ significantly (Least Significant Difference test, $P < 0.05$)

[2)]Grain yield is given for a moisture content of 12.5%

late ripening stage (Fig. 12.13c,d). Crop growth rates during ripening were larger in plants in the D plot due to their larger net assimilation rate and LAI. The dry weights at harvest and grain yields were significantly larger in the D plot than in the W plot in both years; the dry weights but not the grain yields were significantly larger in the D plot than in the C plot in both years (Table 12.3).

Similar phenomena have been observed in wheat production in other places. Grain yield of wheat (cv. 'Norin 61') was negatively correlated with the amount of precipitation from February to April (the panicle formation to flowering stages) based on long-term experiments (16–25 years) at five experimental stations in the Kanto area of Japan, where the same cultivar was grown in the same way throughout the study period (T. Minoda, K. Kobayashi, C. Endo, T. Sekiwa, Y. Mori, T. Hirasawa, unpublished, 2016).

IV. Conclusions

The root functions of water and nitrogen absorption and cytokinin transport to the shoot are essential for photosynthesis of crop plants both in drying soil and in soil with sufficient moisture. These root functions differ among cultivars, and are affected by the precipitation pattern during the growing season and by the soil properties both in rice and upland crops.

Rice growth under flooded conditions could be improved by breeding to take advantage of lines with genetically superior root system development. For upland crops, on the other hand, improving soil drainage during the rainy season and improving soil properties could be combined with genetic improvement to enhance root system development and improve root functions. If we can detect differences among cultivars or research lines in their photosynthetic capacity, in their midday and afternoon reductions in photosynthesis, and in their reduction in photosynthesis during senescence, it may become possible to identify the loci responsible for these traits and improve crop photosynthesis by means of marker-assisted selection both for rice and upland crops.

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Chapter 13

Photosynthesis in Poor Nutrient Soils, in Compacted Soils, and under Drought

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Summary

Plants require the uptake of nutrients (in most cases via roots) and their incorporation into plant organs for growth. In non-woody species, 83% of fresh weight is water, 7% is carbon, 5% is oxygen, with the remaining 5% including hydrogen and such nutrients. In natural ecosystems, availability of nutrients in soils is heterogeneous, and many species often adapt their growth to the amount of nutrients that roots can take up by exploring the available soil volume. In agricultural areas, the lack of some nutrients is frequent. In both cases, plants must also face periods of drought and soil compaction. These environmental stresses are therefore not uncommon in natural ecosystems and crops, and the stressed plants often experience a decrease in photosynthetic CO_2 fixation. In this chapter, we review changes observed in photosynthesis in response to nutrient deficiencies, soil compaction, and drought. The current knowledge on photosynthesis in carnivorous plants, as a special case of plant species growing in nutrient poor soils, is also included. Pigment limitations (chlorosis and/or necrosis), stomatal limitations, ultrastructural effects and mesophyll conductance limitations, photochemistry (primary reactions), carboxylation and Calvin-cycle reactions, and carbohydrate metabolism and transport will be discussed. With regard to nutrients, we have focused on the most common nutrition-related stresses in plants, the deficiencies of macro- (nitrogen, phosphorous, and potassium) and micronutrients (iron, manganese, copper, and zinc). Other nutrient deficiencies (or toxicities, both in the cases of essential nutrient excess or heavy metals) are not reviewed here. For other nutrient deficiencies and toxicities, and the role of the above-mentioned, and other nutrients (such as calcium and magnesium) in gas exchange, and as intracellular signal transducers, enzyme activators, and structure and function stabilizers of biological membranes, readers are referred to papers published elsewhere (Marschner H, Mineral nutrition of higher plants. Academic, London, 1995; Cakmak I, Kirkby EA, Physiol Plant 133:692–704, 2008; Morales F, Warren CR, Photosynthetic responses to nutrient deprivation and toxicities. In: Flexas J, Loreto F, Medrano H (eds) Terrestrial photosynthesis in a changing environment: a molecular, physiological and ecological approach. Cambridge University Press, Cambridge, pp 312–330, 2012; Hochmal AK, Schulze S, Trompelt K, Hippler M, Biochim Biophys Acta 1847:993–1003, 2015).

I. Limiting Nutrients

A. Photosynthesis and Nitrogen Deficiency

Nitrate and ammonium are the two major sources of inorganic nitrogen (N) available for uptake from the soil by plant roots. Plants require a N concentration of 2–5% dry mass (DM), depending on species, phenological stage, and tissue (Marschner 1995). Under suboptimal N, senescence of older leaves is enhanced, N is retranslocated from mature to young leaves, and new growing leaves are thicker and smaller (Marschner 1995). As a consequence, growth is delayed. Sage and Pearcy (1987a) ascribed the restricted development of N-deficient plants to low leaf expansion rates, rather than to declines in photosynthesis. In some species, however, N shortage does result in markedly lower rates of photosynthesis. This is not unexpected, because the photosynthetic apparatus contains approximately half of the total leaf N (Makino and Osmond 1991). Often, photosynthetic capacity and leaf N per unit leaf area are correlated (Field and Mooney 1986; Sage and Pearcy 1987b; Walcroft et al. 1997). Broad, almost universal, correlations between maximum rates of photosynthesis (A_{max}) and N among species come from sev-

eral large data sets (Field and Mooney 1986; Reich et al. 1997). However, the A_{max}-N correlations differ among species and functional groups (Evans 1989; Reich et al. 1998), and are composed of a series of nested correlations with increasing slopes as specific leaf area (defined as the ratio of leaf area to dry mass, and used to estimate the reproductive strategy of a particular species based upon, among other factors, light and moisture levels), and usually N, increases (Reich et al. 1998). On the other hand, the strength of these correlations generally decreases as the N range decreases (Reich 1993), making relationships within functional groups rather weak.

In cotton, leaf N, photosynthesis, and stomatal conductance are linearly and positively correlated (Lokhande and Reddy 2015). Also, N deficiency resulted in lower stomatal conductance in eggplant (Flores et al. 2015) and winter wheat (Wang et al. 2015a). However, Sage and Pearcy (1987b) reported that the ratio of sub-stomatal to external CO_2 concentration either does not change or even increases under N limitation in *Chenopodium album* and *Amaranthus retroflexus*, indicating the absence of stomatal limitations. In line with these findings, no changes in stomatal conductance under N deficiency were observed in *Pelargonium sidoides* (Mofokeng et al. 2015). Li et al. (2009) attributed the enhanced photosynthesis of plants grown under high N to higher chloroplastic CO_2 concentrations, associated with a higher CO_2 mesophyll conductance due to an increased chloroplast size. Warren (2004) showed that diffusional limitations (including both stomatal and mesophyll ones) increase with N deficiency. There is, furthermore, increasing evidence for N deficiency-mediated decreases in CO_2 mesophyll conductance (Urban et al. 2008; Bown et al. 2009; Li et al. 2009; Yin et al. 2009).

Nitrogen deficiency-mediated changes in different components of the photosynthetic apparatus are well coordinated. For instance, both chlorophyll (Chl) and Rubisco decrease with N deficiency. Plants use N most efficiently when the light-harvesting (i.e., Chl) and carboxylation (i.e., Rubisco) capabilities are matched (von Caemmerer and Farquhar 1981). Also, irrespective of nutrient supply, the maximum carboxylation velocity of Rubisco and the maximum electron transport rate contributing to ribulose-1,5-bisphosphate (RuBP) regeneration derived from the CO_2 response of photosynthesis are closely coupled (Wullschleger 1993; Walcroft et al. 1997). The above-mentioned findings support that there is tight control in the allocation of N between light harvesting (Chl and pigment-protein complexes) and carboxylation capacity (Rubisco and other Calvin cycle enzymes). In many cases, part of the decreased photosynthetic capacity induced by N deficiency can be ascribed to the diminished amount of Calvin cycle enzymes (Terashima and Evans 1988), because effects on Rubisco and other photosynthetic enzymes are often larger than those on Chl (Ferrar and Osmond 1986; Evans and Terashima 1987). Chloroplasts are fewer in number (Al-Abbas et al. 1974), the number of thylakoids per chloroplast is fewer, and thylakoid protein per chloroplast is less (Evans and Terashima 1987; Terashima and Evans 1988) under reduced N availability.

Moreover, there is strong evidence that N deficiency induces sink limitation within the whole plant, due to decreased growth (Paul and Driscoll 1997; Logan et al. 1999), leading in turn to a feedback down-regulation of photosynthesis. This phenomenon, now widely recognized, has been known for almost 5 decades (Neals and Incoll 1968).

Another factor to be considered for how plants perform at any given N supply is the photosynthetic N use efficiency (PNUE), i.e., the rate of photosynthesis per unit N. A general observation is that species growing in poor nutrient (and water limited) habitats have lower PNUE than those from nutrient rich (and water sufficient) habitats. The question is why this occurs, and the answer is not simple. Multiple factors contribute to a low

PNUE in stressed plants. A smaller fraction of N is allocated to photosynthetic functions in species from oligotrophic and/or xeric habitats (Field and Mooney 1986; Hikosaka et al. 1998). Leaves that are structurally reinforced can also enhance stress tolerance (Coley 1988), and this has an impact on photosynthesis. Generally, a greater allocation of C to structural tissues (e.g., collenchyma and sclerenchyma) increases leaf density, decreasing the amount of photosynthetic mesophyll tissue, the contents of water and N per unit dry mass, and the N allocation to Rubisco (Morales and Warren 2012). In addition, a low PNUE can be the consequence of low levels of CO_2 within the leaf mesophyll tissue (C_i), due to more conservative water use (a higher water-use efficiency, WUE), although this may not occur in N-deficient plants (Field et al. 1983).

Leaf respiration rates were reduced in the case of N limitation in pepper (González-Meler et al. 1997) and poplar plants (Ibrahim et al. 1998). Respiration was also positively associated with leaf N concentration in N-deficient *Eucalyptus globulus* seedlings (Sheriff and Nambiar 1991). As a consequence, night leaf respiration is an important component of daily C balance for leaves with low N (Sheriff and Nambiar 1991). The abundance of many proteins in both immature and mature leaves decreased with N deficiency in creeping bent grass, including those involved in photorespiration (Xu et al. 2011), in line with the finding that photorespiration rate (on an area and a mass basis) decreased in low-N cassava plants as compared with high-N ones (Cruz et al. 2003).

In summary, N poor soils may affect photosynthesis through several ways (Table 13.1). Chlorophyll, Rubisco, and the amount of Calvin cycle enzymes, as well as CO_2 mesophyll conductance, are decreased, and the reduction in overall growth induces a C sink limitation that can limit the upregulation of photosynthesis in source leaves.

B. *Photosynthesis and Phosphorous Deficiency*

The total P content in soils is generally very high, but only a small fraction (that which is present as $H_2PO_4^-$ and HPO_4^{2-}) is directly available to the plants for uptake. For opti-

Table 13.1. Plant photosynthetic responses to nutritional deficiencies (including carnivorous plants), compacted soils, and drought. Symbols indicate main effect (☑), effect described several times (✓), occasionally (✓), or not described in the literature (✘), and no effect (≈)

	Limitations					
			Non-stomatal limitations			
Stress factor	Pigment limitations (chlorosis or necrosis)	Stomatal limitations	Mesophyll conductance limitations and ultrastructural effects	Primary reactions	Carboxylation and Calvin cycle reactions	Carbohydrate metabolism and transport
-N	✓	✓	✓	✓	☑	✓
-P	≈	✓	✓	✓	✓	✓
-K	✓	✓	✓	✓	✓	✓
-Fe	✓	✓	✓	☑	✓	✓
-Mn	✓	≈	✓	✓	✓	✘
-Cu	✓	✓	✓	✓	✓	✘
-Zn	✓	✓	✓	✓	✓	✓
Traps of carnivorous plants in nutrient poor soils	✓	✓	✓	✓	✓	✘
Compacted soil	✘	✓	✘	✘	✘	✓
Drought	✓	☑	✓	✓	✓	✓

mal plant growth, P requirement is in the range 0.3–0.5% DM (Marschner 1995; Frydenvang et al. 2015). Under adequate P supply, 85–95% of the plant total inorganic P (P_i) is located in vacuoles and its release is usually slow. In a plant with optimal P nutrition, P_i concentration in the cytosol is approximately 6 mM. In the stroma of the chloroplasts, severe inhibition of starch biosynthesis occurs below 5 mM P_i (Marschner 1995).

Low P crop nutrition reduces yield, and supply of P to crops is seldom sufficient to properly maintain physiological processes. A deficient P supply is manifested first in growth and then in photosynthesis (Halsted and Lynch 1996). Hence, inadequate P availability typically reduces plant growth (Bottrill et al. 1970; Plesnicar et al. 1994; Yan et al. 2015) due to the inhibition of leaf cell expansion and division (Terry and Ulrich 1973b). Often, but not always (Yan et al. 2015), Chl concentrations are affected little or even increase (Rao and Terry 1989) under P deficiency, with leaves showing anthocyanin accumulation (Sánchez-Calderón et al. 2006) and a dark green color as cell and leaf expansion are more affected than chloroplast and Chl formation (Hecht-Buchholz 1967). This may, or may not, be accompanied by decreased photosynthetic rates (Bottrill et al. 1970; Brooks 1986; Lauer et al. 1989; Yan et al. 2015). At least in some species, for example in *Eucalyptus globulus*, A_{max} reductions due to sub-optimal P supply can be as large as 55% (Turnbull et al. 2007).

Some studies suggested an impact of P deficiency on photosynthesis through altered stomatal functioning. This may be because ATP biosynthesis requires P_i, and ATP is involved in the control of stomatal opening (Agbariah and Roth-Bejerano 1990). Accordingly, P deficiency results in significantly lower stomatal conductance in many cases (Brooks 1986; Rao and Terry 1989; Xu et al. 2007; Flores et al. 2015; Yan et al. 2015). In pea, however, plants grown with an adequate P supply had 20% lower stomatal conductance than those grown in a P-deficient soil (Jin et al. 2015). On the other hand, CO_2 mesophyll conductance is affected very little by P deficiency (Bown et al. 2009).

The simplest explanation for positive relationships between photosynthesis and leaf P concentrations is that P_i is one of the primary substrates of photosynthesis (Walker and Sivak 1986). In that sense, there are dramatic and rapid changes in internal concentrations of P_i when P-deficient sugar beet plants are re-supplied with P (Rao and Terry 1995). These P deficiency-mediated decreases in photosynthesis were accompanied by lower Rubisco contents, specific activity, and carboxylation efficiency (Brooks 1986; Lauer et al. 1989; Yan et al. 2015). It is also known that the activation of Rubisco is accelerated in the presence of P_i (Sawada et al. 1992). All these reports suggest that P_i limitation has a large effect on carbon reduction. Some effects have been reported on photosystem (PS) II (Singh and Reddy 2015; Yan et al. 2015) and PS I (Frydenvang et al. 2015) photochemistry, decreasing in the former both photochemical quenching and the intrinsic PS II efficiency. Also, a low stromal P_i limits ATP synthase proton conductivity (Takizawa et al. 2008). Both accumulation of starch (and/or sucrose) in leaves, due to reduced sink demand (Pieters et al. 2001), and insufficient P_i for operation of triose phosphate transport (Herold 1980) can result in decreased rates of photosynthesis. Moreover, photosynthetic metabolism is particularly sensitive to P_i, insofar as both the RuBP pool size (Brooks 1986; Fredeen et al. 1989) and its regeneration efficiency (Brooks 1986; Plesnicar et al. 1994; Yan et al. 2015) can be reduced in P-limited plants. Nevertheless, the amount (and fraction of P) involved in the primary processes of photosynthesis is variable and often small (Bieleski 1973), which makes unclear why the correlations between photosynthetic rates and P concentrations are so strong. With regard to this, levels of P_i that reduce photosynthesis in sucrose-accumulating species hardly influence photosynthesis

in the species accumulating starch (Walker and Sivak 1985). Starch biosynthesis liberates P_i from reduced C, making P_i available again for different metabolic reactions.

In many cases, positive relationships between photosynthesis and P are best explained by leaf P content (Singh and Reddy 2015), not by the active (cytoplasmic located) P pool (Turnbull et al. 2007). This may be because, at least in some cases, P affects photosynthesis indirectly via effects on N allocation to the photosynthetic apparatus. For example, there is a strong positive correlation between P supply and P stored as orthophosphate and N allocated to Rubisco in *Pinus pinaster* (Warren and Adams 2002). This may be because, at longer time scales, P deficiency causes carbohydrate accumulation that, in turn, provides feedback regulation resulting in the down-regulation of genes coding for the photosynthetic machinery (Krapp and Stitt 1995).

Plant respiration may be affected under P deficiency because P_i is itself a substrate for mitochondrial respiration (excluding the alternative oxidase, which is not regulated by adenylate level). Early in the 1970s, Bottrill et al. (1970) and Terry and Ulrich (1973b) reported that both respiration and photorespiration decrease with P deficiency. However, since Rubisco catalyzes both carboxylation and oxygenation (Ogren and Bowes 1971), early reports that photorespiration decreases with P deficiency should be evaluated with care. In fact, it has been reported that the fraction of electron transport used for photorespiration was increased in P-deficient rice leaves (Weng et al. 2008) and that the electron transport rate to photosynthesis ratio was increased under P deficiency in cotton (Singh and Reddy 2014). In stress situations, the latter ratio may reflect either an increased level of photorespiration in C_3 species (Flexas et al. 1999, 2002) or an increased level of the Mehler reaction in C_4 species (Fryer et al. 1998).

In summary, P poor soils induce plant P deficiency, affecting plant growth but not always photosynthesis. The latter may be largely dependent on whether the plant is a starch- or sucrose-accumulating species. In cases where P limitation results in reduced rates of photosynthesis, the mechanisms underlying such lower rates have been reported to include altered stomatal functioning, PS II and PS I photochemistry, ATP synthase, Rubisco contents and specific activity, RuBP pool size and regeneration, and sugar transport (Table 13.1).

C. Photosynthesis and Potassium Deficiency

The K content in soils, present almost exclusively in inorganic form, is usually between 0.3 and 3%. Potassium may limit growth on some soils, especially those with sand, due to high K mobility and large plant requirements. Potassium constitutes 1–2% of the total plant DM (Marschner 1995), being the most abundant univalent cation in plant cells. Cytoplasmic K concentrations are maintained in a narrow range (100–120 mM), whereas in the chloroplasts they are more variable (between 20 and 200 mM; Marschner 1995). In many species, K deficiency decreases both growth and photosynthesis (Bottrill et al. 1970; Basile et al. 2003; Kanai et al. 2007; Weng et al. 2007). In some species, these effects are associated with low Chl concentration (Erel et al. 2015), impaired PS II photochemistry (Wang et al. 2015b), chloroplasts with an altered ultrastructure, and restricted sugar long-distance transport (Hu et al. 2015; Hao et al. 2016).

Potassium plays a significant role in the regulation of stomatal function (Macrobbie 1998), with light-dependent K uptake into the guard cells being a critical step for stomatal opening (Schroeder 2003). Increases in leaf temperature of approximately 0.5–1.5 °C were reported in K-deficient sugarcane when compared to the K-sufficient controls (Gates 1970), suggesting closure of stomata. Terry and Ulrich (1973a) reported that K deficiency reduces photosynthesis in

sugar beet by decreasing CO_2 stomatal conductance. Also, stomatal closure in response to K deficiency has been reported in soybean (Wang et al. 2015b) and cotton (Hu et al. 2015).

In other plant species, stomatal closure does not appear to be the origin of the decreases in photosynthesis with K deficiency. In barley, transpiration increased with insufficient K (Lösch et al. 1992), in olive trees K starvation increased stomatal conductance (Arquero et al. 2006), and in eggplant no statistically significant differences in stomatal conductance were found with K deficiency (Flores et al. 2015). Protein synthesis requires large amounts of K (Leigh and Wyn Jones 1984), and this is probably responsible for the strong correlations found between proteins such as Rubisco and K concentrations (Flaig and Mohr 1992). Recently, decreased Rubisco activity and contents have been reported in response to K deficiency (Erel et al. 2015; Hu et al. 2015; Wang et al. 2015b).

Regarding respiration, the effects of K deficiency are contradictory. Markedly elevated levels of respiration were reported in K-deficient spinach (Bottrill et al. 1970), but Terry and Ulrich (1973a) showed lower levels of leaf respiration in sugar beet K-deficient with respect to the controls. Photorespiration was found to be lower in K-deficient sugar beet with respect to the controls (Terry and Ulrich 1973a; however, see commentary above about the photorespiration measurements in the early 1970s).

In summary, plants growing in K poor soils usually exhibit impaired growth and lower rates of photosynthesis. In some cases, alteration in chloroplast ultrastructure and Chl content has been reported (Table 13.1). In some species, K deficiency induces stomatal closure, but in other species CO_2 stomatal conductance increases with K deficiency. In the latter cases, decreases in photosynthesis are mediated by decreases in Rubisco concentration and activity (Table 13.1). Finally, under K deficiency some impacts on photosynthesis can be interpreted as the result of a lower level of long-distance transport of sugar from the source leaves to plant sinks preventing the full upregulation of photosynthesis to meet the genetic potential of a given species (Table 13.1).

D. Photosynthesis and Iron Deficiency

Generally, soils are quite rich in Fe, with an average Fe content of 3.2% (Murad and Fisher 1988). However, in high pH soils (calcareous and alkaline), Fe occurs in very low solubility forms, including oxides and hydroxides. In these soils, the free Fe concentration is 10^{-10} M, whereas plants require levels close to 10^{-7} M (Loeppert 1986). As a consequence, Fe deficiency is quite common in these soils. The threshold for Fe deficiency in plants is in the range 50–150 μg Fe g^{-1} of leaf DM (Marschner 1995). Within the plant, chloroplasts contain *ca.* 80% of the whole plant Fe, with Fe being a constituent of cytochromes, Fe-S centers, and others (Terry and Abadía 1986). Under Fe deficiency, chloroplast and thylakoid structure is altered, the amount of thylakoid membranes per chloroplast decreases, and this is accompanied by decreases in all membrane components, including the photosynthetic pigments Chls and carotenoids (Morales et al. 1990, 1994), proteins with which they are associated in PS I and PS II (Timperio et al. 2007), and the electron carriers of the photosynthetic electron transport chain (Terry and Abadía 1986). Among them, PS I is a prime target of Fe deficiency (Terry and Abadía 1986; Timperio et al. 2007). Iron deficiency also decreases RuBP carboxylation capacity, both through reduced Rubisco enzyme activation (Taylor and Terry 1986) and down-regulation of gene expression (Winder and Nishio 1995). In Fe-deficient leaves, the Fe deficiency-mediated decreases in light harvesting, electron transport, and CO_2 fixation capacities are well coordinated (Winder and Nishio 1995; Larbi et al. 2006). As a consequence of

all these impacts, Fe-deficient leaves have low photosynthetic rates. Not only photosynthesis but also leaf respiration rates are lower in Fe-deficient leaves when compared to the Fe-sufficient controls (Morales et al. 1998). No photorespiration data have been reported to date in plants grown under Fe deficiency (see discussion below for some problems when trying to use combined gas exchange and Chl fluorescence methodology). Carbohydrates, including sucrose, glucose, and starch, are drastically depleted in Fe-deficient sugar beet leaves, and this suggests that an imbalance in the source to sink ratio is not responsible for the decreased photosynthesis under Fe deficiency in this species (Arulanantham et al. 1990). On the other hand, sugars accumulate in the shoots of *Arabidopsis thaliana* plants under Fe deficiency (Zargar et al. 2015).

In summary, Fe deficiency affects photosynthesis through well-coordinated decreases of light harvesting, photosynthetic electron transport, and CO_2 fixation (Table 13.1).

E. Photosynthesis and Manganese Deficiency

Although soils can contain relatively large amounts of Mn, only a small fraction is readily available to the plants. In nature, Mn can have several redox states, but only Mn^{+2} is taken up by plants. Regardless of plant species or prevailing environmental conditions, critical Mn deficiency levels are in the range 10–20 μg g^{-1} DM (Marschner 1995). Growth is impaired in Mn-deficient plants when compared with the Mn-sufficient controls (Ohki 1985; Yu et al. 1998). Manganese deficiency decreases leaf Chl and carotenoid content (Henriques 2003; Carrasco-Gil et al. 2016). Although Terry and Ulrich (1974) reported chlorosis in young leaves, chlorosis generally occurs in the oldest ones, with the green color remaining only in the main veins.

Photosynthetic rates are generally decreased under Mn deficiency (Bottrill et al. 1970; Terry and Ulrich 1974; Ohki et al. 1981; Ohki 1985; Kriedemann and Anderson 1988; Henriques 2003). Respiration decreased (Bottrill et al. 1970) or did not change (Terry and Ulrich 1974; Ohki et al. 1981), whereas photorespiration decreased under Mn deficiency (Terry and Ulrich 1974; see above comment about photorespiration measurements at that time).

Causes for the Mn deficiency-mediated impaired photosynthesis are very well established. Due to the participation of Mn in water photolysis, photosynthetic electron transport is likely to be affected by Mn deficiency. Thus, in most species, the capacity to evolve O_2 is clearly reduced under Mn deficiency (Kriedemann et al. 1985). Possibly associated with the disappearance of the thylakoid grana, these changes were accompanied by an important loss of PS II reaction centers (Abadía et al. 1986). Analysis of the Kautsky induction curve revealed that in addition to the usual O-J-I-P steps, clear K and D steps developed in a Mn-inefficient barley genotype under Mn deficiency, indicating damage to PS II (Husted et al. 2009). Evidence for PS II damage came from perturbations in the photosynthetic apparatus that clearly involved loss of the PS II core protein PsbA (Husted et al. 2009). In pecan leaves, however, the maximum potential PS II efficiency decreased only slightly with increasing Mn deficiency (Henriques 2003). In this species, the reduction in photosynthesis was associated with a decreased number of PS II units, due to a decreased number of chloroplasts per unit leaf area (Henriques 2003).

A decreased CO_2 availability at the carboxylation sites with Mn deficiency has been ruled out, because no reductions in stomatal conductance or transpiration rates were observed (Terry and Ulrich 1974; Ohki et al. 1981; Ohki 1985; Henriques 2003). On the other hand, Terry and Ulrich (1974) reported decreased CO_2 diffusion rates from intercellular spaces to the chloroplast stroma in Mn-deficient plants. This can now be interpreted as a decreased CO_2 mesophyll con-

ductance. Both the initial slope and CO_2-saturated phases of photosynthesis versus C_i curves were similarly affected under Mn deficiency, suggesting decreases in Rubisco activity and RuBP regeneration rates (Kriedemann and Anderson 1988).

In summary, Mn deficiency results in lower rates of photosynthesis mainly through decreased rates of photosynthetic electron transport, owing to the participation of Mn in water photolysis (Table 13.1). Effects of Mn deficiency on electron transport are due in most species to decreases in PS II efficiency, and only in pecan due to the number of PS II units (maintaining reasonably constant PS II efficiency). If there are CO_2 limitations, they are mediated by decreases of CO_2 mesophyll conductance but not by stomatal closure (Table 13.1). Decreased Rubisco activity and RuBP regeneration rates have been also reported under Mn deficiency (Table 13.1).

F. Photosynthesis and Copper Deficiency

In non-contaminated soils, the Cu content is between 2 and 40 mg kg^{-1} soil. Plants take up Cu as Cu^{+2} in aerated soils or as Cu^{+} in soils with little O_2 or under flooding. Copper deficiency generally appears in plants in the range 3–5 μg g^{-1} DM, with the range being dependent on plant species, tissue, developmental stage, and N supply (Marschner 1995). Plant growth is reduced under Cu deficiency (Yu et al. 1998). Copper deficiency decreases photosynthetic rates at least in part due to stomatal closure (Bottrill et al. 1970; Kriedemann and Anderson 1988). Leaf respiration was not affected under Cu deficiency (Bottrill et al. 1970), and no reports can be found in the literature about effects of Cu deficiency on photorespiration.

Large effects of Cu deficiency on leaf Chl were reported in sugar beet (Henriques 1989), whereas the effects were moderate in cereals (Kriedemann and Anderson 1988), and absent in spinach (Bottrill et al. 1970). The Chl *a*/Chl *b* ratios decline under Cu deficiency in cereals (Kriedemann and Anderson 1988), but not in spinach (Bottrill et al. 1970) or sugar beet (Henriques 1989). Also, Cu deficiency interferes with pigment and lipid biosynthesis (Barón et al. 1995), and consequently with chloroplast ultrastructure, reducing the number of thylakoids per granum, and with most stacks showing some swelling (Henriques 1989).

Some effects on photochemistry have been reported under Cu deficiency. During the Kautsky induction curve, Chl fluorescence quenching after the maximum was slight in Cu-deficient plants, so that fluorescence remained close to the maximum (Kriedemann and Anderson 1988). Since plastocyanin is a Cu-containing protein, and plays a key role as a carrier in PS II to PS I electron transport and in cyclic electron flow around PS I, this may reflect a slower re-oxidation of PS II. This is consistent with a larger decrease in PS I activity compared to that of PS II under Cu deficiency (Henriques 1989). In the same line, it has recently been reported that decreased photosynthetic and electron transport capacities correlated with a reduction in plastocyanin levels in Cu-deficient leaves (Shahbaz et al. 2015). Beyond impacts on photosynthetic electron transport, similar decreases in both initial slope and CO_2-saturated phases of photosynthesis versus C_i curves were observed under Cu deficiency (Kriedemann and Anderson 1988), suggesting a general reduction in Rubisco activity and rates of RuBP regeneration.

In summary, the effects of Cu deficiency on plant photosynthesis depend largely on species. Some species experience growth reduction and chlorosis, whereas others show no changes in Chl (Table 13.1). Cu-deficient plants have decreased photosynthetic rates, associated with one of the following processes: thylakoid degradation and swelling, reduced stomatal conductance to CO_2, decreased PS I (more than PS II) activity, and/or lower Rubisco activity and RuBP regeneration (Table 13.1).

G. Photosynthesis and Zinc Deficiency

The soil Zn content in non-contaminated soils is between 10 and 80 mg kg^{-1}. Plant roots take up Zn predominantly as the divalent cation Zn^{2+}, although it is presumably also taken up as a monovalent cation ($ZnOH^{+}$) in high pH soils (Marschner 1995). The critical deficiency levels in plants are approximately 15–20 μg Zn g^{-1} leaf DM (Marschner 1995). Zinc deficiency reduces plant growth and leads to reduced rates of photosynthesis in many plant species (Bottrill et al. 1970; Yu et al. 1998; Schuerger et al. 2003; Chen et al. 2008; Mattiello et al. 2015). Plant respiration is also reduced under Zn deficiency (Bottrill et al. 1970), but, similar to other micronutrient deficiencies, no data are available about the effects of Zn deficiency on photorespiration.

Increases in light intensity rapidly induced chlorosis and necrosis in Zn-deficient plants, Chl concentrations declining with increasing light intensity (Marschner and Cakmak 1989). In the deficient plants exposed to high light intensity, severe symptoms of chlorosis and necrosis occurred, and partial shading of the leaf blades either prevented or at least drastically delayed development of chlorosis and necrosis in the shaded areas (Marschner and Cakmak 1989). Decreases in the leaf Chl (Bottrill et al. 1970; Schuerger et al. 2003; Wang and Jin 2005; Chen et al. 2008; Fu et al. 2015) and carotenoid (Schuerger et al. 2003) concentrations were found in leaves affected by Zn deficiency. Zinc deficiency generally affects the level of Chl *a* more than Chl *b*, resulting in decreased Chl *a*/Chl *b* ratios (Bottrill et al. 1970; Schuerger et al. 2003; Chen et al. 2008), although this ratio has been reported to increase in Zn-deficient maize (Wang and Jin 2005). Zinc deficiency is commonly accompanied by thylakoid disorganization and degradation (Henriques 2001; Fu et al. 2015). As Zn deficiency aggravated, cellular deficient characteristics like abnormal cell arrangements and disorganization of thylakoids became more exaggerated; visible external symptoms of Zn deficiency appeared with internal alterations, including photosynthetic apparatus damage accompanied by subsequent chloroplast and grana disintegration (Fu et al. 2015).

Zinc deficiency-mediated inhibition of photosynthesis was associated with a decreased stomatal conductance (Mattiello et al. 2015), although C_i decreased in cauliflower (Sharma et al. 1995) but increased in rice (Chen et al. 2008). In Zn-deficient rice, more excitation energy was distributed to PS II than to PS I (Chen et al. 2008). Possibly related to this imbalance, Zn deficiency has been reported to diminish PS II photochemistry (Schuerger et al. 2003; Chen et al. 2008; Fu et al. 2015). Effects of Zn deficiency on carbonic anhydrase (Ohki 1976) and Rubisco (Marschner 1995) enzymes suggest that carboxylation reactions may be, at least in part, the origin for the observed decreases in photosynthesis. Finally, a feedback down-regulation of photosynthesis under Zn deficiency cannot be excluded, since an accumulation of sugars in leaves has been related to decreases in photosynthetic CO_2 fixation (Marschner 1995).

In summary, Zn deficiency may impair photosynthesis through several ways. Stomatal conductance, PS II photochemistry, carbonic anhydrase and Rubisco were affected in Zn-deficient plants (Table 13.1). Also, an accumulation of leaf sugars may induce photosynthetic feedback down-regulation under Zn deficiency (Table 13.1). Altered chloroplast and cell membranes, decreases in the efficiency of PS II, chlorosis (lower foliar levels of both Chl and carotenoids), and degradation of chloroplast thylakoids have all been reported in Zn-deficient plants (Table 13.1).

II. Photosynthesis in Compacted Soils

Highly compacted soils are common in heavily used recreation areas, construction sites, urban areas, timber harvesting sites, fruit

orchards, agroforestry systems, and tree nurseries (Kozlowski 1999). Worldwide, it is estimated that 68 million ha of arable land are degraded by soil compaction (Hamza and Anderson 2005), with 33 million of them in Europe (Van den Akker and Canarache 2001). Compaction occurs naturally by soil settling or slumping or may be induced by tillage tools, heavy machinery, pedestrian traffic, animal trampling or fire (Kozlowski 1999). Soil compactness severity is usually measured through quantification of soil porosity, bulk density, or mechanical impedance (Colombi and Walter 2016, and references therein). Compaction typically alters soil structure and hydrology, increasing soil bulk density, breaking down soil aggregates, decreasing soil porosity and pore connectivity (macro- and meso-pores disappear), aeration (gas diffusivity) and water infiltration capacity, and increasing soil strength, water runoff, and soil erosion (Kozlowski 1999; Colombi and Walter 2016).

Regarding effects on plants, soil compaction induces changes in the amount and balance of plant growth hormones, particularly increasing abscisic acid and ethylene (Kozlowski 1999). As soils become increasingly compacted, the risk of waterlogging increases and root respiration shifts towards an anaerobic state (Kozlowski 1999; Colombi and Walter 2016). Thus, inadequate O_2 supply in the root zone induces the occurrence of aerenchyma (Colombi and Walter 2016) and is a constraint to plant performance, particularly in heavy, compacted soils (Bhattarai et al. 2004). In a growth chamber experiment with *Pinus contorta* seedlings, soil compaction increased bulk density, penetrometer resistance, and soil CO_2 and ethylene (Conlin and van den Driessche 1996). Soil compaction in the field may benefit or inhibit plant growth, although, as a consequence of the above-mentioned facts, harmful effects on plant growth are more common (Kozlowski 1999; Tubeileh et al. 2003; Polanco et al. 2008; Grzesiak et al. 2015). Also, the tolerance towards compaction may differ among species (Kozlowski 1999), and genetic variability may exist within species (Grzesiak et al. 2015).

Soil compaction (with soil bulk densities ranging from 1.2 to 1.7 g cm^{-3} at 0–20 cm depth, equivalent to 0.8 to 2.4 MPa soil strength measured with a soil penetrometer) reduced growth in sunflower, due to slower leaf expansion rates and smaller maximum size of individual leaves (Andrade et al. 1993). In greenhouse experiments, soil compaction also caused reductions in total biomass production in snap bean (30%) and cabbage (14%) but had no significant effects in cucumber and sweet corn (Wolfe et al. 1995). In *P. contorta* seedlings, compaction had a small effect on height growth, with the tallest seedlings occurring at the largest (8.0 MPa) compaction, but decreased needle length and root DM as compaction increased from 0.1 to 8.0 MPa (Conlin and van den Driessche 1996). Deep ripping following soil compaction increased height (25%) and diameter (51%) growth and DM per needle (30%) in lodgepole pine, whereas in Douglas-fir it increased stem diameter (51%) but not height (9%) growth (Kamaluddin et al. 2005). Soil compaction (bulk density of 1.45 g cm^{-3} in the compacted soil *vs.* 1.30 g cm^{-3} in the loose one) markedly hampered root elongation and delayed leaf appearance rate in maize, decreasing plant height, shoot and root DM, and leaf area (Tubeileh et al. 2003). Increasing soil bulk density from 1.0 to 1.5 g cm^{-3} reduced shoot length, total leaf area, leaf size, and DM of leaves, shoots, and roots of container-grown apple trees (Ferree et al. 2004). In grapevine, shoot growth, leaf area, number of inflorescences, and leaf DM decreased linearly as soil bulk density increased above 1.4 g cm^{-3} (Ferree and Streeter 2004). Under field conditions, the average total marketable yield was reduced in cabbage, snap bean, cucumber, and sweet corn in direct-seeded compacted plots (Wolfe et al. 1995). The magnitude of yield response to compaction in the field, when compared to greenhouse conditions, was associated with

species sensitivity to secondary effects of compaction, such as prolonged flooding after rainfall events, reduced nutrient availability or uptake, and/or prolonged and more severe pest pressure (Wolfe et al. 1995).

Lower rates of photosynthesis in plants growing in compacted soils are associated with both stomatal (Kozlowski 1999; Tubeileh et al. 2003) and non-stomatal (Kozlowski 1999) inhibition. Whole plant photosynthesis is reduced not only as a result of decreasing photosynthetic rates but also by reduction in total leaf area (see above). Increased soil compaction was associated with decreased photosynthesis in *P. contorta* seedlings (Conlin and van den Driessche 1996), maize (Tubeileh et al. 2003), and American elm (Polanco et al. 2008) and with increased rates of shoot respiration in *P. contorta* seedlings (Conlin and van den Driessche 1996). In soybean and cotton, air injection in a heavy clay soil increased photosynthesis but had no effect on stomatal conductance and transpiration rate (Bhattarai et al. 2004), suggesting non-stomatal limitations. Conversely, photosynthesis and transpiration rates were reduced when soil bulk density was increased in apple trees (Ferree et al. 2004), suggesting stomatal limitations.

In some species, however, soil compaction has been shown to maintain or even increase photosynthesis. In sunflower, photosynthesis and leaf N were either unaffected or significantly higher in compacted treatments, with leaf water and osmotic potential or leaf turgor not being significantly affected (Andrade et al. 1993). In snap bean and cabbage, soil compaction-mediated growth reductions could not be attributed to effects on soil water status, leaf turgor, nutrient deficiency, or photosynthesis (Wolfe et al. 1995). In an experiment over two consecutive years, grapevine photosynthesis and transpiration rates increased linearly as soil bulk density increased the first year, whereas in the second one a non-linear pattern was observed, with the highest rates found in plants grown in 1.3–1.4 g cm^{-3} bulk density soils (Ferree and Streeter 2004).

Photosynthetic rate was 2-fold higher in the trampling-tolerant *Eleusine indica* (a C_4 monocot) than in *Plantago asiatica* (a C_3 dicot; Kobayashi et al. 1999). The authors concluded that *E. indica* communities develop more commonly than the *P. asiatica* communities in summer at heavily trampled sites because of these differences in photosynthesis. The maximum net photosynthetic rate, stomatal conductance, and transpiration rate were likewise higher in *E. indica* than in the trampling-sensitive *Digitaria adscendens*, both summer annual C_4 grasses (Kobayashi and Hori 2000).

Often, but not always, compaction reduces water absorption and leads to leaf water deficits (Kozlowski 1999). Soil compaction reduced American elm root hydraulic conductance (Polanco et al. 2008). Soil-to-leaf hydraulic conductance, which probably plays a role in water absorption from the trampled compact soil, was higher in *E. indica* than in *P. asiatica* (Kobayashi et al. 1999). There were no differences in soil-to-leaf hydraulic conductance and leaf osmotic potential at full turgor, but the bulk modulus of elasticity in cell walls was higher (i.e., less elastic) and leaf water potential was much lower in *E. indica* than in *D. adscendens* (Kobayashi and Hori 2000). In trampled habitats, the decreased leaf water potential in *E. indica* might play a role in water absorption from the compacted soil (Kobayashi and Hori 2000).

Generally, compaction of surface soils and sub-soils decreases absorption of the major mineral nutrients (Kozlowski 1999). Apple trees growing in compacted soil (soil bulk density of 1.5 g cm^{-3}) had reduced leaf concentrations of N, Ca, Mg, Mn, Na, and Zn, and increased concentrations of P, K, B, and Fe (Ferree et al. 2004), whereas in grapevine compacting soil to a bulk density of 1.5 g cm^{-3} was associated with increases in leaf N, Ca, Mg, Al, Fe, Mn, Na, and Zn concentrations and decreases in P, K, B, and Mo

concentrations (Ferree and Streeter 2004). Soil compaction also reduced Na concentration in American elm (Polanco et al. 2008). In *P. contorta* seedlings, shoot concentrations of mineral nutrients decreased as soil compaction increased (Conlin and van den Driessche 1996).

It was concluded that early growth reduction of sunflower plants grown on compacted soil was more sink- than source-limited with regards to water, N, and carbon supply (Andrade et al. 1993). In maize, the main effect of soil compaction on carbon partitioning was on the level of root carbon exudation, which increased considerably to the detriment of the root (Tubeileh et al. 2003). The latter authors hypothesized that increasing soil resistance to root penetration induced a sink limitation in roots and this increased carbon release into the soil. Increasing soil bulk density in apple trees caused a decrease in the leaf concentration of sucrose that was accompanied by an increase in the levels of its two monomers fructose and glucose (Ferree et al. 2004).

In summary, soil compaction reduces growth. In species in which the rate of photosynthesis of plants that grow in very compacted soils is decreased, both stomatal and non-stomatal inhibitions have been reported (Table 13.1). Often, but not always, compaction reduces plant water absorption, leading to leaf water and mineral nutrient deficits. In apple trees, increases in soil bulk density caused changes in the leaf sugar concentrations.

III. Photosynthesis Under Drought

In large agricultural areas of the world, soil drought limits crop production. Irrigation is very likely the oldest crop management technique aimed to increase yield. More than 1 billion ha of cultivated soil are irrigated worldwide (Toenniessen 1984). Water shortages in many cultivated areas in America and Europe are not uncommon, while water availability is a major constraint in developing countries. Drought periods are also of extreme importance in natural habitats. Thus, the water availability and droughts have caused dramatic impacts on many human communities in the last several decades, and climatic change trends could make it even more important in the future.

Under drought, species such as barley, coffee, and grapevine maintain high leaf Chl concentrations, and therefore have high light-harvesting capacity (Da Matta et al. 1997; Flexas et al. 2002; Flexas and Medrano 2002, and references therein). Conversely, other species decrease the absorption of light by decreasing the leaf Chl concentrations in response to water stress (Kyparissis et al. 2000; Munné-Bosch and Alegre 2000).

Drought markedly decreases plant photosynthesis (Dubey 1997; Medrano et al. 1998; Flexas and Medrano 2002). In a cork oak woodland, ecosystem CO_2 net sequestration (in g C m^{-2} $year^{-1}$) was reduced under low water availabilities (Costa-e-Silva et al. 2015). Under full irrigation and saturating light, photosynthesis and photorespiration utilize 12–35% and 6–9% of the light absorbed by PS II, respectively, with the rest (54–72%) being dissipated thermally (Morales et al. 2006; data from Flexas and Medrano 2002). Under mild drought, photorespiration increases somewhat at the expense of photosynthesis, with no change in the extent of thermal dissipation (Flexas and Medrano 2002). Although re-assimilation of CO_2 evolved from photorespiration may increase as drought progresses (Haupt-Herting et al. 2001), estimated rates of dissipation are similar whether or not re-assimilation is considered (Flexas and Medrano 2002). This is probably the most common situation in crops and in nature in plants growing in Mediterranean habitats. Irrespective of soil water availability, high vapor-pressure deficits lead to transient leaf water deficits in summer at midday. When drought is moderate or severe, both photosynthesis and photorespiration decrease and

plants may thermally dissipate up to 70–92% of the absorbed light (Flexas and Medrano 2002). Under severe drought, respiration was unaffected in *Nothofagus dombeyi*, a drought-tolerant species, but decreased in the less drought-tolerant species *Nothofagus nitida* (Sanhueza et al. 2015). In *Gossypium hirsutum* (upland cotton or Mexican cotton), respiration was exceptionally sensitive to progressive drought, more so than photosynthesis (Snider et al. 2015). In a recent report, Dahal et al. (2015) compared respiration and photosynthesis of *Nicotiana tabacum* wild-type plants with that of transgenic lines over-expressing the alternative oxidase (AOX), a mitochondrial terminal oxidase that uncouples the consumption of reducing power from ATP synthesis. Results indicate that AOX amount influences respiration in the light, particularly during severe drought, when cytochrome pathway respiration may become increasingly restricted. AOX overexpression dampened photosystem stoichiometry adjustments and losses of key photosynthetic components that occurred in the wild-type plants, suggesting that the development of biochemical limitations to photosynthesis are dampened in plants with increased non-energy conserving respiration in the light.

Decreases in photosynthetic rates with drought are initially due to stomatal closure (Flexas et al. 2002). When water availability is restricted, mutants with less than half of their normal, wild-type complement of stomata are highly drought tolerant (Hepworth et al. 2015). Stomatal closure, while avoiding water loss, reduces the entrance of CO_2 inside the leaves (Flexas et al. 2002). Carbon sequestration decreases under low water availabilities in cork oak woodlands should be ascribed to stomatal regulation or photosynthetic limitations, and to a lesser extent to leaf area reductions (Costa-e-Silva et al. 2015). Mesophyll conductance to CO_2 also decreases in response to reduced water availability (Flexas et al. 2002), leading to the proposal that one of the major limitations to photosynthesis under drought arises from the low chloroplast CO_2 availability (Flexas and Medrano 2002).

When drought progresses, both diffusional limitations (stomatal closure and decreased CO_2 mesophyll conductance; Flexas et al. 2002) and decreases in PS II photochemistry (Filek et al. 2015) contribute to the decreases in photosynthesis. When the water stress is moderate or severe, decreases in photosynthesis are possibly due to a decreased RuBP availability and/or decreased Rubisco activity (Lawlor 1995).

Finally, water stress has been reported to lead to an accumulation of sugars (Jin et al. 2015; Kebbas et al. 2015) and a feedback down-regulation of photosynthesis (Souza et al. 2004; Silva et al. 2012).

In summary, drought decreases plant photosynthesis, and causes for such decreases depend on the extent of water stress. Under mild water stress, diffusional limitations (both stomatal and mesophyll conductance to CO_2) dominate over the non-diffusional ones (Table 13.1). When drought is moderate or severe, decreases in PS II photochemistry may contribute to the decreases in photosynthesis, in addition to the diffusional causes (Table 13.1). Also, some reports indicate that water stress may down-regulate photosynthesis through sugar accumulation (Table 13.1).

IV. The Case of Carnivorous Plants

Carnivorous plants have evolved independently nine times in nutrient poor, sunny, and wet habitats (Givnish 2015). Soils where the carnivorous plant species grow are usually deficient in nutrients, mainly N, P, and K (Ellison 2006). These plants overcome this problem by trapping animal preys that are rich sources of the deficient nutrients. For instance, the N and P concentrations in an insect body are 5- to 10-fold higher than in carnivorous plant tissue, and the plant is able to take up these nutrients efficiently (Pavlovič

et al. 2014). For this purpose, carnivorous plants have evolved leaves into trap devices that attract, capture, digest, and absorb mineral nutrients from preys. Five basic traps can be recognized in carnivorous plants:

1. Pitfall traps (found in *Brocchinia*, *Catopsis*, *Cephalotus*, *Darlingtonia*, *Nepenthes*, *Paepalanthus*, *Sarracenia*, and *Heliamphora*). These trap prey into a modified pitcher or leaf rosette that contain a pool of digestive enzymes or bacteria. The rim of the pitcher (peristome) is slippery when moistened, causing insects to fall into the trap. Pitcher plants may also contain waxy scales, cuticular folds, inward and downward pointing hairs, guard-cell-originating lunate cells on the inside of the pitcher, and viscoelastic digestive fluid to ensure that insects cannot climb out (Bohn and Federle 2004; Gaume et al. 2004; Gorb et al. 2004; Gaume and Forterre 2007; Bazile et al. 2015).
2. Flypaper traps (found in *Byblis*, *Drosera*, *Drosophyllum*, *Philcoxia*, *Pinguicula*, *Roridula*, and *Triphyophyllum*). The leaf of flypaper traps is studded with mucilage-secreting glands that may be short (like those of the *Pinguicula*) or long and mobile (like those of *Drosera*). Some species have active traps with tentacles and leaf bending reactions (*Drosera*), but some others have passive traps that do not move in response to prey capture (*Triphyophyllum*).
3. Snap traps (found in *Aldrovanda* and *Dionaea)*. These traps utilize rapid leaf movements for prey capture. The rapid trap closure is triggered by action potentials generated by touch of sensitive hairs on the trap lobes (Volkov et al. 2008).
4. Bladder suction traps (found in *Utricularia*). They pump ions out of their interiors and water that follows by osmosis generates a partial vacuum inside the bladder (bladder resetting). The bladder has a small opening, sealed by a hinged door. Aquatic invertebrates touch trigger hairs and deform the door by lever action, releasing the vacuum. The invertebrate is sucked into the bladder where it is digested (Vincent et al. 2011a, b; Adamec 2012).
5. Eel traps (found in *Genlisea* and *Sarracenia psittacina*). This trap is easy to enter but exit is obstructed by inward-pointing bristles (Adamec 2003).

All carnivorous plants are green and able to fix CO_2 (photoautotrophy), although there is also evidence for carbon uptake from prey (facultative heterotrophy; Michalko et al. 2013). Although carnivorous plants can digest polysaccharide and take up the sugars (Michalko et al. 2013), the carbon can also be absorbed as N-bearing amino acids (Dixon et al. 1980; Rischer et al. 2002; Kruse et al. 2014). The ecological significance of carbon uptake from prey is not known but is probably very small for terrestrial carnivorous plants. Therefore, in contrast to what occurs in parasitic plants, the main source of carbohydrates in carnivorous plants appears to be photosynthesis. Carnivorous plants utilize C_3 photosynthesis, but not very efficiently (except in the case of aquatic carnivorous plants, see Ellison and Adamec 2011). First, they have very low foliar N, P, and K concentrations in comparison to non-carnivorous plants from different habitats (Méndez and Karlsson 1999; Ellison and Farnsworth 2005; Ellison 2006), although these concentrations are comparable to those found in co-occurring non-carnivorous plants in the same habitat (Osunkoya et al. 2007; van der Ent et al. 2015). The low foliar elemental concentrations are indicative of the nutrient-poor environments. Since leaf photosynthetic capacity is related to foliar N content (Evans 1989), this may partially account for the low rate of photosynthesis observed in many terrestrial carnivorous species. However, the fact that PNUE is also reduced indicates that there must be some other reason for the decreased photosynthetic rate.

It has been proposed that carnivorous plants have lower rates of photosynthesis as a result of adaptation to carnivory (Givnish et al. 1984). This is more obvious in carnivorous species that produce leaves that can

photosynthesize but do not capture prey (photosynthetic laminae or phyllodes) and/or produce traps that capture prey and photosynthesize little or not at all (e.g., *Cephalotus*, *Nepenthes*, *Utricularia*, *Sarracenia*, *Genlisea*; Fig. 13.1a). However, this is difficult to recognize in carnivorous species with leaves that can both photosynthesize and capture prey (e.g., *Pinguicula*, *Drosera*, *Heliamphora*, *Darlingtonia*; Fig. 13.1b). Reworking leaf morphology and physiology to carnivory apparently reduces the efficiency of photosynthesis in the traps (Givnish et al. 1984; Ellison and Gotelli 2001).

The extent to which the trap is photosynthetically inefficient varies among genera. For example, the bladders of terrestrial bladderworts, which are often buried in substrates, are achlorophyllous and exhibit no net CO_2 uptake (Adamec 2006). Although the genus *Genlisea* has not been studied yet, it is almost certain that its colorless traps in the soil would have very low or no photosynthesis at all. Aquatic bladderworts have traps containing Chl but, in comparison to leaves, photosynthesis is significantly lower (Knight 1992; Adamec 2006). Very low photosynthesis, close to zero, was also documented in the pitcher trap of several species of *Nepenthes* (Pavlovič et al. 2007, 2009, 2011b; Karagatzides and Ellison 2009; He and Zain 2012). On the other hand, the pitchers of the genera *Cephalotus*, *Sarracenia, Darlingtonia* and probably also *Heliamphora* have much higher photosynthetic rates (Ellison and Farnsworth 2005; Karagatzides and Ellison 2009; Hájek and Adamec 2010; Pavlovič 2011). Several morphological, anatomical, and biochemical characteristics of the trap organs are responsible for lower rates of photosynthesis, and this has been best described in the carnivorous genus *Nepenthes* (Fig. 13.2). First, the three dimensional structure of the pitcher trap is not optimal for efficient light capture in comparison to a flat leaf. Lower Chl content also contributes to decreased light absorption (Pavlovič et al. 2007). Once Chl in PS II captures light, the efficiency of its photochemical conversion (actual PS II efficiency) is also lower (Pavlovič

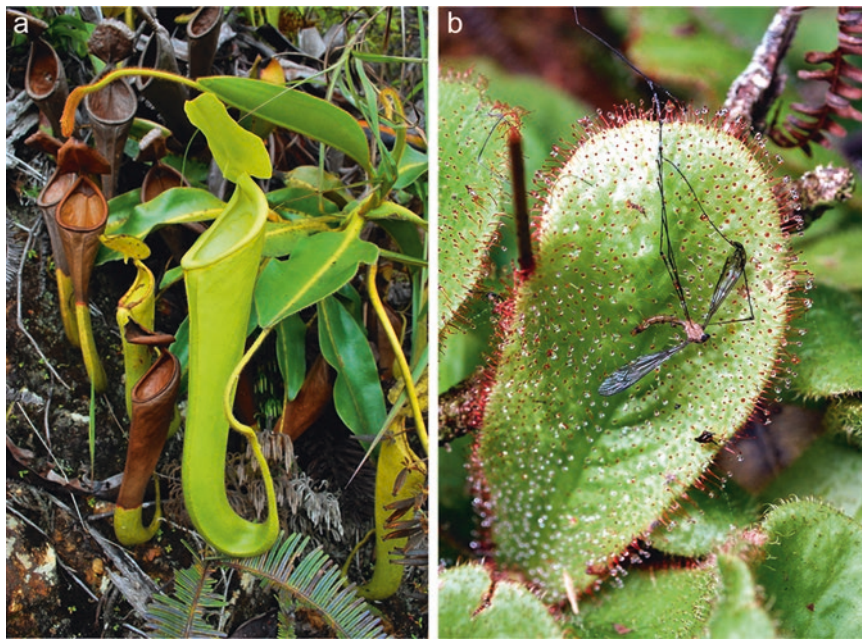

Fig. 13.1. Carnivorous plants with (**a**) leaves differentiated into photosynthetic lamina and trap (*Nepenthes chaniana*) and (**b**) leaves serving for both photosynthesis and prey capture (*Drosera schizandra*)

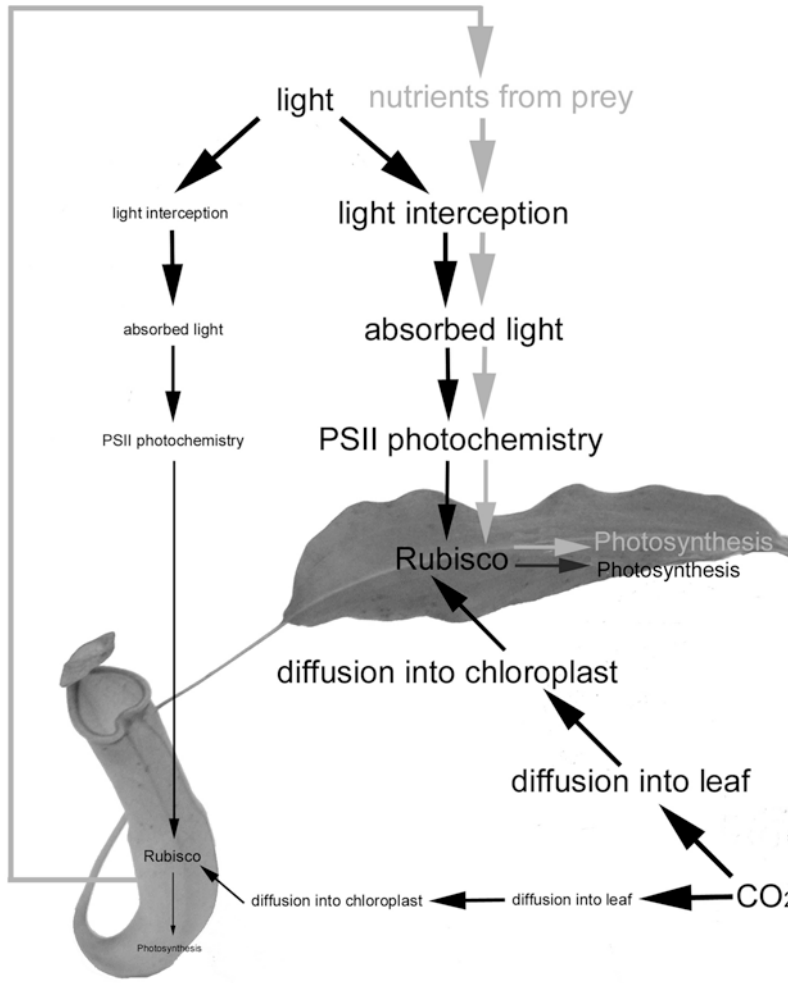

Fig. 13.2. Schematic representation of photosynthetic cost (black arrows) and benefit (grey arrows) in carnivorous pitcher plants of the genus *Nepenthes*. Components of the scheme are: light interception (a measure of projected leaf area), absorbed light (related to Chl concentration), PS II photochemistry (estimated as actual PS II efficiency), diffusion into leaf (stomatal conductance), diffusion into chloroplast (mesophyll conductance), Rubisco (Rubisco concentration) and CO_2 fixation (rate of photosynthesis). The arrow thickness and font size depicts the intensity of the given process. (According to studies of Pavlovič et al. 2007 and Pavlovič and Saganová 2015)

et al. 2009, 2011b). The decreased stomatal density on the trap surface results in lower stomatal conductance that contributes to lower levels of CO_2 diffusion into the trap. The compact mesophyll lacks palisade parenchyma and has only a small volume of intercellular spaces, which is important for symplastic transport of prey-derived nutrients, but results in a low mesophyll conductance for CO_2 (Pavlovič et al. 2007). The pitchers have lower N and protein content and a decreased amount of total soluble proteins invested into Rubisco, and thus low carboxylation activity (Galmés et al. 2014; Pavlovič and Saganová 2015). Conversely, in conventional plants Rubisco is present at very high levels in photosynthesizing cells and may contribute up to 50% of soluble leaf proteins (Feller et al. 2008). It is tempting to assume that the decreased content of Rubisco in carnivorous plants is due to the

investment of proteins into other functions related to carnivory (e.g., production of lures, enzymes, transporters, structural proteins, etc.) and is a consequence of the low N concentrations, because the proportion of total N in Rubisco decreases with decreasing leaf N (Evans 1989).

Pavlovič and Saganová (2015) analyzed data from a wide range of studies and concluded that in 16 out of 18 cases photosynthesis was significantly lower in the trap compared to the assimilatory part of the leaf. Despite the lower trap photosynthetic rate, recent studies have shown that some traps are metabolically active and their photosynthetic rates undergo spatial-temporal changes. For example, the bladders of *Utricularia* showed very high respiration rate during resetting (ion pumping) of the bladders, resulting in low rates of photosynthesis (Adamec 2006). Photosynthetic reactions were strongly inhibited during propagation of action potentials in the trap lobe of the Venus flytrap and probably also in *Aldrovanda* (Pavlovič et al. 2010a, 2011a). At the same time, respiration rate was increased, reflecting the metabolic costs of electrical signaling. Separated tentacles of *Drosera prolifera* also had high rates of respiration due to electrical signaling activities (Adamec 2010), indicating that all active traps bear significant energetic costs during their active periods.

The traps of carnivorous plants are expensive structures, because C and N invested in the traps does not return as much C as it would if it was invested in assimilatory tissue. However, nutrients taken up from prey via the trap can enhance photosynthesis and growth (Fig. 13.2; Adamec 2008; Farnsworth and Ellison 2008; Pavlovič et al. 2009, 2011b, 2014; Kruse et al. 2014). The mechanisms underlying increased photosynthesis after feeding involve increased Chl and foliar tissue N concentrations as well as increases in photosynthesis-related proteins, mainly Rubisco, which may account for 20–30% of total leaf N in conventional plants, and Chl-binding proteins (Givnish et al. 1984; Feller et al. 2008; Pavlovič et al. 2016). The extent to which photosynthesis can be enhanced by the increased mineral input from prey clearly depends on environmental conditions. If factors like light or water are in short supply, then they can limit photosynthesis and the extent to which nutrients added by carnivory can elevate photosynthesis. This may explain the occurrence of carnivorous plants not only in nutrient poor but also sunny and wet habitats, because only in such environments can they have a competitive advantage over non-carnivorous plants (Givnish et al. 1984). Whenever carnivorous plants grow in nutrient-rich soil or in sub-optimal water and light, they can give up carnivory temporarily: the genera *Nepenthes* and *Cephalotus* cease producing pitchers, *Sarracenia* forms non-carnivorous phyllodia instead of traps, *Dionaea* decreases trap excitability, and *Drosera* and *Pinguicula* decrease leaf stickiness (Zamora et al. 1998; Ellison and Gotelli 2002; Thorén et al. 2003; Pavlovič et al. 2010b; Escalante-Pérez et al. 2011). The production of carnivorous organs is phenotypically very plastic, under the genetic control of cell division, and we are just at the beginning of understanding how deadly traps are formed (Fukushima et al. 2015).

In summary, carnivorous plant species are characterized by low foliar nutrients (N, P, and K), a consequence of growing in habitats with nutrient poor soils. They have differentiated a variety of traps for the capture of prey, from which carnivorous plants obtain large amounts of nutrients. Photosynthesis in these species is not very efficient, with the exception of aquatic carnivorous plants. Causes for such low efficiency are summarized in Table 13.1, highlighting the low foliar N (and putatively Rubisco) and the low Chl concentration in traps (some of them achlorophyllous).

V. Conclusions

Mineral nutrients are elements acquired primarily in the form of inorganic ions from soil. The area of roots, root distribution in soil profile, and ability to absorb nutrients at low concentration from the soil water solution make absorption of ions by plants a very important process. The mineral ions (macro- and micro-nutrients) after being absorbed by roots are retranslocated to the various plant parts where they are used in many physiological processes.

In agricultural areas and natural habitats, plants require availability of a variety of nutrients, optimal soil water content and favorable soil compaction because species adjust their growth, development, and yield to actual environmental conditions. Usually, the decrease in photosynthetic rate as a result of such stresses occurs later than cell elongation growth. When plants grow in nutrient poor soils (including carnivorous plants), in compacted soils, and under drought, rates of photosynthesis may be lower for a variety of reasons (Table 13.1). In responses to some stresses, such as P deficiency and in some species with water stress, Chl concentration generally does not change. In response to other common nutrient stresses, such as reduced availability of N, K, or micronutrients (Fe, Mn, Cu or Zn), Chl concentrations generally decrease. However, decreases in Chl may or may not lead to a lower rate of photosynthesis, depending on the extent of the Chl concentration decrease and the consequent decrease in light absorption. In the case of Fe-deficient leaves, the relationship between Chl concentration and light absorption is curvilinear, being linear at low Chl concentrations and showing only small changes in absorption at the highest ones (Morales et al. 1991). In plants growing in different environments under Fe deficiency, light absorption, photochemistry, and carboxylation are downregulated in a coordinated way (Winder and Nishio 1995; Larbi et al. 2006).

Stomatal limitations for photosynthesis are well documented in plants grown under low water availability, and are also frequently reported in those grown in compacted soils or under P, K, Cu, and Zn deficiencies (Table 13.1). When concluding about stomatal limitations for photosynthesis, based simply on lower rates of transpiration of lower stomatal conductance, one must be wary. Lower rates of photosynthesis result in higher internal levels of CO_2, which will lead to decreased stomatal opening. In other words, a lower rate of transpiration could result from a lower rate of photosynthesis, rather than stomatal limitations being responsible for the lower rate of photosynthesis. In any case, when stomatal closure occurs and limits photosynthesis, usually is not the only diffusional limitation for CO_2 fixation. Once CO_2 has entered the leaf and is in the sub-stomatal chamber, it has to cross the mesophyll to reach the carboxylation sites in the chloroplast. There is increasing evidence for decreases in mesophyll conductance to CO_2 in water-stressed plants and under certain nutrient deficiencies, such as limiting N and Mn (Table 13.1). Lower rates of photochemistry (primary reactions) limits photosynthesis in Fe-deficient plants, and this has been occasionally reported with K deficiency (Table 13.1). Carboxylation, Calvin cycle reactions, carbohydrate metabolism, and transport limitations are more frequently reported under N and K deficiencies, whereas they are occasionally reported in some micronutrient deficiencies, in compacted soils, and with drought (Table 13.1).

When investigating the effects of nutrient levels on photosynthesis, it should be taken into account their possible interactions. For instance, applying P to P-deficient soils increases not only plant P uptake (1.5-fold) but also N uptake (70%, Jin et al. 2015). The nitrate transporter NRT1.1, which functions as a dual-affinity nitrate transporter and nitrate signaling sensor, is down-regulated by Fe deficiency (Liu et al. 2015a). However, most of the interactions occur at the micro-

nutrient level. When planning nutrient deficiency experiments, one should always consider the presence of other nutrients and/or toxic elements, which may induce deficiencies or counteract putative responses. For instance, the presence of Cd in the nutrient solution or adding Zn in excess causes Fe (Larbi et al. 2002) and Mn (Van Assche and Clijsters 1986) deficiency, respectively. Cadmium inhibits the water-splitting reaction in tomato, but the effect disappears after Mn supply (Baszynski et al. 1980). Poplar or wheat leaves recover from Cd stress when an excess of Fe (Solti et al. 2008) or P (Arshad et al. 2016) is supplied. Also, supplying Fe in excess to plants grown under excess Cu almost completely recovered both the physiological (Ouzounidou et al. 1998) and biochemical (Kumar et al. 2008) traits of control plants. Silicon, considered an important element in several crops, has a significant role in limiting Fe deficiency responses by improving photosynthesis and composition of thylakoid protein complexes (Muneer and Jeong 2015) and improving drought tolerance (Ahmed et al. 2016).

Changes in photosynthetic metabolism that occur when plants experience nutrient deficiencies over days or weeks may well be very different from those seen by otherwise incubating chloroplasts in solutions containing different nutrient concentrations for some minutes (discussed for P by Brooks 1986). Similar conclusions can be putatively obtained for other macro- or micronutrients. The exception could be drought, since independently of the duration or intensity of water stress, or growing plants in the field or in pots, responses seem to always be triggered by the low CO_2 concentration attained in the chloroplasts (Flexas and Medrano 2002). One question arises: are responses observed under water limitation always due to a water deficit or at least in part to possible side effects? In potato, for instance, deficit irrigation reduced plant P uptake 22% compared to full-irrigated plants (Liu et al. 2015b), and in field-grown pea, drought decreased P and N uptake 17% and 13% respectively (Jin et al. 2015). It would not be surprising if this should happen with other nutrients.

One aspect that deserves further investigation is the interaction of plants with soil microorganisms. Soil compaction reduced photosynthesis in non-ectomycorrhizal American elm plants, but when inoculated with ectomycorrhizal fungi compaction had little effect on growth and physiological parameters (Polanco et al. 2008). Thus, ectomycorrhizal associations may be beneficial to plants growing in sites with compacted soil (Polanco et al. 2008). In a similar way, the association of arbuscular mycorrhizal fungi with *Populus cathayana* increased photosynthesis and photosynthetic electron transport, especially under drought (Li et al. 2015).

When performing measurements of photosynthesis, researchers often fix leaves perpendicularly to sunlight. Some species, however, change the orientation of their leaves towards light during the day. For instance, both wild soybean (Kao and Tsai 1998) and *Arbutus unedo* (Gratani and Ghia 2002) grown with low water availability have more vertical leaf angles at midday, resulting in lower leaf light absorption. On the other hand, one of the best-known plant movements, phototropic solar tracking in sunflower, is a shade-avoidance response that maximizes light-driven CO_2 assimilation, playing a major role in solar tracking populations of competing sunflower plants (Kutschera and Briggs 2016). The importance of mechanisms such as leaf movements, taking into account probable para-heliotropic leaf movements and leaf folding, should be a matter of future research.

The existence of major gradients within the leaf (Sun et al. 1996b; Nishio 2000) in studies of photosynthesis is an often overlooked fact. The cell composition of superficial cell layers and that of the inner ones is different, and the former are also exposed to higher light intensities than the latter (Sun

et al. 1996a). This within-leaf heterogeneity may cause some difficulties. On the one hand, techniques such as gas exchange or pigment composition, often used to study plant responses to stress, give integrative results of all cell layers within the leaf. On the other hand, Chl fluorescence gives inherently more weight to the effects of stress on the superficial cell layers. Therefore, interpreting data obtained using these very different techniques is not always straightforward. Further work in delineating the varying contribution of the differently assessed metrics is needed to fully understand them.

As an example, the combined use of gas exchange and Chl fluorescence techniques (Valentini et al. 1995; Medrano et al. 2002) was successful for separating the electron transport rate into fractions dedicated to photosynthesis, respiration, and photorespiration in water-stressed plants (Flexas and Medrano 2002). However, it has not yet been possible to use this approach with some plants grown under stress, such as N- or Fe-deficient plants. The reason is that the ratio between photosynthetic electron transport and the sum of net photosynthesis, respiration, and photorespiration decreases below the theoretical value of 4.5–5 (von Caemmerer and Farquhar 1981) with Fe and N deficiency (data recalculated from the literature and our unpublished observations). It is probable that the uppermost cell layers, contributing more to the electron transport rate (estimated with Chl fluorescence), may be more affected than deeper cell layers, which contribute more to the estimation of the photosynthetic rate (measured with gas exchange techniques). In plants affected by drought, the ratio between photosynthetic electron transport and net photosynthetic rates never decreases below 5 (Flexas et al. 1999).

Under some circumstances, reports clearly evidence an altered chloroplast structure and function (as in the case of K-deficient plants and plants deficient in micronutrients; Table 13.1) that can be ascribed to damage. But, could the alteration actually reflect acclimation to the condition? This is a matter that deserves further investigation.

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Chapter 14

The Role of Leaf Movements for Optimizing Photosynthesis in Relation to Environmental Variation

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Summary

There is an amazing array of leaf movements among plants, elicited by a wide variety of environmental signals. Leaf movements may occur over developmental time scales, involving growth processes, or over the scale of milliseconds involving rapid depolarization of membranes and ion fluxes. This chapter covers primarily leaf movements in response to light, although mechanical stimuli, temperature, and water are also discussed. The focus on phototropism, heliotropism, and thermonasty is due to the large effects these movements have on rates of photosynthesis. Heliotropism, whether it is due to differential growth on opposite sides of a plant stem, or turgor changes within a motor organ such as a pulvinus, has direct and large effects on light interception. Diaheliotropic, paraheliotropic, or a combination of the two movements may act to increase photosynthesis by increasing light interception, or optimize resource use efficiency by modulating incident light at intermediate levels. Paraheliotropic and wilting movements are major adaptive mechanisms in response to water deficits, high light, high temperatures, or a combination of these stresses. Thermonastic movements have been shown to play a major role in reducing damage to the photosynthetic machinery during low temperature periods, or conversely in high temperature periods. Despite the mounting number of case studies, a comprehensive understanding of the signal cascades and regulatory genetic pathways controlling rapid, reversible leaf movements has yet to be accomplished. An understanding of the phylogeographic distribution of rapid leaf movements is also lacking. The presence of rapid leaf movements in a number of commercially important crop species suggests that incorporation and modification of these properties may have potential to improve both productivity and stress resistance in cultivated species.

I. Introduction and Overview

Many organs of plants have the potential for photosynthesis including leaves, petioles, stems, fruits, and roots. Unarguably, leaves are the most universal organs utilized for photosynthesis in higher plants. Many leaf structural and functional characteristics have an impact on net photosynthesis rate (A), light use efficiency, and A response to environmental conditions. In fact, some leaf traits at the species level have been identified, which define a globally significant theory called "The Leaf Economic Spectrum" (Reich 2014) reviewed in Chap. 16 of this volume. Although the leaf economic spectrum is an elegant model of leaf function on a species level basis, there is significant variation in that relationship and the leaf economic spectrum doesn't define short-term patterns of photosynthesis. Leaf movements can be particularly important for regulation of A over a time period shorter than the time needed to change leaf physiology, morphology, or anatomy. Factors such as light intensity and variability, position of the solar direct beam, temperature, water balance, nutrient supply, and gravity can induce a myriad of changes in leaf lamina orientation. Many of these induced changes in leaf lamina orientation can be particularly significant to leaf A. In fact, leaf movements can be one of the most important ways that leaves can optimize A and resist damage during rapid environmental change.

The overall goal of this chapter will be to review the current knowledge about leaf lamina movement and its relationship with A. Our philosophy will be to stay away from dwelling on physiological processes that regulate leaf movement because these have been reviewed extensively elsewhere (Ueda and

Nakamura 2007; Luettge and Hertel 2009). The focus of this review will be the significance of those leaf movements to instantaneous rates of photosynthesis, resource use efficiency, and avoidance of stress-induced damage to the leaf's photosynthetic apparatus.

Following an introduction and term definitions, we will consider how leaf movements can regulate incident solar radiation on leaves, which in turn will affect leaf temperature, transpiration, and *A*. Next, we will describe the effects of leaf movements as stress avoidance and resource use efficiency responses. Finally, we sum up the state of knowledge and what ought to be known in order to more fully understand the relationship between leaf movements and *A*.

II. A Classification of Leaf Movements

Plant-induced leaf movements are diverse and occur in response to many environmental stimuli. Therefore, a leaf movement classification system could be founded on the environmental stimulus that induces the movement, the plant response that causes the movement, or the putative functional significance of the movement. We set the stage for our chapter with the following classification of leaf movements; (1) those that are induced by differential cell growth rates within the organs that support the leaf lamina (petioles, shoots), (2) those that are induced by short term changes in cell water balance (turgor pressure), or (3) those that are induced because of the anatomical structure of the leaf lamina or petiole. The particular environmental stimulus will be a second order classification in our system. Moreover, we will use a well-established terminology that defines movements as directional to the stimulus (tropic) or non-directional (nastic). All leaf movements will be included in our classification (Table 14.1), but only those movements that have pertinence to *A* will be covered in our discussion. A focus of our discussion will be the orientation of the leaf lamina with respect to the solar direct beam referred to as the angle of incidence (Fig. 14.1).

A. Leaf Movements as a Consequence of Differential Cell Growth

Differences in the rates of cell expansion on each side of an organ can induce the organ to bend, which can lead to leaf lamina reorientation. Irreversible cell elongation (plastic expansion) is primarily regulated by turgor pressure. In developing plant cells, when turgor pressure exceeds the yield threshold of the cell wall, cells will expand. Based on the Lockhart equation (Lockhart 1965) the rate of expansion equals the extensibility times the difference between turgor pressure and the yield threshold. Thus, the rate of cell expansion and the extent of expansion will depend on the maintenance of turgor pressure and the extensibility of the cell wall. Auxin, an important plant growth regulator, is well known to increase cell wall extensibility in above ground tissues (Kutschera and Niklas 2013). Therefore, differential distribution of auxin activity in the developing leafy shoot or petiole may cause changes in leaf orientation (movement) as long as turgor potential is adequate. Some evidence suggests that auxin inhibitors on the sunny side of the shoot may be the cause of the differential cell growth (Bruinsma and Hasegawa 1990; Hasegawa et al. 2004) rather than differential auxin concentration. This class of leaf movement ranges from short-term, diurnal movements, such as found in *Helianthus* and *Malva* species (Koller 1990, 2011) to long-term, developmental periods during the growth of the leaf support structure (Darwin and Darwin 1896) - and can have significant implications to leaf lamina *A*. In addition, a complex set of regulation processes can induce return movement by differential cell growth on the opposite sides of the shoot during the night so that leaves face east prior

Table 14.1 A classification of leaf movements in plants

	Plastic expansion		Elastic expansion		Anatomy	
Environmental stimulus	Tropic	Nastic	Tropic	Nastic	Tropic	Nastic
Light	Phototropic Heliotropic	Epinastic	Heliotropic	Nyctinastic	Compass Vertical	
Gravity	Gravitropic					
Temperature						Thermonastic
Water				Hydronastic	Hydronastic (wilting)	
Touch	Thigmotropic			Thigmonastic		Leaf flutter
Darkness/shade		Hyponastic				
		Nyctinastic				

Plastic expansion refers to differential irreversible cell expansion. Elastic expansion refers to temporary expansion or shrinkage of specialized cells at the axis of leaf movement. Anatomy refers to the general anatomical structure of the organ under consideration. Tropic indicates movement to or away from the environmental stimulus. Nastic movement is not directional to the environmental stimulus

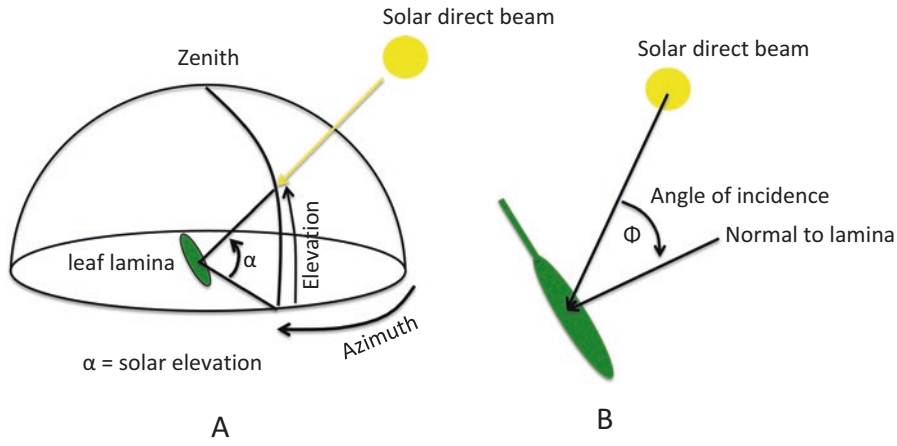

Fig. 14.1. (**a**) A representation of the geometric relationships between the leaf lamina and the solar direct beam in relationship to a geospatial dome of solar inclination over the leaf lamina. α = the solar angle between the position of the sun in the sky and horizontal. (**b**) The geometric relationships between the solar position and a vector horizontal (normal) to the leaf lamina. The angle of incidence is used to calculate the cosine of incidence, an important metric for leaf movement

to the sun rising (Vandenbrink et al. 2014). In the following, we define the major types of leaf movement determined by differential cell growth.

1. Phototropism

One of the earliest recognized plant movement processes, phototropism, defines movement of shoots or coleoptiles of plants in controlled environments based on the position of the main source of radiation (Darwin and Darwin 1896). Differential irreversible cell expansion, between the shaded and sunlit sides of the shoot, induces the shoot to bend toward the solar source. The end result of this movement is a leaf lamina more perpendicular to the solar direct beam. Once the subject cells have matured, and stem growth has ceased, the position of the leaf lamina becomes fixed. Thus, the leaf position will be sustained over a long term as long as the position of the main source of radiation does not change. If the source of radiation changes (potted plant is rotated, or the light source is moved), new growth is needed for the shoot to adjust leaf lamina toward the new direction of the radiation source. The effect of the new orientation will be a new direction of stem growth away from the previous curvature. If the original position of the radiation remained for long enough for the stem tissue to mature and lignify, then the original stem curvature remains. Therefore, older leaves will not change their orientation, but leaves developed after the shift in direct beam will be more perpendicular to the new direct beam angle. Phototropism is usually related to seedling reorientation toward light due to differential cell expansion, but it can occur by changes in cell turgor as well over a shorter duration.

2. Gravitropism

Roots penetrate down and shoots bend upward in response to gravity even in the absence of light. The gravitropic response is usually used to describe root growth in response to amyloplast location (sedimental amyloplasts) and spatial variation in the distribution of auxin in roots. However, gravitropism applies to shoot angle as well (Okamura et al. 2015), which will influence leaf lamina position in relation to the direct radiation beam. Sedimental amyloplasts

have been observed in stems, coleoptyles, hypocotyls, and pulvinules, all of which have the ability to reorient in response to gravity (Hashiguchi et al. 2013). Particularly in grasses, gravitropism determines the angle of the tiller in relation to horizontal. When starch synthesis is low, the gravitropic response is limited and tiller angle becomes less erect allowing more light to penetrate into the grass canopy in morning and afternoon hours, when sun elevation is low, but less light penetration at midday, when the sun elevation is at its highest (Okamura et al. 2015). Therefore, gravitropism is one process that determines leaf lamina angle through its long-term effects on shoot angle and canopy architecture.

3. Hydrotropism

Roots can bend toward a water source in a process termed hydrotropism. There is some controversy whether this is actually a version of gravitropic or thigmotropic response. Therefore, the nature of the hydrotropic assay is critical to determine the existence of the hydrotropic response (Eapen et al. 2015). We will not consider hydrotropism further in this report because it is only known to occur in roots. It may have long term consequences for whole plant *A* in allowing greater water uptake as soils dry. This would act to maintain greater stomatal opening and photosynthetic activity relative to plants without hydrotropic root growth.

4. Thigmotropism

Shoot response to mechanical forces such as wind is often invoked as the cause of "wind swept" canopy architecture. This canopy architecture has been shown to be the result of wood growth and meristem damage rather than a physiologically determined movement of stems away from the predominant wind direction (Telewski 2012). The relationship between mechanical stimuli, primarily wind, and photosynthetic processes is most important in short-term responses such as leaf fluttering, which is described later in this chapter.

5. Epinastic Growth

Differential growth of adaxial versus abaxial cells of an organ may induce the organ to change its orientation. Higher growth rates of adaxial cells of the petiole compared with abaxial cells can induce the leaf lamina to be pointed down (pendent). Pendent leaves have less exposure to radiation at midday than horizontal leaves, which can have a significant effect on leaf carbon gain. Epinastic growth can be an indication of pathogen damage (Lee et al. 2008) and may be primarily due to calcium induced changes in auxin concentration. Epinastic movement is generally not directional to light.

6. Compass Plants

A 'compass plant' is characterized by leaves held in a vertical position as well as a high proportion of leaf lamina oriented in a specific compass direction (Jurik et al. 1990). The vertical position makes the leaf lamina perpendicular to the solar direct-beam in the morning and evening hours, but parallel to the solar direct-beam at midday hours (Fig. 14.2). The term compass plant could also apply to shoots that are pointing predominantly in one compass direction such as that for *Larrea tridentata* in the Sonoran Desert of California (Neufeld et al. 1988). A similar advantage of vertical orientation is obtained by species with photosynthetic stems (Nilsen 1995), particularly suffrutescent shrubs such as species of *Cytisus, Spartium*, or *Senna* (Nilsen et al. 1993; Nilsen and Sharifi 1994). In all cases, the defined leaf or stem orientation is established during development resulting in a permanent position at maturity.

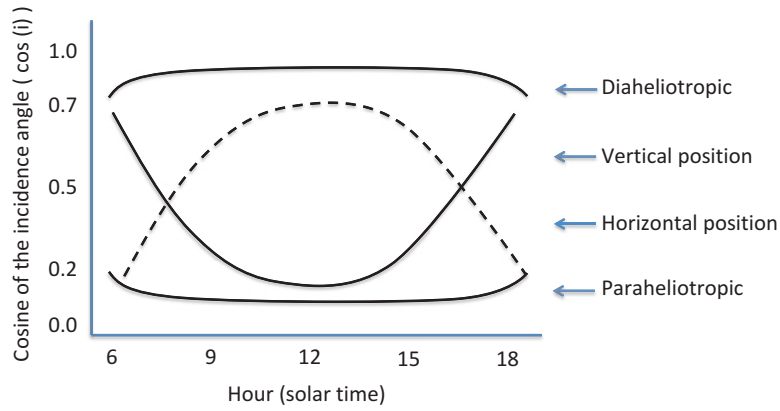

Fig. 14.2. Comparison of the cosine of incidence (representing incident photon flux density) over a diurnal period for leaves with paraheliotropic movement, diaheliotropic movement, a vertical orientation, or a perpendicular orientation to the ground surface. A larger cosine of incidence correlates with a higher proportion of the direct beam photon flux incident on the leaf lamina

B. *Movements Due to Cell Physiological Changes*

Many leaf movements influence *A* over a short-term duration between a few hours and a fraction of a second because the leaf lamina movement is temporary and reversible. These leaf movement patterns are usually associated with rapid changes in turgor pressure, similar to that which control stomatal aperture and induced by an environmental signal. In such cases, changes in turgor pressure in specialized cells (e.g., buliform cells, pulvinus) can regulate the leaf movement patterns. The factors that cause changes in turgor pressure, which induce short-term leaf movement, have been effectively reviewed in the past (Koller 1990; Lüttge 2003; Lüttge and Hertel 2009; Forterre 2013). The following is a broad classification of these short-term leaf movements.

1. *Tropic Movements Toward or Away from a Stimulus – Heliotropism*

Leaves of many species maintain their leaf lamina perpendicular (diaheliotropic) to or parallel (paraheliotropic) to the solar direct beam (Forseth 1990) over a diurnal cycle. The angle of incidence is the angle formed between the solar direct beam and a vector normal (Fig. 14.1b) to the leaf lamina (Fu and Ehleringer 1989). The cosine of incidence, an index of the angle of incidence, is 1.0 when the leaf lamina is normal to the direct solar beam and 0.0 when the leaf lamina is parallel to the solar beam. Leaf lamina position and its variation over the diurnal cycle have a large effect on lamina exposure to PPF (Fig. 14.2). In general, heliotropism is leaf movement in coordination with daily changes in the solar elevation and azimuth (Fig.14.1) in order to maintain a relatively constant cosine of incidence (Fig. 14.2).

Heliotropism can be considered broadly as any leaf or shoot movement in response to the solar angle. However, this term is usually applied only to reversible leaf orientation and excludes phototropism or vertical orientation. Heliotropic leaf movement occurs relatively slowly over the day cycle (12–15 degrees per hour) and leaves cycle back to their morning position over night. The movements are repeatable from day to day and paraheliotopism is often more pronounced as the water availability becomes limiting (Forseth and Ehleringer 1983b). Most leaf heliotropisms occur because of turgor changes in pulvini. However, heliotropic shoot movement, such as that of sunflower,

can also occur because of differential shoot growth (Vandenbrink et al. 2014). Some species with palmately compound leaves having pulvini at the leaf and leaflet level (e.g., *Lupinus arizonicus*) show mixtures of diaheliotropic and paraheliotropic movements, thus demonstrating a fine control of light interception that responds to water availability, temperature, and light (Forseth and Ehleringer 1982). Cultivated legumes such as *Phaseolus* and *Glycine* show a less directed paraheliotropic movement primarily through leaf angle adjustments that maintain the cosine of incidence between 0.5 and 0.8 throughout much of the day, i.e., an angle of incidence of 45–60 degrees (Berg and Hsiao 1986; Fu and Ehleringer 1989; Kao and Forseth 1992a, b). Heliotropic movements may occur at different extents among the leaves in the same plant canopy, which may optimize leaf lamina exposure to radiation and whole plant carbon gain (Ehleringer and Forseth 1989; Niinemets and Fleck 2002). This response is compromised at times because the light sensor is usually located in the pulvinus of most legumes. Shading the pulvinus by leaves, especially in dense agricultural plantings, reduces the effectiveness of heliotropic movements for controlling light interception by the leaf lamina (Koller 2011).

2. *Nastic Movements in Response to a Stimulus*

Thermonastic

Some leaves change their orientation in response to increasing (Fu and Ehleringer 1989; Bielenberg et al. 2003; Zhu et al. 2015) or decreasing (Nilsen 1987) temperatures. Included in this response are heliotropic plants whose paraheliotropic responses are altered by high pulvinus or leaf temperatures (Koller 1990; Kao and Forseth 1992b). The alteration generally acts to reduce solar radiation interception (i.e., paraheliotropism is increased and/or leaf angles become more vertical). Some of those movements occur because of the effects of air temperature on leaf water relations (Nilsen 1987), while others respond to supra-optimal temperatures (Kao and Forseth 1992b) or critically low temperature (Nilsen 1985). However, leaf temperature is dependent on both PPF and air temperature, which makes it difficult to separate thermonastic and heliotropic leaf movements.

Nyctinastic

Commonly called "sleep movements", leaves of some species change orientation at dusk and return to their original position the next dawn. Nyctinastic movements have been the subject of detailed scrutiny particularly in relation to regulation of plant circadian clocks (Satter and Galston 1981; Moran 2007). Usually the leaflets of a compound leaf fold up or down at dusk pressing the opposite leaflets together. These movements have little significance to photosynthetic rate because they occur from dusk to dawn. Yet, their influence on nocturnal transpiration, venting high intercellular CO_2, avoiding low temperatures due to radiative cooling (in temperate floras), leaf area loss to herbivores, or nutrient leaching due to nighttime rainstorms may secondarily influence whole canopy carbon gain and water use efficiency (WUE) over the long term.

Hyponastic

Leaves on plants with a rosette, such as *Arabidopsis thaliana*, can adjust the angle of the leaf in relation to the ground by hyponastic leaf movement. Petioles elongate and leaves move up into a steeper orientation at low radiation and move to a lower angle at times with higher radiation. These movements are considered part of the shade tolerance syndrome (Pierik and de Wit 2014) and may have some implications for *A* when competing plants reduce PPF through shading.

Thigmonastic

Mechanical disturbances of leaves can induce rapid responses. The best known case of thigmonasty is the "sensitive plant" *Mimosa pudica* (Scorza and Dornelas 2011). These responses are usually the result of rapid changes in turgor pressure due to ion fluxes into specialized motor cells (Scorza and Dornelas 2011). Some genetic factors for motor cell function have been identified (Chen et al. 2012). Thigmonastic leaf movement can interfere with photosynthesis while the leaves are folded (Amador-Vargas et al. 2014). We will not be discussing this leaf movement pattern further because there is little implication of thigmonasty to leaf photosynthesis, other than interference and long-term effects due to avoiding reductions in leaf area due to herbivory.

3. *Movements Due to Anatomical Structure*

Leaf Fluttering

Leaves that have a large leaf lamina compared with the petiole support structure tend to flutter when they experience windy conditions. The fluttering movement can be rapid and may have significant implications to leaf energy budget and canopy carbon gain (Roden and Pearcy 1993; Roden 2003). Leaf fluttering is enhanced when petioles are relatively long and twisted. In this case, the leaf movement is not associated with rapid changes in turgor pressure of motor cells, but rather due to the interaction of petiole anatomy and wind forces on leaf lamina.

Hydronastic

Changes in leaf angle relative to the direct beam can be just due to bulk turgor reduction in a succulent petiole. For example, leaf laminas of many temperate *Rhododendron* species become more pendant when either cold or drought induces reduced bulk turgor pressure in the petiole (Nilsen 1987). Moreover, stems or leaves of some grass species curl in response to bulk tissue water loss (Moulia et al. 2006; Alvarez et al. 2008). These changes in angle, or curling in response to water loss, can have significant effects on photosynthetic parameters and leaf level WUE (Bao and Nilsen 1988; Lebkuecher and Eickmeier 1993; Russell et al. 2009).

Low water potential induced by excessive transpiration or an inhibited supply of water can result in leaf drooping due to loss of turgor and the subsequent wilt. Also, rapid increases in radiation in an otherwise low radiation condition (sun patches) can induce leaf wilting (Young and Smith 1979; Smith and Hughes 2009) in some species. Wilting refers to leaf drooping, which can reduce the solar load on the leaf at midday and minimize damage by high temperature and excessive radiation. The likelihood of wilting at bulk tissue turgor loss is dependent upon the rigidity of the leaf lamina and petiole. Species with rigid anatomical structure of leaf lamina and petiole will not wilt when bulk turgor loss occurs. Therefore, the impact of wilting (a form of hydronastic leaf movement) on A is determined by the interactions among leaf or petiole anatomy, leaf water relations, and photosynthetic parameters.

III. Relationships Between Leaf Photosynthesis and Leaf Movements

Most types of leaf movement have a direct or indirect relationship with photosynthesis (Table 14.2). In the following we discuss the relationship of each type of leaf movement with photosynthetic optimization or stress avoidance. In our discussion, we highlight some case studies for each type of leaf movement and its interaction with A. We hope this provides the reader with a starting point to access the breadth of excellent examples available in the literature.

Table 14.2 Representative case studies for the interaction between leaf movement patterns and photosynthesis, including species, habitat, and source in the primary literature

Habitat	Movement	Species	Source
Temperate understory	Thermonastic	*Rhododendron maximum*	Nilsen (1985)
	Paraheliotropic	*Oxalis oregana*	Powles and Bjorkman (1981)
	Heliotropic	*Amphicarpa bracteata*	Prichard and Forseth (1988a)
	Hyponastic	*Arabidopsis thaliana*	van Zanten et al. (2010)
	Gravitropism (shoot)	*Arabidopsis thaliana*	Hashiguchi et al. (2014)
Temperate shrubland	Epinasty	*Rhamnus croliniana*	Stewart and Graves (2004)
Temperate field	Compass plant	*Lactuca serriola*	Werk and Ehleringer (1985)
	Diaheliotropic	*Malva parviflora*	Greer and Thorpe (2009)
	Diaheliotropic	*Lavatera cretica*	Koller (1981)
Temperate ag. field	Heliotropic	*Glycine max*	Kao and Forseth (1992b)
	Hydronastic rolling	*Sorghum bicolor*	Corlett et al. (1994)
	Diaheliotropic	*Gossypium hirsutum*	Ehleringer and Hammond (1987)
	Heliotropic	*Phaseolus vulgaris*	Berg and Hsaio (Berg and Hsiao 1986)
Temperate beach	Heliotropic	*Strophostyles helvola*	Prichard and Forseth (1988b)
Temperate aquatic	Paraheliotropic, Diaheliotropic	*Marsilea quadrifolia*	Kao and Lin (2010)
Temperate canopy	Flutter	*Populus tremuloides*	Roden (2003)
Sub-alpine	Wilting in sunpatch	*Penstemon parryi, Verbascum thapsus*	Knapp and smith (1988)
Alpine	Thermonastic	*Saxifraga paniculata*	Neuner et al. (1999)
Sub-tropical, montane	Heliotropism	*Pueraria montana*	Forseth and Teramura (1987)
Sub-tropical ag. field	Vertical position	*Oryza sativa*	Nobel et al. (1993)
Tropical dry savanna	Vertical position	*Styrax camporum*	Feistler and Habermann (2012)
	Nyctinasty	*Albizzia julibrissin*	Hillman and Koukkari (1967)
	Thigmonasty	*Mimosa pudica*	Amador-Vargas et al. (2014)
Tropical understory	Hydronastic rolling	*Ctenanthe setosa*	Nar et al. (2009)
	Paraheliotropic	*Calathea lutea*	Herbert and Larsen (1985)
Tropical canopy	Paraheliotropic (leaf-folding)	*Bauhinia tenuiflora*	Huang et al. (2012)
Desert-winter annuals	Diaheliotropism	Multiple species, *Malvastrum rotundifolium*	Mooney and Ehleringer (1978), Ehleringer and Forseth (1980), Forseth and Ehleringer (1983a)
	Paraheliotropism	*Lupinus arizonicus*	(Forseth and Ehleringer 1983a)
Desert summer annuals	Diaheliotropism	Multiple species	Ehleringer and Forseth (1980)
	Diaheliotropism	*Amaranthus palmeri*	Ehleringer (1983)

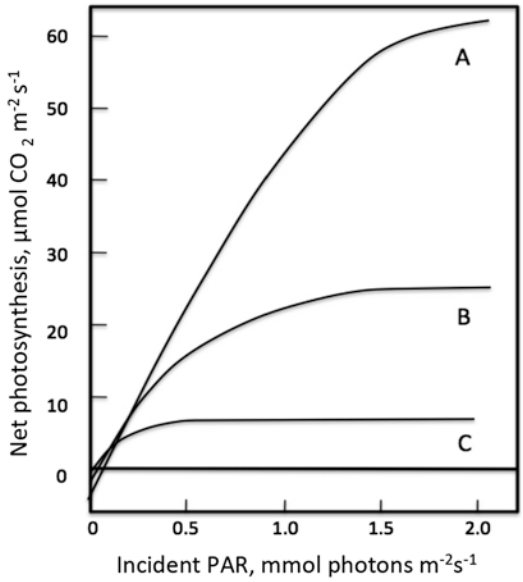

Fig. 14.3. Net photosynthesis (*A*) versus photosynthetic photon flux (PPF) for three different photosynthetic capacities. Curve A represents some desert, high light annuals that do not photosaturate. Diaheliotropic movements act to increase carbon gain for this type of response. Curve B represents a more typical curve seen in many temperate and tropical C_3 species. Incident light levels above the inflection point (photosaturation) would not increase photosynthesis, hence diaheliotropic movements would increase photosynthesis only in morning and afternoon hours. Paraheliotropic movements may increase resource use efficiency by keeping incident light levels near the point of photosaturation. Curve C represents an understory plant or a plant with low photosynthetic capacity due to environmental limitations such as water stress. Here, diaheliotropic movements would have little effect on *A*, while paraheliotropic movements may limit excess excitation energy, reducing high light associated damage and supra-optimal leaf temperatures

A. *Effects of Leaf Movements on Diurnal Patterns of Leaf Photosynthesis*

Incident solar radiation on a leaf and the leaf's photosynthetic capacity, i.e., the photosynthetic light response curve (Fig. 14.3), will determine the potential effects of leaf movement on carbon gain. Depending upon the biochemical carboxylation capacity and the environment, e.g., water and nutrient supply, temperature, CO_2 concentration, and light intensity, photosynthesis will photosaturate at a range of incident PPF (Fig. 14.3). Generally, leaf development in lower light levels, water deficits, low nutrient availabilities, and supra or sub-optimal temperatures will lead to lower light saturated levels of photosynthesis (Curve C in Fig. 14.3). Therefore, the effectiveness of diaheliotropic leaf movements in increasing leaf carbon gain will generally be greater for plants with a higher light saturation point.

The sun's elevation in the sky will vary with time of day and season and this, coupled with leaf orientation, will determine the solar radiation angle of incidence for the leaf (Fig. 14.1). Diaheliotropism, because it acts to maintain leaves perpendicular to direct solar radiation, has two primary effects on the interception of solar radiation for a leaf. First, it will maximize solar radiation integrated over the day; second, it will increase morning and afternoon incident radiation relative to that of a horizontal leaf (Fig. 14.2). Given the ability of plants to use these higher radiation levels for photosynthesis (curve A in Fig. 14.3), diaheliotropism will increase total daily photosynthetic carbon gain relative to a stationary, horizontal leaf. Estimates for this increase vary, from 10–30%, depending upon the species and environment (Shell and Lang 1976; Mooney and Ehleringer 1978; Forseth and Ehleringer 1983a; Greer and Thorpe 2009). Forseth and Ehleringer (1983a) calculated that daily carbon gain advantages of diaheliotropism are highly sensitive to leaf temperature and water potentials. Higher temperatures and lower water availability reduce the total daily enhancement of carbon gain for diaheliotropic leaves because these variables reduce maximal photosynthetic rates and photosaturating light levels (Curves B and C, Fig. 14.3).

The largest contributions of diaheliotropism to daily photosynthesis occur primarily at low solar elevation angles, i.e., temperate latitudes and morning and afternoon hours (Fig. 14.2). Greer and Thorpe (2009) calculated the daily carbon gain benefit of diahe-

liotropism for *Malva* in midsummer to be 13% at 15 degrees latitude and 21% at 65 degrees latitude. For desert winter annuals of the southwestern United States, growth begins during late winter/early spring when ambient temperatures are sub-optimal for photosynthesis. Diaheliotropism under these conditions not only increases light interception, but also raises leaf temperatures to more optimal levels in cool morning and afternoon hours. In addition, cooler temperatures in morning and afternoon hours result in lower leaf to air vapor pressure deficits, the driving gradient for transpiration. Increasing light interception and photosynthesis during periods of lower vapor pressure deficits increases leaf level WUE. Thus, for desert annuals, diaheliotropism provides advantages in both photosynthesis and WUE, particularly early during the growing season.

In contrast, paraheliotropism will reduce light interception, and potentially photosynthesis, depending upon the photosaturating level of PPF for photosynthesis. For many C_3 species, photosynthesis is photosaturated at 1/3 to 1/2 full-midday sunlight (Curve B in Fig. 14.3). For leguminous species (e.g., *Phaseolus, Glycine*, *Strophostyles)* that show combinations of diaheliotropism in the morning and afternoon, with paraheliotropism at midday, incident radiation levels are maintained close to the photosaturating light level for greater periods of the day than for static, horizontal leaves (Kao and Forseth 1992b). These movements are modulated by environmental factors, and vary along with photosynthetic response. For example, factors that reduce photosynthesis, such as supra-optimal temperatures, reduced water availability, and lower nutrient supplies will increase paraheliotropism and reduce incident light levels (Fu and Ehleringer 1989; Kao and Forseth 1992a). Lower leaf temperatures and transpiration due to leaf movement reductions in light interception will increase WUE and reduce potential damage to the photosynthetic apparatus. These effects act to compensate for any marginal reductions in *A* due to reduced light interception.

B. Avoiding Stress on Photosynthetic Performance

1. Avoiding over Excitation of the Photosystems

Environmental factors that reduce *A* efficiency or damage the leaf *A* apparatus are associated with leaf movements that reduce incident radiation, i.e., paraheliotropism and wilting (Koller 1990). Supersaturation of photosystems with photons can result in damaged photosystems and reduced photosynthesis (Adams et al. 2006). Therefore, to minimize damage to photosystem functionality, excessive photon dose on the leaf lamina must be avoided. Excess is a relative term because excess results from the interaction of PPF and photon energy use. Limitations of down-stream processes are important to determining what level of incident PPF constitutes excessive dose (Demmig-Adams and Adams 2006). If down-stream processes such as CO_2 fixation by a carboxylating enzyme, enzyme activity, substrate availability in the Calvin-Benson cycle, triose phosphate use, or sucrose transport are inhibited due to environmental factors or plant developmental stages, then lower PPF may become excessive. For example, low temperature conditions reduce many enzymatic processes, which lead to an excessive PPF for evergreen leaves during the winter (Russell et al. 2009; Verhoeven 2014). In addition, drought or high salinity induced decrease in stomatal conductance (g_s) often leads to excessive PPF (Ashraf and Harris 2013).

It is well known that plants have several physiological mechanisms for dissipating excess photon energy such as photoinhibition, photooxidation, and heat dissipation through the xanthophyll cycle (Adams et al. 2006). Moreover, several possible physiological processes can reduce the buildup of reactive oxygen species that is a consequence

of excessive excitation of photosystems (de Pinto et al. 2015; Derks et al. 2015; Gururani et al. 2015; Noctor et al. 2015). These processes are important for avoiding deleterious effects of excessive PPF, yet an alternative (or complementary) approach is to alter leaf lamina position relative to the direct solar beam, which can reduce PPF and avoid excessive photon energy absorption. Huang et al. (2012) found that the paraheliotropic plant *Bauhinia tenuiflora* had lower capacities for dissipation of excess PPF than did a stationary-leaved species, *Microcos paniculata*. However, the leaf movements in *B. tenuiflora* compensated for the reduced capacity for physiological dissipation. Similar results were found for *Robinia pseudoacacia* (Arena et al. 2008). Paraheliotropic movements prevented photoinhibition and heat damage in *Macropitilium atropurpureum* leaves (Ludlow and Björkman 1984). Also, diaheliotropic species may show a higher capacity for physiological dissipation of excess light energy. In a comparison of 9 diaheliotropic species with 4 static-leaved species, there was greater midday depressions in photosystem II quantum efficiencies in the static-leaved species than in the diaheliotropic species (Sailaja and Das 1996).

Paraheliotropic leaf movement can minimize solar direct beam PPF absorption. Such leaf movement can occur over the whole day cycle, or a portion of the day, in the whole canopy, or they can occur only in a portion of the canopy leaves (Zhu et al. 2015). Leaf movement patterns, such as paraheliotropism, are coordinated with physiological tendencies. For example, paraheliotropic movements are more likely to occur in species that optimize photosynthesis by modulating g_s and nitrogen content, while diaheliotropic movements are most likely to occur in those species that avoid photosynthetic damage through photon energy dissipating mechanisms (Zhang et al. 2011).

Static, vertical leaf orientation can maximize PPF in morning and late afternoon hours and minimize PPF at mid-day hours, thereby having large effects on photosynthetic rates and water use efficiencies (Werk and Ehleringer 1985). For leaves with erect angles and leaf lamina oriented east and west (compass plants), light interception is highest in morning and afternoon hours, and lowest at midday (Fig. 14.2). This increases photosynthesis during the lower temperatures and vapor pressure differences of morning and afternoon, which in turn increases daily integrated WUE for the plant. Lower midday light interception acts to decrease potentially damaging midday leaf temperatures and lower transpiration rates. Active paraheliotropic leaf movement is more successful at minimizing PPF on a leaf than a permanent vertical position, especially during periods of high temperatures and low water availability (Fig. 14.2). Leaves can be permanently fixed in a vertical orientation during development (Feistler and Habermann 2012) or only become vertical under certain environmental conditions (Nilsen 1985, 1986; Niinemets and Fleck 2002). For example, limiting resource availability such as soil nitrogen can induce vertical leaf position as a mechanism to minimize photosynthetic damage from excessive PPF (Close and Beadle 2006). Moreover, in some cases only a fraction of the leaves in a canopy of one plant are held in a vertical position (Feistler and Habermann 2012), yet there can be a measurable advantage of the few vertical leaves to the whole plant carbon gain during excessive PPF conditions.

Leaves are highly vulnerable to excessive PPF if they develop under low light conditions. For example, the light environment around plants growing under a canopy of other plants (understory) is dominated by low PPF. In order to optimize photosynthesis, such plants produce leaves that are designed to maximize PPF absorption (large leaf areas and horizontal display). However, under some situations the PPF can dramatically increase. For example, seasonal leaf fall, tree falls creating canopy gaps, insect defoliations, timber harvesting, forest edges,

or natural small canopy gaps (resulting in sunflecks) can all induce high PPF that persists for various amounts of time. Photoinhibition will occur when excess PPF is harvested compared to the ability of chloroplasts to fix carbon dioxide and make sugars (Adams et al. 2013). If the high PPF persists for a long duration, such as that occurring after logging a site, then understory plants will either die due to excessive water loss and overheating, or produce a new leaf population that optimizes photoprotection mechanisms. However, if the high PPF is shorter term, such as in sunflecks, sun patches, or seasonal canopy openness, then photoprotective mechanisms including leaf orientation of the extant leaf population become important.

Wilting during sunflecks or sunpatches (Knapp and Smith 1990; Smith and Berry 2013) can result in a decrease in PPF on the leaf lamina over a short time frame. Leaves on understory plants can have high g_s in order to minimize diffusion resistance to CO_2 (Knapp and Smith 1990). During a sunfleck, leaf temperature increases and ambient vapor pressure decreases, which together increase transpiration rate (Knapp and Smith 1988). In order to manage water potential and minimize embolism, g_s decreases after the onset of the sunfleck. When g_s decreases during a high PPF event, excessive photon absorption may occur particularly because the understory leaf has been constructed to optimize photon absorption. Therefore, there is a selective advantage of having a more vertical leaf orientation during a sunfleck to minimize the potential for photooxidative damage. Leaves that tend to wilt during drought also minimize PPF when they experience a long duration sun-fleck (sun-patch). Thus, wilting that results in a vertical leaf angle can help minimize damage to photosystems when leaves are unable to provide sufficient transpirational cooling. There are some understory species that show rapid, reversible leaf orientation to near vertical leaf angles during sunflecks. This includes *Oxalis oregana* (Bjorkman and Powles 1981; Powles and Bjorkman 1981) in the redwood forests of the western United States, and *Amphicarpa bracteata* in the eastern deciduous forests of the U.S. (Prichard and Forseth 1988a). The vertical orientation during sunflecks reduces the probability that leaves will increase in stored sugar, which is highly correlated with photoinhibition and down regulation of photosynthesis (Adams et al. 2014). Calculations of the effects of rapid orientation to near vertical leaf angles in *A. bracteata* during a several minute sunfleck showed a reduction in leaf temperatures of 4 °C, and a 31% reduction in transpiration compared to a horizontal leaf (Prichard and Forseth 1988a).

Low temperature can result in vulnerability to excessive photon absorption and engagement of the xanthophyll cycle for non-photochemical dissipation of absorbed photon energy as heat (Verhoeven et al. 1999). Photosystems absorb incoming photons at low temperature when many down stream enzymatic processes are inhibited from using the energy input. For example, understory evergreen plants in temperate regions maintain leaf lamina during the winter. The highest PPF of the year for these leaves often occurs during the winter because the overstory is deciduous. Thus, the coincidence of low winter temperature and high radiation can lead to excessive photon excitation of the photosystems (Verhoeven 2013). Physiological characteristics of the potential damage and plant responses have been reviewed recently (Verhoeven 2014; Verhoeven et al. 2005). Here we focus on the significance of leaf movements to photoprotection of evergreen understory plants.

Thermonastic leaf movements can contribute to the photoprotection of evergreen understory leaves. That contribution has been best studied for temperate understory species of *Rhododendron* (Nilsen 1992). Thermonastic leaf movement in *Rhododendron* species (Fig. 14.4) can be divided into two movements: (1) decrease in leaf angle at low temperature (Fig. 14.4a) and

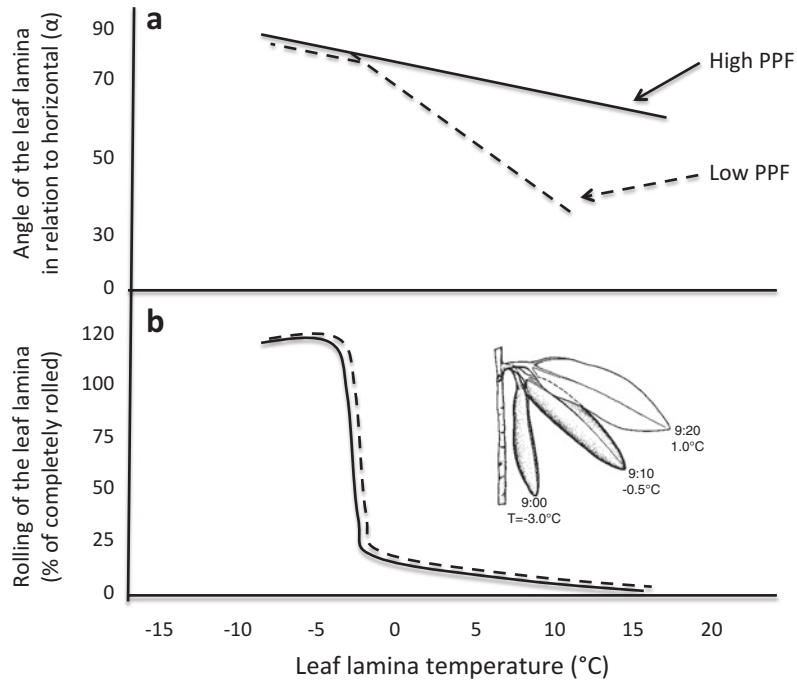

Fig. 14.4. (**a**) The relationship between the α (angle between the leaf lamina and horizontal to the ground surface) where 90° is perpendicular to the ground surface. Under relatively high PPF conditions (under a deciduous canopy in the winter), leaf angle (α) at temperature above –2° C is larger than under low PPF conditions (under an evergreen canopy in the winter). (**b**) The relationship between leaf rolling and leaf lamina temperature for leaves in relatively high or low PPF conditions. Complete rolling occurs when opposite margins of the leaf come into contact with each other. Values for leaf rolling above 100% indicates that opposite margins of the leaf are overlapping in the rolled state. The insert is a line diagram of a thermonastic *Rhododendron* leaf in a temperate habitat. The angle of the leaf in relation to horizontal (α) and the relative rolling of the leaf both respond to leaf temperature increase as the morning proceeds from 9:00 AM to 9:20 AM and the leaf lamina temperature increases from –3 °C to 1 °C

(2) increase in leaf rolling at low temperature (Fig. 14.4b). Manipulative experiments that prevent each or both of these movements during the winter indicate that the primary photoprotective effect is from the change in leaf angle rather than the leaf rolling movement (Bao and Nilsen 1988; Russell et al. 2009). However, the sum of both leaf movements can significantly increase spring carbon gain (Russell et al. 2009), minimize photooxidative damage to chloroplasts (Nilsen and Bao 1988), and maintain leaf photosynthetic capacity for a greater proportion of leaf life (Nilsen 1986, 1992). Thermonastic leaf rolling occurs in response to a critical leaf temperature and is unrelated to water relations traits (Nilsen 1990; Nilsen et al. 2014). The extent of thermonastic leaf rolling is highly correlated with relative winter tolerance of *Rhododendron* species (Nilsen 1991, 1992). Thus, a reduction in leaf lamina exposure to PPF during cold conditions is an important mechanism of photoprotection of overwintering leaves, which promotes spring photosynthesis and allows for extended leaf longevity.

2. *Avoiding Overheating or Nocturnal Freezing*

Coupling of leaf lamina temperature with air temperature is determined by the energy balance. Extreme leaf temperature is most important for larger entire leaves. Temperature of small leaves or divided com-

pound and lobed leaves tends to be more coupled with air temperature. The temperature of large, entire leaves exposed to high PPF can become higher than air temperature, particularly during drought conditions. Heat loss by conductive and convective means tends to be less significant for large leaves and heat loss by latent heat exchange (transpiration) is more important. Therefore, under low water availability, when plants are conserving water by reducing transpiration (decreasing g_s), increasing leaf temperature is likely to occur. Reducing PPF by leaf movement can be a major mechanism of minimizing overheating the leaf lamina (Forseth and Norman 1993) and protecting photosynthetic enzymes from denaturation or photosynthetic membranes from becoming too fluid for proper function. Even with high water availability, reductions in leaf temperatures for the large-leaved species *Pueraria montana* occurred when comparing horizontally restrained leaves to naturally oriented leaves during midday, summer conditions (Forseth and Teramura 1987). Comparison of the temperature of horizontally restrained leaves to photosynthetic temperature response curves showed that the restrained leaves were well above optimal temperatures, and approached temperatures that cause irreversible damage.

Nocturnal leaf freezing may be a problem for species, particularly those with larger leaf size in open habitats, at the northern edge of their distribution. Exposure to a cold sky during the night can cause leaf temperature to drop below air temperature. Horizontal leaf position yields the highest potential for nocturnal leaf lamina freezing. Therefore, vertical leaf position at night can reduce or avoid the possibility of leaf lamina freezing by minimizing exposure to the cold sky temperature. Also, nyctinastic movement and permanent vertical leaf position may serve to prevent nocturnal lamina freezing and thereby prevent damage to photosynthetic membranes by ice crystal formation.

3. *Avoiding Low Water Potential, Conserving Water, and Maintaining WUE*

Several types of leaf movement have an effect on or are related to water relations of the leaf or whole plant. The significance of these leaf movements is accentuated during low water availability. We have already discussed the significance of leaf wilting to minimizing PPF and damage to photosystems during short-term increases in PPF. However, the potential photoprotection by wilting will also help maintain WUE over a longer term when conditions return to normal (Zhang et al. 2010).

Leaf movement can increase water conservation and help maintain higher whole plant water potential (Ehleringer and Forseth 1980; Forseth and Ehleringer 1980). Water conservation induced by reduced transpiration often includes a decrease in g_s. In the absence of leaf movement, relatively high PPF on the leaf lamina during stomatal closure will result in elevated leaf temperature and potential photosystem damage. Also, elevated leaf temperature, relative to air temperature, results in an increase in the vapor pressure gradient between the leaf and the air and counteracts the water saving potential of reduced g_s (Kao and Forseth 1992b). Therefore, paraheliotropic leaf lamina movement can minimize vapor pressure difference between the leaf and turbulent air, which can conserve water loss and increase WUE.

Most leaf rolling and leaf curling movements reduce the percent of the leaf lamina exposed to PPF and decreases the exposure of the abaxial leaf surface (location of stomata) to the turbulent air layer (Kadioglu and Terzi 2007). Although often equated with each other, leaf rolling refers to movement of leaf margins toward the midvein resulting in a cylinder shaped formation (Nar et al. 2009; Nilsen et al. 2014). In contrast, leaf curling should be used to refer to an increased curvature in the midvein of the leaf resulting in an arched leaf form (Soares et al. 2008). Pathogens can induce leaf curling (Sugio

et al. 2015). Thus, leaf curling can be a consequence of pathogen attack and not an adaptation to environmental stress. Yet, leaf curling in the outer sun lit leaves can increase PPF penetration into a canopy to maximize whole plant carbon gain and WUE (Lebkuecher and Eickmeier 1993; Simioni et al. 2013).

Although leaf rolling can be caused by insects and pathogens, it can also occur during exposure to extreme temperature, low water availability, or high salinity. Therefore, leaf rolling is considered an adaptation for both water stress tolerance (Moulia 1994; Kadioglu et al. 2012) and for avoiding high PPF during periods of extreme temperature (Nilsen et al. 2014). A rolled leaf has minimal exposure to direct PPF because most of the leaf lamina has a low cosine of incidence and because a portion of the leaf lamina is overlapped by other leaf lamina. Moreover, the connection between stomata and turbulent air is broken if stomata are on the inside of the curvature. The latter advantage is most important for drought induced or high temperature induced leaf rolling, while there is no significance of leaf rolling to g_s during low temperature induced leaf rolling (Nilsen 1987).

IV. Conclusions

This review highlights a diversity of leaf movements in plants, most of which relate to *A* and WUE (Fig. 14.5). Light intensity, quality, and variation are the primary stimuli for both tropic and nastic leaf movements. Yet many other climatic conditions (temperature, hydration, nutrients, gravity, electrical signals) accentuate or refine the interaction between leaf movement and light attributes. One functional significance of leaf movement is optimization of *A* on an instantaneous level (Fig. 14.5a), or over a longer duration such as a day or season. However, avoidance of stress, usually related to weak down-stream photosynthetic processes, is another important functional significance of leaf movement. Moreover, several leaf movements moderate leaf temperature resulting in improved WUE (Fig. 14.5b). Leaf movements can compensate for ineffective physiological processes for stress resistance, or complement those physiological processes. However, some leaf movement processes, such as thigmonastic or epinastic movements, can interfere with *A*. The broad significance of leaf movement to optimizing *A*, WUE, and stress resistance is a likely reason why leaf movements are so diverse and common in plants. Moreover, the multitude of examples in species found in temperate climates, arid environments, and tropical dry forests is a testament to the significance of leaf movements to stress tolerance.

Although much is known about the physiological causes and functional significance of many leaf movement processes, much can yet be learned. For example, biogeographic variation in leaf movements across the range of a species has not been well characterized, yet this information may provide important insight on the interaction of leaf movement patterns and plant fitness. Utilizing phylogenetic approaches to study types of leaf movement patterns across broad taxonomic groups may provide valuable information about the evolution of leaf movements. Meshing the knowledge of genetic regulation of leaf movement with physiological functional significance of those movements should result in new paradigms based on the synergy of these two fields. Little is known about the signal cascade from light interception to the organ effecting the movement, and even less about how the various environmental factors that have been found to affect leaf movements interact with this signal cascade. It is interesting to speculate how *A* may be optimized and stress resistance increased by incorporating a combination of diaheliotropic and paraheliotropic movements into cultivated species. One initial obstacle may be refining the light sensor location, as the location of the sensor in the pulvini of legumes

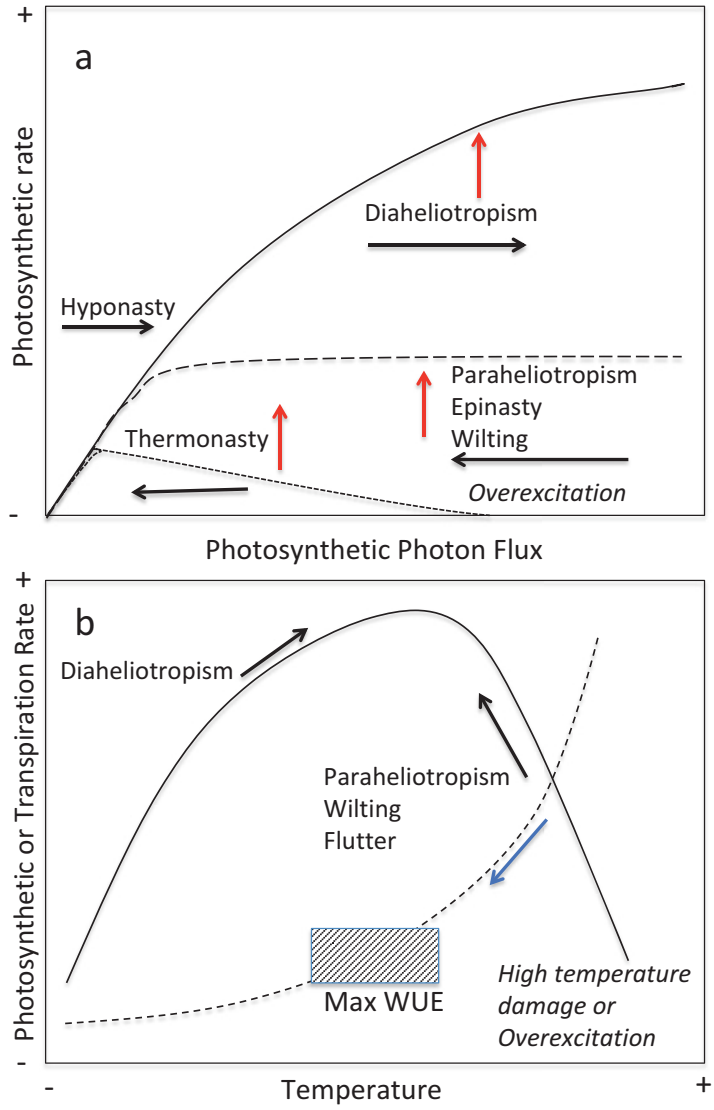

Fig. 14.5. The roles of several leaf movements in optimizing photosynthesis are summarized in panels **a** and **b**. Panel **a** shows a typical response curve for photosynthesis plotted versus incident photosynthetic photon flux (solid line). Diaheliotropism increases incident photon flux (black arrow), which will increase photosynthesis (red arrows) up to the light saturation point. Environmental stress due to soil water deficits, low nutrients, or other environmental factors will reduce the capacity of the leaf to use light in photosynthesis, resulting in the dashed response curve. In these cases, paraheliotropism, epinasty, or, in severe cases, leaf wilting can reduce incident light (black arrows), when excess light is harvested in relation to the amount that can be used to fix carbon into sugars by chloroplasts (represented by the dotted line response curve, red arrow). During low winter temperatures, thermonasty can also reduce damage due to excess light conditions, maintaining photosynthetic capacity (red arrow). Hyponasty can increase PPF under low PPF conditions resulting in increased *A*. Panel **b** shows the consequences for photosynthesis (solid line) and transpiration (dashed line) due to leaf movement effects on leaf temperature. For leaves below the temperature optima for photosynthesis, diaheliotropism may increase leaf temperatures through its effects on incident light level, increasing photosynthesis (e.g., desert winter annuals). For leaves in supraoptimal temperatures, paraheliotropism, leaf flutter, and leaf wilting may decrease temperatures, maintaining them closer to the optimum for photosynthesis, and also reducing transpiration. This also acts to increase water use efficiency (*A*/E) due to the exponential increase in transpiration with increasing temperatures, given constant leaf conductances. With stomatal closure due to water deficits, maintaining lower leaf temperatures is even more important to avoid excess water loss and/or irreversible damage to the photosynthetic apparatus due to overexcitation by excess incident light (Colour figure online)

clearly limits the effectiveness of leaf movements in a dense canopy where many pulvini experience different light levels than the leaf lamina. Lupines may serve as a model in this regard as the light sensor is located both in the pulvini and in basal regions of the leaf lamina (Koller 2011).

Although the molecular regulation and evolution of leaf movements are yet to be completely understood, it is clear that knowledge of the relationships between environmental conditions and leaf lamina position should be included in any study of the factors that determine *A* and leaf or canopy carbon gain. We have shown that leaf physiological limitations can be compensated by leaf movement patterns or physiological stress tolerance mechanisms can be complemented by leaf movement patterns. Further research on the interactions between physiological processes (signal reception, transduction) and leaf movement patterns ought to lead to a much better understanding of the relationship between environmental conditions and photosynthetic processes.

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Chapter 15

Photosynthetic and Photosynthesis-Related Responses of Japanese Native Trees to CO_2: Results from Phytotrons, Open-Top Chambers, Natural CO_2 Springs, and Free-Air CO_2 Enrichment

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Summary

We explore the effects of elevated CO_2, in relation to other environmental factors, on leaf photosynthesis, the functioning of other organs, and the plant as a unit, primarily in tree species and herbs common to cool temperate forests in northeast Asia. First, results of a series of chlorophyll fluorescence and gas exchange studies using white birch as a model tree species are discussed. Excess energy appears to be suppressed by enhancing photosynthetic capacity or thermal dissipation, depending on the availability of nitrogen in both current and elevated CO_2 levels. Next, evidence suggests adaptation of wild plants to CO_2 near springs. If some adaptation occurs, plants will not necessarily respond like current plants to future environmental change. Finally, physiological ecology of woody plants grown in open top chambers and Free-Air CO_2 Enrichment (FACE) is summarized in relation to the changing environment. This summary emphasizes that effects of future environments on plants should be examined by paying attention not only to CO_2 but also to various environmental components, such as soil types, nutrient availability, herbivores, mycorrhizae, ground level O_3, and methane emission.

I. Introduction

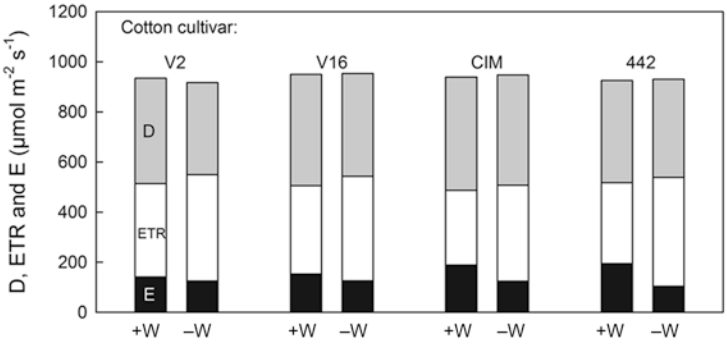
Here we summarize ecophysiological processes of terrestrial plants, ranging from the leaf to stand levels, under elevated CO_2 and other related climatic factors. The studies were mainly conducted in the northeastern part of Asia. Investigations into the effects of elevated CO_2 on crops started in 1970s, when improved cultivation techniques were needed in greenhouses. The term "CO_2 fertilization" is now common in Asia (Yabuki 2004). Additional studies were initiated when the International Biological Program (IBP) started in 1968. The program aimed at estimating the biological production capacity of the Earth. Most living organisms, including humans, depend on the primary production of terrestrial plants. The topic of "plant productivity" was reviewed by Japanese groups (Kira and Shidei 1976; Monsi and Saeki 1978) with a special focus on CO_2 and plant productivity (Yabuki 2004). Totsuka (1966) suggested an essential role of CO_2 in the estimation of photosynthetic production in relation to atmospheric pollution.

Many studies have been carried out in free air CO_2 enrichment (FACE) systems (Hendrey 1992) and the results summarized (Nösberger 2006) to better understand plant functioning in a world of elevated CO_2 (Canadell et al. 2007). We provide a summary of traits of photosynthetic acclimation of Japanese wild plant species to CO_2, along with accompanying morphological and ecological changes at individual plant or stand level. Plant CO_2 responses at the molecular and cellular levels have been detailed elsewhere (e.g., Terashima et al. 2014). In this chapter, results of a series of chlorophyll fluorescence and gas exchange studies in relation to elevated CO_2 using white birch as a model tree species (Koike 1995) are shown. Next, studies on wild plants growing near CO_2 springs are discussed, and an adaptation to elevated CO_2 is argued (Hikosaka et al. 2016). Finally, physiological ecology of woody plants grown in open-top chambers (OTCs) and FACE systems is summarized in relation to the changing environment as suggested by Emberson et al. (2004) and Kobayashi (2015).

II. Sensitivity of Japanese White Birch Leaves Grown under Elevated CO_2 and Long-Term Drought to PS II Photoinactivation

A. Introduction

Although light is essential for plant growth, excessive light energy neither consumed by electron transport nor dissipated as heat may cause photoinactivation of photosystem (PS) II (Kato et al. 2003; Kornyeyev et al. 2010). Plants can regulate excess energy by a functional coordination of electron transport and xanthophyll-related thermal energy dissipation (Demmig-Adams et al. 1996). For example, leaves within a Japanese oak canopy can keep excess energy below a certain level at the maximum irradiation during sunflecks throughout the canopy (Kitao et al. 2012, 2018). To study the fate of absorbed light energy, we use chlorophyll fluorescence parameters; "excess energy" (E) calculated as $(1-qP)\ F_v'/F_m' \times 0.5 \times$ leaf absorptance $\times$ PFD, which has a close relationship with the rate of photoinactivation of PS II, "electron transport rate" (ETR) estimated as $qP\ (F_v'/F_m') \times 0.5 \times$ leaf absorptance x PFD, and light energy dissipated as heat, i.e. "thermal energy dissipation" (D) as $(1-F_v'/F_m') \times 0.5 \times$ leaf absorptance $\times$ PFD (Kato et al. 2003; Kornyeyev et al. 2010). The chlorophyll fluorescence parameter E can be used as an empirical measure of the sensitivity of PS II to photoinactivation (Kornyeyev et al. 2010). In this section, we discuss how the absorbed light energy is partitioned into ETR, D, and E in plants grown under long-term drought and elevated CO_2.



Effect	Water	Cultivar	Water x Cultivar
D	ns	ns	ns
ETR	***	*	ns
E	***	ns	ns

Fig. 15.1. Thermal energy dissipation (D, gray), electron transport (ETR, white), and excess energy (E, black) in four cultivars of cotton (V2, V16, CIM, and 442) grown under well-irrigated conditions (+W) and drought (−W). Measurements were done in the field under a photon flux density (PFD) of approximately 2000 μmol m^{-2} s^{-1} around noon. The data were subjected to a two-factorial ANOVA. Significant effects of water, cultivar, and the interaction of water and cultivar on D, ETR, and E are indicated in the table by ***: $P \leq 0.001$, *: $P \leq 0.05$ and ns: not significant. (Data were re-analyzed after Kitao and Lei (2007)) (Colour figure online)

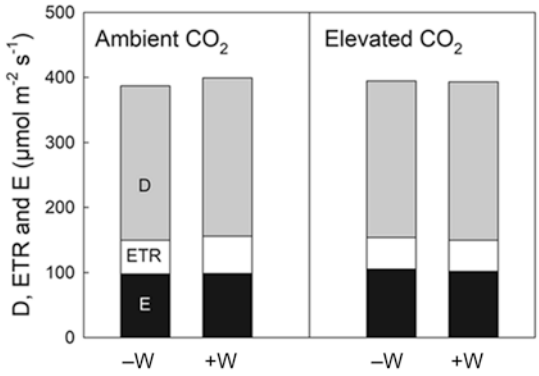
B. *Leaf Physiological Acclimation to Long-Term Drought*

Generally, progressive water stress imposed in leaves that developed under well-irrigated conditions leads to decreases in electron transport rate (Epron and Dreyer 1992; Cornic and Fresneau 2002). Conversely, leaves developed under long-term drought have higher leaf nitrogen (N) with higher photosynthetic capacity, indicated by an increase in the maximum rate of Rubisco carboxylation ($V_{c,max}$) (Kitao et al. 2003; Flexas et al. 2006; Kitao and Lei 2007; Kitao et al. 2007; Aaltonen et al. 2017). Such a photosynthetic up-regulation is considered as an adaptive response to lower sub-stomatal CO_2 concentration (C_i) via stomatal closure under drought stress (Flexas et al. 2006; Snider et al. 2014). Long-term drought reduces plant growth but decreases the ratio of shoots to roots (S:R ratio) mainly due to a greater reduction in shoot biomass, which allows greater N allocation into leaves (Koike et al. 2003; Kitao et al. 2007; Aaltonen et al. 2017).

Based on results from a field experiment, higher electron transport rates were observed in cotton plant leaves that developed under long-term drought in spite of their lower stomatal conductance, as a consequence of photosynthetic up-regulation (Fig. 15.1) (Kitao and Lei 2007). Thermal energy dissipation (D) seemed to have a minor role to circumvent photoinhibition in the leaves developed under long-term drought. Rather, increases in ETR, including both pathways of photosynthesis and photorespiration (Cornic and Fresneau 2002), contributed to keep E at lower levels in the leaves developed under drought. Thus, plants can circumvent photoinactivation under long-term drought by means of changes in carbon (biomass) and N allocation at the whole plant level, and photosynthetic up-regulation at the leaf level.

C. *Excess Energy in Plants Grown Under Long-Term Drought and Elevated CO_2*

Drought causes stomatal closure leading to low C_i (Cornic and Fresneau 2002). Similarly, sub-ambient CO_2 concentration also results in low C_i, but induces opening of stomata (Franks et al. 2013; Temme et al. 2013). In both leaves developed under long-term drought and sub-ambient CO_2, photosynthetic up-regulation along with higher leaf N compensates for the reduced photosynthetic performance under low C_i (Anderson et al. 2001; Kitao et al. 2003; Flexas et al. 2006;



Effect	E	ETR	D
CO_2	ns	ns	ns
Water	ns	ns	ns
CO_2 x Water	ns	ns	ns

Fig. 15.2. Thermal energy dissipation (D, gray), electron transport rate (ETR, white), and excess energy (E, black) in Japanese white birch measured under the growing conditions *in situ*. −W: once-weekly irrigation; +W: daily-irrigation. Measurements were made when soil moisture was at its lowest. PFD of 1000 μmol m^{-2} s^{-1} and 25 °C leaf temperature were used. The data were subjected to a two-factorial ANOVA. "ns" indicates no-significant effect. Area-based leaf N was 0.83, 0.72, 0.74, and 0.66 g m^{-2}; intercellular CO_2 concentration was 209, 300, 387, and 553 μmol mol^{-1} for Ambient-CO_2 − W, Ambient-CO_2 + W, Elevated-CO_2 − W, and Elevated-CO_2 + W, respectively. (Data were re-analyzed after Kitao et al. (2007)) (Colour figure online)

Kitao and Lei 2007; Smith et al. 2012; Pinto et al. 2014). Both long-term drought and sub-ambient CO_2 reduced plant growth, however S:R ratio generally decreased under drought (Koike et al. 2003; Kitao et al. 2007; Aaltonen et al. 2017) but increased under sub-ambient CO_2 (Temme et al. 2013). Conversely, plants grown under elevated CO_2 often show photosynthetic acclimation typically accompanied by a decrease in $V_{c,max}$ with lower leaf N, known as photosynthetic downregulation, particularly under limited N availability (Rogers and Ellsworth 2002; Ainsworth and Long 2005; Kitao et al. 2005; Ruiz-Vera et al. 2017).

Our question is how the absorbed light energy is partitioned into ETR, D, and E in plants grown under long-term drought and elevated CO_2. Seedlings of Japanese white birch were grown under elevated CO_2 (720 μmol mol^{-1} in a phytotron) and long-term drought with limiting N supply. Drought stress was imposed by limiting irrigation frequency to once-a-week (control: everyday irrigation, details in Kitao et al. 2007). Photosynthetic capacity, indicated by $V_{c,max}$, increased under long-term drought with higher leaf N (i.e., photosynthetic up-regulation), whereas elevated CO_2 decreased $V_{c,max}$ with lower leaf N (i.e., photosynthetic down-regulation; Kitao et al. 2007). Lower S:R ratio was observed in plants grown under drought even at elevated CO_2 (Kitao et al. 2007). Thus, both water regime and CO_2 treatment affected photosynthetic capacity in the opposite directions. When drought stress was severely imposed, i.e., the day before regular irrigation, photosynthetic performance under the respective growth conditions was not significantly different among the treatment combinations, where no significant differences were observed in ETR, D, and E (Fig. 15.2). Different photosynthetic capacities combined with different C_i under the treatment combinations resulted in the same extent of E in plants grown under the combinations of different CO_2 and water regimes, suggesting that leaves acclimated to long-term drought and elevated CO_2 could circumvent PS II photoinactivation.

D. Impacts of Drought and Elevated CO_2

The fate of absorbed light energy by leaves was studied in plants grown under long-term drought and elevated CO_2. It is noteworthy that ETR, D, and E remained constant as a consequence of drought-induced photosyn-

thetic upregulation and elevated-CO_2-induced downregulation, which compensated for the difference in C_i among treatment combinations. It is remarkable that plants can cope with drought by a proper adjustment of leaf N investment even under future elevated CO_2. Such a stability observed in E also suggests that keeping E below a certain level to prevent PS II photoinactivation is essential for plants to live under various environmental conditions (Kitao et al. 2012; Kitao et al. 2018).

III. Effects of Long-Term Exposure to High CO_2 Springs in Japan

A. Introduction: CO_2 Springs

Plant response to elevated CO_2 in a time scale over generations is important to predict ecosystem changes under climate change. However, experiments in which ecosystems are exposed to elevated CO_2 for a long time, such as FACE, require considerable economic cost and effort, and may be influenced by artificial effects. Vegetation around natural CO_2 springs has been exposed to high CO_2 concentrations for a long time (Koch 1994; Miglietta et al. 1994; Kohut 2003; Onoda et al. 2007).

Detailed field studies of natural CO_2 springs were started in Italy in the early 1990s. Miglietta and Raschi (1993) and Miglietta et al. (1993a) described several CO_2 springs where CO_2 had been emitted from vents in the Mediterranean climate. Studies on plant responses to elevated CO_2 levels have been carried out at CO_2 springs with respect to photosynthesis (Jones et al. 1995), photoinhibition (Stylinski et al. 2000), water relations (Chaves et al. 1995; Jones et al. 1995; Tognetti et al. 1996, 1998, 1999), non-structural carbohydrate (Körner and Miglietta 1994), isoprene emission (Rapparini et al. 2004; Scholefield et al. 2004), growth (Miglietta et al. 1993b), tree-ring expansion (Hättenschwiler et al. 1997; Tognetti et al. 2000), and litter quality (Gahrooee 1998; Cotrufo et al. 1999). Although a number of studies have been conducted in Italy, only a few comparable studies have been carried out in different climatic regions (e.g., New Zealand, Newton et al. 1996; Iceland, Cook et al. 1998; Venezuela, Fernandez et al. 1998; Slovenia, Vodnik et al. 2002). Differences in precipitation and temperature may influence the effect of elevated CO_2 levels on plants (Long 1991; Idso and Idso 1994; Drake et al. 1997). Furthermore, interspecific variations in CO_2 responses may be evident across plant communities that were established at different CO_2 springs (Poorter 1993). Therefore, studies on natural CO_2 springs in different climates are crucial to generalize plant response to long-term elevated concentrations of CO_2.

The Japan Archipelago is situated on the Pacific volcanic belt and has at least 82 CO_2 springs (Kimbara 1992; Cook et al. 2000). Onoda et al. (2007) first searched Japanese CO_2 springs that were suitable for biological research and described the CO_2 environment to establish study sites. A search for CO_2 springs followed former geological investigations (Kimbara 1992; Tsurumi et al. 1999; Cook et al. 2000). The investigators visited many springs and assessed whether they could be used for biological research with respect to the following aspects: (1) the CO_2 concentrations around the springs are continuously at high levels, (2) toxic gasses for plants (e.g., H_2S, SO_2) are not emitted or are at levels too low to affect plant growth, (3) the springs are cold, and (4) natural vegetation is abundant around the CO_2 spring. Onoda et al. (2007) described environmental characteristics of three CO_2 springs: Ryuzin-numa, Yuno-kawa and Nibu (Nyuu).

Ryuzin-numa, which is characterized by the presence of a pond, is a semi-open space surrounded by a birch–oak forest with more than 20 species of shrubs and herbs (Fig. 15.3a). A gradient of CO_2 concentrations was observed along a distance from the vent. Mean daytime CO_2 was about

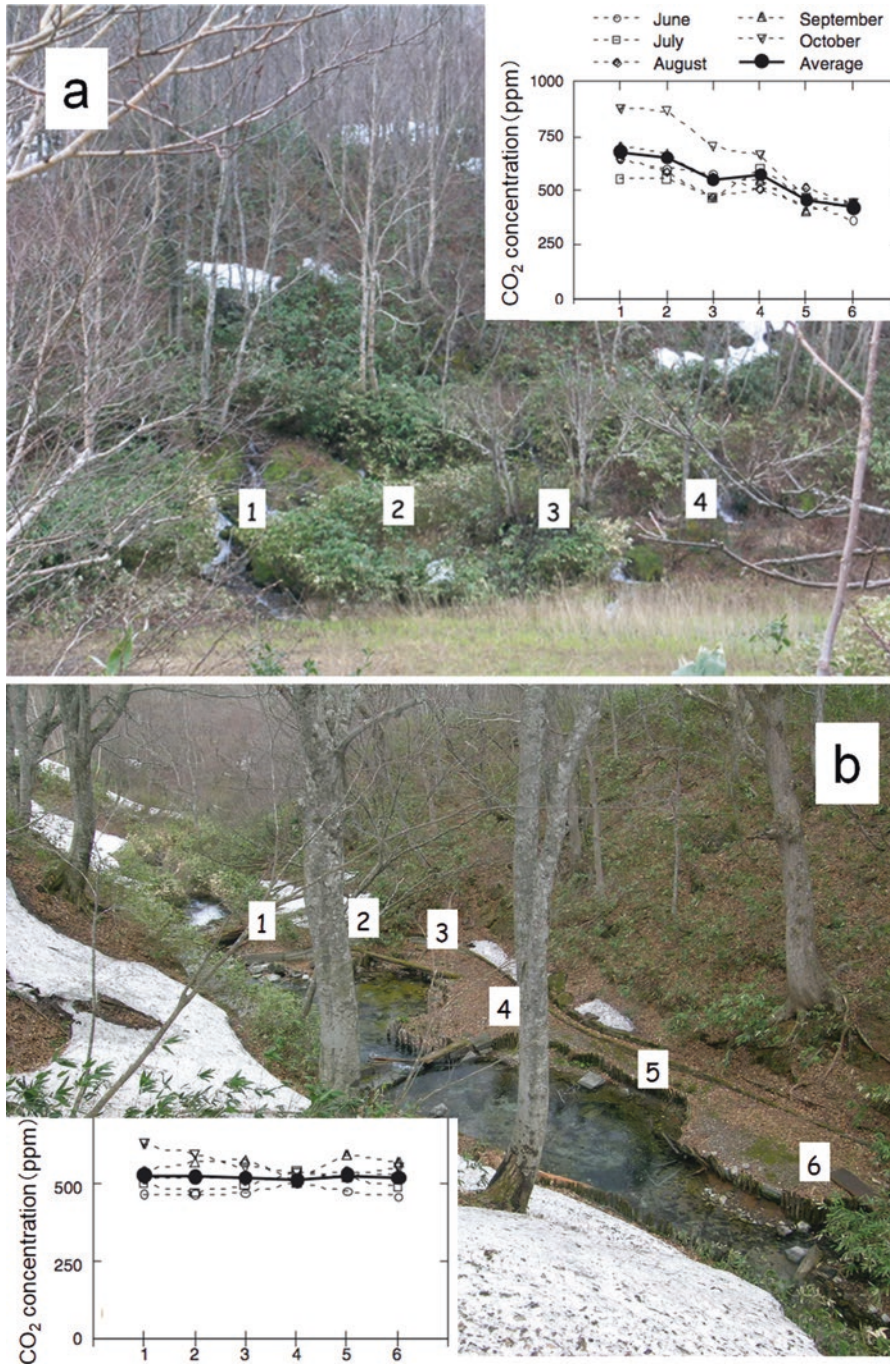


Fig. 15.3. Photographs and CO_2 concentrations in Ryuzin-numa (**a**) and Yuno-kawa (**b**). CO_2 concentrations were taken from Onoda et al. (2007). X-axis in the graphs denotes positions where CO_2 concentration was determined in the photographs. (Photographs were taken by Kouki Hikosaka)

680 μmol mol^{-1}. There was no clear seasonal trend in CO_2 concentrations during the growth season. The area with CO_2 above 500 μmol mol^{-1} was approximately 300 m^2.

Yuno-kawa is located in a gentle valley area with mature beech–oak forests (Fig. 15.3b). The CO_2 concentration here was relatively stable and stayed at around 530 μmol mol^{-1}. The vertical gradient of CO_2 was fairly small from 0.1 m to 5 m above ground, and no diurnal change in CO_2 concentration was observed (data not shown). CO_2 concentration was high along the stream and decreased gradually with distance from the springs but was still higher than ambient CO_2 300 m downstream from the spring. The approximate area where mean CO_2 was above 500 μmol mol^{-1} was greater than 400 m^2.

Nibu is located on a gentle hill-slope facing west and is covered by a species-rich beech-oak forest. CO_2 emissions at this site were the largest among the three CO_2 springs, and a large area with high CO_2 levels was maintained. The stream of CO_2-rich water also resulted in an adjacent open area having high CO_2 levels. CO_2 concentration in this open area fluctuated, due to varying wind speeds, but was higher than that of ambient CO_2 with a gradual reduction with distance from the vent. CO_2 concentration decreased gradually with height, but it was still higher than ambient at 5 m above the ground surface.

Tsurumi et al. (1999) described another high CO_2 area called "gas hollow". The hollow (Hollow A) has CO_2 vents in several positions and its size is 22 m long × 20 m wide × 7 m deep. CO_2 was emitted from the vents (2 tons day^{-1}) and the maximum CO_2 concentration of the source gas was 24% (240,000 ppm). CO_2 concentration was highest at the bottom and lowest at the edge of Hollow A.

Onoda et al. (2005) studied species composition in Hollow A. There is another hollow (Hollow B, 18 × 18 × 4 m) 60 m away from Hollow A, which has no CO_2 vent. Because of a fatal accident in Hollow A in 1997, almost all trees and ground vegetation in and around both hollows were cut down in 2000 except for a few beech (*Fagus crenata*) trees (4 trees at Hollow A and 1 tree at Hollow B). The original vegetation in the hollows consisted of mature beech and oak trees with bamboo (*Sasa kurilensis*) as a dominant vegetation on the forest floor. After the original vegetation was removed, the light intensity at ground level increased and more than 50 species naturally started growing (secondary succession). Onoda et al. (2005) investigated the cover of seven species in the two hollows. CO_2 increment significantly affected two species positively and one species negatively. This result suggests that elevated CO_2 modified the structure of a plant community. There was a C_4 species (*Miscanthus sinensis*) in the two hollows, which is considered to be relatively less advantageous at high CO_2 than C_3 species, but its cover was not influenced by CO_2 concentration.

B. *In situ Ecophysiological Traits in CO_2 Spring Plants*

It is known that biological responses of plants to elevated CO_2 vary depending on the time scale. For example, in the short-term, elevated CO_2 increases photosynthetic rate and decreases stomatal conductance. When plants are grown at elevated CO_2 for weeks or months, photosynthetic capacity often decreases, which partly offsets the short-term effect on photosynthetic rates. CO_2 springs provide a unique opportunity to test elevated CO_2 effects over decades. Several studies have examined the traits of wild plants growing around natural CO_2 springs, where geological CO_2 is emitted from vents or mineral springs (Miglietta et al. 1993a, b; Newton et al. 1996; Vodnik et al. 2002; Onoda et al. 2007).

One disadvantage of studying plants around CO_2 springs is the difficulty in establishing appropriate controls, because envi-

ronmental conditions are heterogeneous and often concomitantly change with CO_2 around the springs (Koch 1994; Miglietta et al. 1994). Most previous studies have investigated the effect of CO_2 on plant traits by making pairwise comparisons between high CO_2 sites around the springs and nearby control sites (e.g., Körner and Miglietta 1994; Jones et al. 1995; Cook et al. 1998; Fernandez et al. 1998; Tognetti et al. 1998; Stylinski et al. 2000; Onoda et al. 2007). However, environmental factors such as irradiance and soil N availability often vary widely in natural environments and can directly influence plant traits. Furthermore, plant responses to high CO_2 may be sensitive to these environmental factors (Curtis and Wang 1998); for example, the degree of down-regulation of photosynthesis is often pronounced under low N availability (Ainsworth et al. 2003; Ainsworth and Long 2005). To obtain a comprehensive understanding of plant responses to elevated CO_2 in varying environmental conditions, it is important to assess how plants respond to elevated CO_2 across other environmental factors under field conditions.

Osada et al. (2010) selected 22 stands of a perennial herb *Polygonum sachalinense*, in which not only CO_2 but also irradiance and soil nitrogen availability varied among the stands. They applied multiple regressions and analyzed the dependence of leaf traits on environmental factors. Among the studied traits, irradiance was the most influential factor (Fig. 15.4). CO_2 and its interaction with irradiance and soil N availability affected several leaf traits. Leaf mass per unit area increased and N per mass decreased with increasing CO_2 and irradiance (data not shown). Leaf N per area increased with increasing soil N availability at higher CO_2. The photosynthetic rate under growth CO_2 increased with increasing irradiance and CO_2, and with increasing soil N at higher CO_2. The maximal velocity of ribulose 1,5-bisphosphate carboxylation (V_{cmax}) was affected by the interaction of CO_2 and soil nitrogen, suggesting that down-regulation of photosynthesis at elevated CO_2 was more evident at lower soil N availability. These results indicate that the long-term effect of elevated CO_2 vary depending on other environmental variables.

C. Genetic Variation in Plant Traits Between High and Normal CO_2 Plants

Genetic differentiation in plant communities occurs across natural environmental gradients, such as light, water, temperature, nutrients, and heavy metals (Linhart and Grant 1996). Such differentiation can be found on an even smaller scale (within a few meters), although gene flow between plant communities somewhat counteracts genetic differentiation (Linhart and Grant 1996; Kelly et al. 2003; Jump and Peñuelas 2005). By the same token, plant communities around natural CO_2 springs provide us with a unique opportunity to explore the micro-evolutionary response of wild plants to elevated CO_2 (e.g., Miglietta and Raschi 1993; Körner and Miglietta 1994; Raschi et al. 1999; Kohut 2003). At these sites, plant communities have been exposed to a CO_2-enriched atmosphere over many generations (e.g., Miglietta and Raschi 1993; Onoda et al. 2007); therefore, selection of some genotypes that have a higher fitness at elevated CO_2 could have occurred around natural CO_2 springs (Cook et al. 1998). Several studies revealed genotypic differences in plants grown under the selective pressure of a CO_2 spring: for example, plants originating from high-CO_2 areas show a lower sugar accumulation (Schulte et al. 2002), higher relative growth rate (Fordham et al. 1997), higher shoot/root ratio (Polle et al. 2001), and a large number of small-sized seeds (Andalo et al. 2001).

Onoda et al. (2009) also conducted a common-garden experiment with three pairs of plants from high and normal CO_2 areas using plants from three CO_2 springs. They tested three hypotheses for adaptation to high-CO_2 conditions: a higher photosynthetic nitrogen use efficiency (PNUE); a higher photosyn-

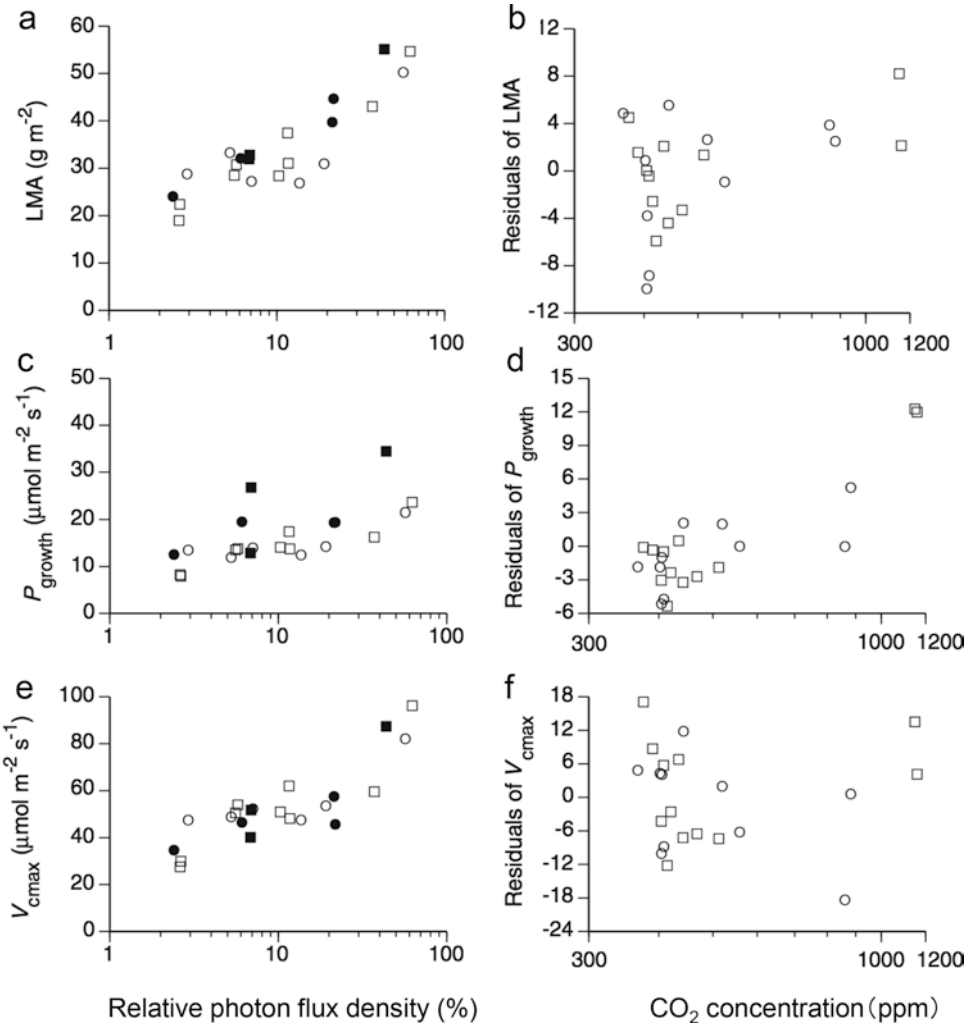


Fig. 15.4. Leaf dry mass per unit area (LMA; **a**, **b**), photosynthetic rate determined at growth CO_2 concentration (P_{growth}; **c**, **d**), and maximum carboxylation rate (V_{cmax}; **e**, **f**) as a function of relative photon flux density (log scale) and CO_2 concentration for leaves of *Polygonum sachalinense* around natural CO_2 springs. **a**, **c**, **e** *Closed symbols* above 500 ppm atmospheric CO_2 concentration, *open symbols* below 500 ppm CO_2 concentration; **a** through **f** *squares* above 10 μg/membrane soil nitrogen availability, *circles* below 10 μg/membrane soil N availability. **b**, **d**, **f** Residuals of variables are plotted against CO_2 concentration. Each symbol denotes mean of stand (three to six leaves). (Redrawn from Osada et al. (2010))

thetic water use efficiency (WUE); and a higher capacity for carbohydrate transport from leaves. Although elevated growth CO_2 enhanced both PNUE and WUE, there was no genotypic improvement in PNUE. However, some spring plants had a higher WUE, as a result of a significant reduction in stomatal conductance, and also a lower starch concentration. Higher natural variation (assessed by the coefficient of variation) within populations in WUE and starch concentration, compared with PNUE, might be responsible for the observed population differentiation. These results support the concept that atmospheric CO_2 elevation can act as a selective agent on some plant traits in natural plant communities.

Nakamura et al. (2011) studied a perennial herb *Plantago asiatica* from Nibu CO_2 spring. Young plants were collected from five quadrats with different CO_2 conditions. Their common-garden experiment revealed that plants transferred from habitats with higher CO_2 had greater leaf to root ratios (Fig. 15.5c), lower photosynthetic rates (Fig. 15.5a), and lower stomatal conductance (Fig. 15.5b) as well as higher relative growth rates (data not shown). The habitat-dependent differences were partly heritable because a similar trend of the leaf to root ratio was found among their offspring grown under normal CO_2 levels (data not shown). Genetic analyses indicated that selfing or biparental inbreeding might promote local adaptation in areas with high CO_2 despite substantial gene flow across the CO_2 gradient. These results indicate that phenotypic and genetic differences have occurred between high and normal CO_2 populations.

D. Implications

Many researchers have conducted elevated CO_2 experiments using 'current plants' to simulate plant growth in a future high CO_2 world. The prediction based on these data is valid if 'future plants' respond to elevated CO_2 in the same manner as current plants. However, studies using CO_2 spring plants demonstrated that such an assumption is not necessarily correct. Future plants that have been subjected to elevated CO_2 for a long time, over generations, may have different traits from current plants as a result of selection. However, studies using CO_2 spring plants are too scarce and it remains unclear whether such differences are common across various species. Since the number of species available for evolutionary study is quite limited, artificial selection experiments are needed to generalize the evolutionary influence of elevated CO_2 and to predict possible evolutionary effects on community structure and ecosystem functions.

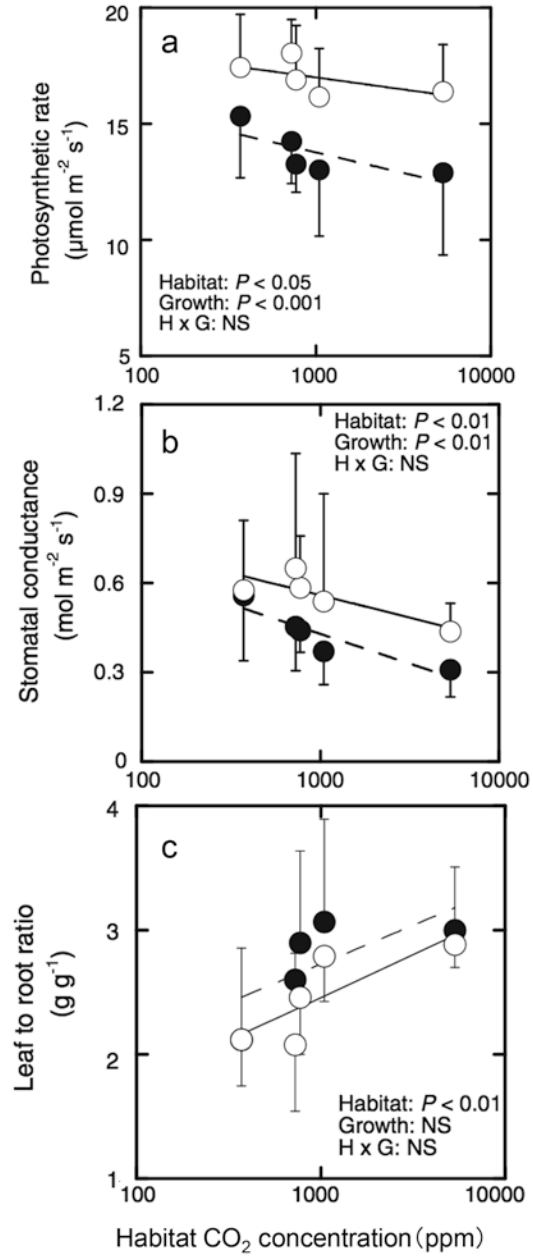


Fig. 15.5. Traits of plants transplanted from different plots as a function of habitat [CO_2]. Photosynthetic rate (**a**) and stomatal conductance (**b**) determined at growth CO_2 and leaf to root ratio (**c**). The closed and open circles denote the means with standard deviations (bars) for individuals that were grown under elevated and ambient [CO_2] conditions, respectively. The results of the analysis of deviance with the generalized linear model are indicated. Lines are linear regression of variables on log-transformed habitat [CO_2], which are shown when the effect of habitat [CO_2] was significant. (Redrawn from Nakamura et al. (2011))

IV. Photosynthesis and Other Processes in Young Deciduous Trees Grown under Elevated CO_2

A. Introduction

Trees have a long lifespan during which they acclimate to a changing environment. Trees have evolved features that allow them to compete above ground for light and space and below ground for water, nutrients, and space (Bader et al. 2009; Wang et al. 2016a, b). Photosynthetic function is mainly regulated by the balance between source and sink activities and allocation of photoassimilates. Photosynthetic production is realized by the activities of individual leaves affected by their leaf lifespan and temporal and spatial changes in the environment (of leaves, branches, and stem) as well as the root system (e.g., Koike et al. 2015; Agathokleous et al. 2016a).

To predict the response of crops and trees to future elevated CO_2, many experiments have been carried out in greenhouses, phytotrons, and controlled environment growth chambers (Aoki and Yabuki 1977; Lemon 1983; Morales et al. 2014). In 1987, Tissue and Oechel found downregulated photosynthesis in a grass grown at elevated CO_2 within an enclosed system established in a field of Alaskan tundra. Arp (1991), who reviewed the literature on CO_2-plant relations, suggested that downregulation of photosynthesis and growth performance of plants raised at elevated CO_2 may be attributed to sink limitation, e.g., root restriction. After the review of Arp (1991), we employed FACE systems (Hendrey 1992) or large OTCs for studies on CO_2 effects on plant growth. To date, important efforts were made to predict the effects of elevated CO_2 on trees and forests using FACE systems. The results of numerous studies on tree responses to elevated CO_2 using FACE systems and other manipulative experiments were reviewed (Norby and Zak 2011; Bader et al. 2013; Irigoyen et al. 2014; Koike et al. 2015). These reviews suggest that CO_2 effects on plants should be mediated by soil fertility, as found, for example, in the Duke FACE (Oren et al. 2001).

Here, we summarize the photosynthetic responses of individual leaves in trees native to northern Japan grown primarily in a FACE (Koike et al. 2015). We also discuss secondary responses, which may result from changes in photosynthetic function, that relate to ecological aspects using this FACE forest stand as a model. Based on these findings, we discuss possible changes in the forest ecosystem of northern Japan in an elevated CO_2 world. We focused on immature volcanic ash soil (VA), which is characterized by deficiency of phosphorous and high porosity, because most soils of northern Japan originated from the activities of volcanoes. In this system we used two soils, brown forest soil (BF: nutrient rich) as the control and BF mixed with VA to simulate soil conditions commonly found in northern Japan.

B. Photosynthetic Responses of Trees to Elevated CO_2

In general, the photosynthetic rate of plants increases under elevated CO_2 for a relatively short period, followed by suppression after a long period of exposure (e.g., Tissue and Oechel 1987; Koike et al. 2015). In fact, downregulated CO_2 uptake was found in seedlings of larch (*Larix sibirica, L. gmelinii* and *L. gmelinii* var. *japonica* x *L. kaempferi*), fir (*Abies sachalinensis*), and birch (*Betula maximowicziana*), all grown in northern Japan (Koike et al. 1996). These plants were grown in pots, with VA as substrate in phytotrons (Yazaki et al. 2005; Watanabe et al. 2014). With addition of N, simulating N deposition in Far East Asia (Yamaguchi and Noguchi 2015), downregulation or photosynthetic adjustment of those plants was not found in most trees except alder (Koike et al. 2015). These findings suggest that N deposition, to some level, may offset CO_2-induced photosynthetic downregulation. Plants gen-

erally cannot expand their root system without limit. Moreover, photosynthetic downregulation is induced by dilution of nitrogen in the plant as a result of CO_2-stimulated acceleration of growth (e.g., Coleman et al. 1993; Norby and Zak 2011).

The FACE experiment in Sapporo showed that elevated CO_2 (=500 μmol mol^{-1}) enhanced growth in two birch species (Monarch birch: *Betula maximowicziana* and white birch: *B. platyphylla* var. *japonica*) and alder (*Alnus hirsuta*) for 3 years, and that clear downregulated CO_2 uptake occurred in birches after 3 years but not in alder. These phenomena, observed in typical light demanding early successional trees, were more clearly found in VA than in BF soil (Eguchi et al. 2008a, b). N content in leaves of birches was lower than those in ambient CO_2 (about 375 μmol mol^{-1} of 2003), whereas foliar N concentration of alder was similar in alder grown in BF and VA soils. Even in FACE, plants cannot expand roots infinitely for obtaining nutrient and water; therefore, birches showed homeostatic adjustment of photosynthesis (Tissue and Oechel 1987). In contrast, alder is a well-known actinorhizal plant and can obtain N from symbionts (*Frankia* sp.), due to enhanced photosynthetic activities of host alder at elevated CO_2 (e.g., Tobita et al. 2011).

C. *Allocation of Photosynthates to Roots*

A certain fraction of photosynthates are allocated to roots, an important process that can regulate rhizosphere dynamics and the CO_2 balance of forest ecosystems in an elevated CO_2 world (e.g., Norby and Zak 2011; McNear 2013; Agathokleous et al. 2016a, b; Wang et al. 2016a). The root production of Siebold's beech (*Fagus crenata*) was studied in trees grown in VA and BF and exposed to ambient (375–390 μmol mol^{-1}) or elevated (500 μmol mol^{-1}) CO_2 during the daytime over the course of 11 growing seasons (Agathokleous et al. 2016b). Elevated CO_2 stimulated the root production of trees grown in VA and increased fine-root production of trees in both VA and BF; these trees displayed an extensive foraging strategy. At the same FACE, roots were studied after a 4-year exposure of three birches (*Betula ermanii, B. maximowicziana,* and *B. platyphylla* var. *japonica*) and one deciduous oak (*Quercus mongolica* var. *crispula*) to the same treatments as Siebold's beech (Agathokleous et al. 2016c). Elevated CO_2 did not increase total root production when the community was grown in BF whereas it did increase total root production in the community grown in VA. Nonetheless, allocation of photoassimilates to plant roots was not affected by elevated CO_2 in either the BF or VA. It was also revealed that trees developed an impressive amount of fine roots in VA at elevated CO_2. Elevated CO_2 induced an increase in the total root biomass of the community grown in VA compare to that grown in BF, perhaps because of a greater need for absorption of nutrients and water through more, or deeper, roots (Bader et al. 2013; Leppälammi-Kujansuu et al. 2014).

Seasonal dynamics of fine roots were studied in Japanese white birch, a typical early successional species with high specific gravity of the xylem (Koike 1991, 1995). Seedlings of Japanese white birch were planted in BF and VA mixed with BF (6:4 in volume). By using mini-rhizotrons (Nakaji et al. 2008; Wang et al. 2016a), it was found that elevated CO_2 decreased the length of living fine roots in BF and VA in all years, except in BF in the first year (Wang et al. 2016b). This is in disagreement with the expectation that the necromass of birch fine roots would increase in response to elevated CO_2 (Karnosky et al. 2003; Wang et al. 2016a). The lifespan of fine roots increased under elevated CO_2 especially in VA at the beginning; however, shorter fine root longevity was observed after 2 years of treatment (Wang et al. 2016b). Hence, it seems that elevated CO_2 does not always stimulate the turnover rate of fine roots, especially during the seedling stage, as it may depend on costs

and benefits of constructing and maintaining or retaining fine roots.

D. Plant Defense: Disease and Insect Herbivores

Under elevated CO_2, the content of carbon-based secondary metabolites often increases with surplus carbohydrates (e.g., Mcelrone et al. 2005; Peltonen et al. 2005). Some secondary metabolites play important roles in tolerance to disease and insect grazing (Lambers et al. 2008). For example, elevated CO_2 reduced the disease severity caused by the fungal pathogen of leaf spot, *Phyllosticta minima*, on red maple, and an increase in secondary metabolites was important for this change (Mcelrone et al. 2005).

Infection by diseases importantly affects forest productivity (Norby and Zak 2011; Koike et al. 2015). Powdery mildew is a common disease of trees and widely infects leaves of oak (Takamatsu et al. 2007). Immature leaves are generally more sensitive to powdery mildew than matured leaves (Edwards and Ayres 1982). Because leaf unfolding in the sprouts is later than ordinary ones, leaves of sprouting seedlings may be particularly infected by powdery mildew.

In order to predict the performance of oak coppice forests in a future changing environment, infection of powdery mildew was examined in leaves of oak sprouts in FACE (Watanabe et al. 2016). In the field, the oak flushes one or two times during the growing season. The average scores for powdery mildew infection in control site and the elevated CO_2 site were 2.0 and 0.6 for 1st flush leaves and 3.8 and 2.6 for 2nd flush leaves, respectively. Growth of sprouts was enhanced and infection of powdery mildew was reduced in the elevated CO_2. Severe infection of oak by powdery mildew clearly reduced the photosynthetic activity of 2nd flush leaves in the control plots compared with elevated CO_2 plots. The growth of oak sprouts in future elevated CO_2 conditions may be enhanced because of reduced infection by powdery mildew and absence of photosynthetic down-regulation with a large root system (Watanabe et al. 2016).

Powdery mildews are favored by relatively dry atmospheric conditions (Aust and Hoyningen-Huene 1986). Although stomatal conductance of leaves is generally lower at elevated CO_2 (e.g., Norby and Zak 2011), it was observed that transpiration in the canopy of oak sprouts tended to increase even at elevated CO_2. The number of leaves generally increases under elevated CO_2 (Koike 1993; Ainsworth and Long 2005). As a result of higher transpiration with a large number of leaves, the relative humidity (RH) within the canopy in the elevated CO_2 may be higher than that of controls. However, no difference was observed in RH between control and elevated CO_2 sites by measuring RH of the canopy. Moreover, the soil moisture content was higher in the elevated CO_2 than the control sites (Eguchi et al. 2005; Kim et al. 2011).

Recently, severe grazing damage of alders (*Alnus hirsute, A. japonica, A. pundula*, etc.) has been occurring in northern Japan and has also been increasing in white birch as compared the period before the 1960s (Tadaki and Akai 1974; Koike et al. 2015). Fajer et al. (1989) suggested insect grazing at elevated CO_2 and higher temperatures may be increased by dilution of nutrients in leaves and an acceleration of growth of larvae based on a feeding experiment with *Plantago lanceolata* grown at elevated CO_2 (700 μmol mol^{-1}) and the specialist insect herbivore *Junonia coenia* (Lepidoptera: Nymphalidae). Poor larval feeding performance on foliage treated with elevated CO_2 may be due to reduced foliar N concentrations (e.g., Coleman et al. 1993). However, as mentioned above, secondary metabolites also increased, as defensive chemicals against insect grazing (Lindroth 1996, 2010; Koike et al. 2006, 2015), irrespective of no change in defense chemicals of leaves of *P. lanceolata*.

The life and life cycle of herbivores depend strongly on host plant leaf quality

and leaf quality changes with elevated CO_2 and N deposition (Koike et al. 2006). Seedlings of four deciduous broad-leaved, two early successional (alder and birch), and two mid-late successional (oak and maple) species were raised under two levels of CO_2 and soil fertility levels in climate-controlled chambers. To predict the relative defense capacity of the plants, a generalist herbivore (wild silkworms; Erisan: *Samia cynthia ricini*, Saturniidae) was utilized in order to avoid data bias. Except alder, larval survival rate and longevity (ML50) were least when fed with leaves obtained from elevated CO_2 (700 μmol mol^{-1}) and infertile soil, and greatest when fed with leaves obtained from ambient CO_2 (360 μmol mol^{-1}) and fertile soil, especially in birch and maple. The longevity (ML50) of Erisan fed with leaves treated at elevated CO_2 ranked as birch>oak>maple. The lifespan of larvae fed with alder leaves was independent of CO_2 treatment, and it was longer when fed with leaves obtained from infertile soil conditions. Alder is actinorhizal and can fix atmospheric N_2 in root nodules formed by the actinomycete *Frankia* sp. Symbiotic microbes can benefit from more photosynthates produced by host plants at elevated CO_2 (Koike et al. 1997; Tobita et al. 2011). Except for alder, leaf chemical traits, particularly the C/N ratio, affected the larval ML50. Decreased leaf N content and increased starch were also found in another FACE experiment where Konara (*Quercus serrata*) and Mizunara (*Q. mongolica* var. *crispula*) oaks were exposed to elevated CO_2 (546 μmol mol^{-1}) and CO_2 + O_3 (O_3 = 52 nmol mol^{-1}, CO_2 = 546 μmol mol^{-1}) (Shi et al. 2016). All these findings suggest that further changes may occur in the interaction among species via insect grazing in an elevated CO_2 world.

E. Roles of Symbionts

Here we should consider the unique responses of alder, an early successional tree that dominates in swampy areas, to elevated CO_2 (Arnone and Gordon 1990; Koike et al. 1997; Tobita et al. 2011). The photosynthetic responses of alder to elevated CO_2 under high phosphorus (P) were considered to be 'photosynthetic acclimation,' while alder presented the obvious 'photosynthetic down-regulation' to elevated CO_2 under low P. Soil P availability affected the growth responses to elevated CO_2 through effects on photosynthetic properties and biomass allocation (Tobita et al. 2011).

The flow of ^{14}C in ECM and non-ECM seedlings of larch and its hybrid larch F_1 was studied (Qu et al. 2004, 2010). Larch seedlings were grown in a greenhouse with larch-forest soil (FM: multiple species of ECM) or *Suillus grevillei* (SM) inoculum, or in the absence of ECM. Plant shoots were exposed to a pulse of $^{14}CO_2$ for 1 h under a PFD of 600 μmol m^{-2} s^{-1}. Seedlings were harvested at 0, 6, and 24 h after the $^{14}CO_2$ pulse. At 24 h, ECM seedlings of both larches allocated ≈2.5% more ^{14}C to roots than non-ECM seedlings. In contrast, FM seedlings of Japanese larch and hybrid larch F_1 allocated 6.5 and 18.0% more ^{14}C, respectively, to the stem than ECM seedlings. P and N content in FM and SM seedlings were significantly higher than in non-ECM seedlings, as was stomatal conductance. In a different experiment, colonization of ECM fungi and species abundance was studied in 2-year-old hybrid larch F_1 seedlings exposed to control (O_3 < 6 nmol mol^{-1}), O_3 (60 nmol mol^{-1}), CO_2 (600 μmol mol^{-1}), and CO_2 + O_3 in OTCs (Wang et al. 2015). Elevated CO_2 increased and CO_2 + O_3 decreased the colonization rate of ECM. Elevated CO_2 has often combined effects with elevated O_3 and may compensate for the negative effects of elevated O_3 (Matsumura et al. 2005; Koike et al. 2012). These results suggest elevated CO_2 may induce shifts in soil biota communities.

Effects of ECM (e.g., *Pisolithus tinctorius*) infection on the growth and photosynthetic traits of red pine (*Pinus densiflora*) seedlings grown at ambient (360 μmol mol^{-1})

and elevated (720 μmol mol^{-1}) CO_2 were investigated (Choi et al. 2005). *Pisolithus tinctorius* inoculation increased the seedling growth and needle phosphate content and net photosynthetic rates at light saturation at both CO_2 treatments. CO_2 and light saturation (maximum assimilation rate) in infected seedlings was also greater than in controls. Seedlings infected with *P. tinctorius* displayed greater WUE and no photosynthetic downregulation at elevated CO_2. It seems that ECM activity was enhanced by some physiological functions related to P and water absorption in seedlings at elevated CO_2. These findings are in agreement with other findings showing that ECM act as a carbon sink and moderate the degree of downregulated photosynthesis (e.g., Qu et al. 2004, 2010).

F. Change in Forest Structure Through Changes in Leaf Area Index

Leaf area index (LAI) is an essential index for estimating photosynthetic production (e.g., Gough et al. 2010). The number of leaves in the top layer was greater in Sapporo FACE compared to many FACEs (Norby and Zak 2011; Koike et al. 2015). As seedlings of the same species were adjacent to each other, species were planted alternately, with a distance of 55 cm between specimens. An increase of LAI was observed until the canopy overstory closed. Particularly, LAI was ≈1.3 times larger at elevated CO_2 than that at ambient CO_2 up to 3 years from the initiation of CO_2 treatment (Eguchi 2008; Hara et al. 2012; Koike et al. 2015). This tendency was well summarized in the previous FACE research for forest trees (Norby and Zak 2011; Koike et al. 2015). This is in agreement with a further experiment where, at elevated CO_2, LAI of a willow stand was 2–3 times greater for less than one month, but sharply decreased thereafter (Koike et al. 1995). It should be mentioned that water availability may drive LAI response in in response to CO_2 (Koike et al. 2015), a phenomenon relating to stomata functioning (Pospíšilová and Ètatský 1999; Eguchi et al. 2005; Eguchi 2008).

Japan usually experiences typhoons from late summer to autumn, hence we simulated the sudden removal of canopy of dominant birches, ash, kalopanax, and basswood in FACE (Watanabe et al. 2016). We monitored leaf photosynthetic traits of these seedlings in a range of light environments (shady to open conditions) in a 10-year FACE experiment. Elevated CO_2 did not affect parameters such as LMA, N content, and biochemical capacity of chloroplasts before removal of the shade trees and after acclimation to open conditions; in fact, a higher net photosynthesis (at light saturation and ambient CO_2) was maintained under elevated CO_2. However, in the year after removal of the shade trees, there was no increase in net photosynthesis under elevated CO_2. In ambient CO_2, LMA and N were higher in the year after removal of shade trees than before, whereas there was no increase under elevated CO_2 conditions. These results indicate that elevated CO_2 delays the acclimation of photosynthetic characteristics of Siebold's beech seedlings to increasing light intensity.

G. Stand Structure and Regeneration Success

Oikawa (1986) predicted that increasing atmospheric CO_2 will increase LAI of the canopy top as long as space permits, and reduce the incident light at the forest floor, based on biomass data in a tropical rain forest. A similar trend was reported in a model tropical stand (Körner and Arnone 1992), a mixed willow stand (Koike et al. 1995), and a spruce stand (Hättenschwiler and Körner 1996). From these studies, it seems that incident light at the forest floor will be decreased or fall below the required levels for survival and growth of regenerated seedlings (Koike 1991). The possibility of acclimatory adjustment of the light compensation point (LCP) of photosynthetic light curves was assessed

in representative tree species native to northern Japan.

LCP of the photosynthetic light curves of eight species of deciduous trees in FACE tended to shift to lower PFD in more shade-acclimated leaves, with an increase of the gradient of the curve at the initial PFD levels (Eguchi 2008; Kitao et al. 2016), as Farquhar et al. (1980) predicted with their theoretical model. The initial slop (Φ_{680}: the value of LED-LiCor at 680 nm, based on incident photons) of the light-photosynthesis curve also increased with increasing shade. From the FACE studies at Sapporo (Eguchi 2008), initial slope at PPF of 680 nm tended to increase in early successional (Monarch birch, white birch) and mid-successional (oak, ash:*Fraxinus mandshurica* var. *japonica*, kalopanax, and basswood:*Tilia japonica*) species, and decrease in late successional species (beech, maple). Based on phytotron (Kitao et al. 2016) and FACE studies, deciduous plants may increase their shade tolerance capacity at high CO_2 as also summarized by Norby and Zak (2011).

Leaf photosynthetic traits were measured in shade-grown seedlings of trees with different successional traits raised under elevated CO_2 and different light conditions. The shade tolerance was evaluated in Monarch birch and white birch as early successional species, oak as a mid-successional species, and maple as a late successional species. LCP decreased in all species when measured under elevated CO_2, accompanied by higher initial slope at PPF of 680 nm and little change in the dark respiration rate (Eguchi 2008, Kitao et al. 2016). LCPs in birches grown under elevated CO_2 were higher than those in oak and maple. However, lower dark respiration rates of leaves raised in elevated CO_2 were observed in oak and maple, suggesting greater shade tolerance in maple in relation to carbon loss during nighttime. Therefore, elevated CO_2 may enhance shade tolerance via lowering LCP but the ranking of shade tolerance related to successional traits was not changed among species.

H. Methane Emission from Forests at Elevated CO_2

With development of canopy at elevated CO_2, incident light at the forest floor is reduced, which results in an increase in moisture content of the soil surface (e.g., Dubbs and Whalen 2010). The effect of elevated CO_2 on atmospheric methane consumption by well-drained forest soil was studied in the Sapporo FACE (Kim et al. 2011). After 3 years of CO_2 enrichment in a mixed broadleaved forest, methane emission from the forest floor was measured in VA and BF soils. The soil methane flux and soil moisture content were measured simultaneously four times (monthly) during each growing season. In both soils, elevated CO_2 decreased methane consumption rate from the soil surface by about half.

We expected methane consumption from VA would be higher than that from BF because of the high porosity of VA (Morishita et al. 2007). Irrespective of soil type, soil methane consumption was observed in the ambient CO_2 plot, but approximately 13% of all sampling points at the elevated CO_2 (FACE) plot (at the highest soil water contents) exhibited methane emission (Fig. 15.6). This evidence suggests that increases in soil moisture affected methane oxidation at elevated CO_2 as found in former studies (Liu and Greaver 2009). With increasing CO_2, an increase in canopy cover (Koike et al. 1995; Eguchi 2008) and litter production can be expected, resulting in an increase in soil moisture content due to inhibition of evaporation from the forest floor. As a result, the forest floor would shift toward anaerobic conditions and emit methane (Dubbs and Whalen 2010; Kim et al. 2011).

I. Further Considerations

An important aspect in CO_2 research is that several factors may influence the experimental outcomes. For example, the effect of elevated CO_2 on radial growth and wood

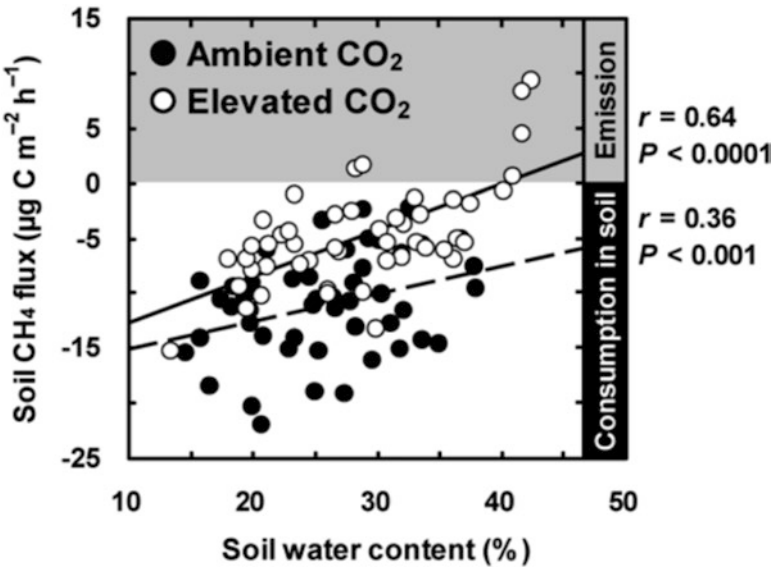


Fig. 15.6. Methane (CH_4) flux between soil beneath a mixed broadleaved forest and the atmosphere in elevated (open circles) and ambient (filled circles) CO_2 as a function of soil moisture content. (See text for additional details. From Kim et al. (2011), with permission from the journal)

structure (i.e., vessel size and distribution) may depend on pot size (Ishizuka et al. unpublished data). Several other studies showed that nutrient availability drives plant response to CO_2 in different plant traits such as anatomy (Watanabe et al. 2010), growth and morphology (Yazaki et al. 2004; Eguchi 2008; Watanabe et al. 2008, 2011; Hara et al. 2012; Koike et al. 2015), leaf physiology (Koike et al. 2015), and root structure and function (Agathokleous et al. 2016b,c). This is in line with findings from other FACE experiments in other parts of the world (Oren et al. 2001; Norby and Zak 2011). Drought may also affect CO_2 effects on plants (Koike et al. 2015). Planting space is an additional factor affecting plant responses to CO_2 and care should be exercised when designing FACE experiments (Agathokleous et al. 2016b). However, O_3, at exposures which are likely to occur in nature, may not readily impact the effects of differing levels of atmospheric CO_2 (Agathokleous et al. 2016a; Shi et al. 2016).

V. Conclusion

Chlorophyll fluorescence studies from a variety of experiments indicate adaptation to stress induced by CO_2, singly or in combination with other factors such as drought, through adjustment of photosynthetic electron transport rate and engagement of thermal energy dissipation. Extensive research conducted near natural CO_2 springs over decades also suggests a photosynthetic adaptation to CO_2. Research conducted in FACE provides support for the assumption of potential adaptation to elevated CO_2. These results, which come from several Japanese species, are in agreement with an extensive body of literature that comes from a variety of species around the globe (e.g., Tissue and Lewis 2012). The present findings suggest that predicting CO_2 effects from short-term experiments (e.g., <3 years) is unrealistic. There is a need for further studies over generations with different species to confirm whether this assumption is unequivocal across species. Artificial selection experi-

ments may provide a fundamental platform for understanding and predicting plant photosynthesis in the future in the context of evolution. In designing such studies, the impact of numerous factors that can influence the response of plants to differing levels of CO_2 must be recognized and mitigated, including pot size (should pots be utilized), nutrient (including N deposition) and water availability, and plant spacing.

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Chapter 16

The Leaf Economics Spectrum and its Underlying Physiological and Anatomical Principles

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Summary

Large variations are found in leaf morphology and physiology across species in nature, reflecting diversity in carbon fixation and growth strategies. These variations in leaf traits are not random; rather, they are tightly coordinated with each other. Leaf traits can be expressed per leaf dry mass or per leaf area. A leaf-mass basis reflects leaf economics, i.e., revenues and expenditures per unit investment of biomass, while a leaf-area basis reflects fluxes in relation to surfaces. Leaf N and P concentrations, and photosynthetic and respiration rates – all considered on a mass basis, are negatively correlated with leaf mass per area (LMA)

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whilst leaf lifespan is positively correlated with LMA. These correlations are summarized into a single major axis called the "leaf economics spectrum" that runs from "quick-return" to "slow-return" species. On the other hand, correlations among area-based traits are less consistent and less understood in relation to leaf economy. LMA was positively correlated with leaf N content but mostly independent from photosynthetic rates per unit leaf area. Given that N is an essential element in photosynthetic proteins and thus photosynthesis, clarifying the mechanisms why the efficiency of photosynthesis (photosynthesis per unit N) decreases with LMA is a major concern in understanding the correlations among area-based traits in relation to leaf economy. Currently available data suggest that greater amounts of cell wall are required for long-lived leaves, which reduces the efficiency of photosynthesis by lowering (1) the fraction of leaf N invested in photosynthetic proteins and (2) CO_2 diffusion rates through thicker and denser mesophyll cell walls. These physiological and structural constraints are a fundamental mechanism underpinning the general correlations among leaf economic traits.

I. Introduction

For more than a century plant scientists have been fascinated by the large variation in leaf morphology and physiology seen across the world's species (e.g., Schimper 1903; Clements 1905; Warming 1909; Shields 1950; Grubb et al. 1975; Chabot and Hicks 1982; Field and Mooney 1986; Grime et al. 1988; Reich et al. 1997; Wright et al. 2004). For example, leaf mass per area varies more than 300-fold among species (Díaz et al. 2016), photosynthetic capacity up to 100-fold (Maire et al. 2015), and leaf nitrogen concentration up to 30-fold (Díaz et al. 2016). These patterns of variation are not random; rather, there is interpretable and at times tight coordination among many leaf traits. This chapter aims to describe general tendencies in leaf trait variation, focusing on leaf photosynthetic traits, and explores the underlying physiological and structural mechanisms.

In the last few decades leaf functional traits such as photosynthetic capacity, nutrient concentrations, and morphology have been measured for species at hundreds of sites worldwide, meaning that we can begin to identify major correlations among leaf traits at a truly global scale (e.g., Field and Mooney 1986; Schulze et al. 1994; Reich et al. 1997; Wright et al. 2004; Kattge et al. 2011; Díaz et al. 2016; Wright et al. 2017). "Glopnet" (Global plant trait network; Wright et al. 2004) was a multi-collaborator project focused on compiling and analyzing leaf trait data, both from the literature and from the coauthors own research. They gathered trait data for *ca*. 2000 species, representing all major biomes on the earth. As described in more detail later, Wright et al. (2004) generalized earlier work from Reich et al. (1997) and others, describing an ecologically-important multi-trait axis that runs across the world's plant species, with key contributions from at least six leaf traits: (1) Leaf mass per area (LMA), indicating the dry-mass construction cost per unit of light-intercepting leaf area; (2) Photosynthetic assimilation rates (A) measured under high light and ambient CO_2; (3) Leaf nitrogen (N), which is an indicator of total amount of leaf protein; (4) Leaf phosphorus (P), which is found in nucleic acids, lipid membranes, and bioenergetic molecules such as ATP; (5) Dark respiration rate (R), which reflects metabolic expenditure of photosynthate in the leaf, such as protein turnover and phloem-loading of photosynthates; and (6) Leaf lifespan (LL), which describes the average duration of the revenue stream from investments in leaf tissue. Quantifying general rules among leaf traits helps to both understand leaf diversity (Reich 2014) and better

represent traits and trait-related processes in vegetation and ecosystem production models (e.g., Moorcroft et al. 2001; Reu et al. 2011; Wang et al. 2012).

Leaf ecophysiological traits such as leaf N, leaf P, A, or R can be expressed per leaf area or per leaf dry mass. Each basis of expression has its merits. A leaf-area basis reflects fluxes in relation to surfaces. It is a natural basis for describing light capture and for expressing transactions through surfaces such as trade-offs between carbon gain and transpiration (e.g., Cowan and Farquhar 1977; Wright et al. 2003; Medlyn et al. 2011). On a mass basis, leaf economics are quantified in terms of revenues and expenditures per unit investment of biomass. For example, leaf N and P concentrations index key aspects of tissue quality, dark respiration rate per unit mass indexes (among other things) tissue maintenance costs, and light-saturated rate of photosynthesis per mass indicates the rate of photosynthetic revenue accrual from a given mass investment in leaf tissue. By definition, area- and mass-based traits can be interconverted via LMA (for example, $N_{area} = N_{mass} \times LMA$). But LMA is far more than a conversion factor (Reich et al. 1998a): many structural, chemical, and physiological properties of leaves vary systematically with LMA (Hikosaka et al. 1998; Poorter and Evans 1998; Hikosaka 2004; Wright et al. 2004; Poorter et al. 2009) and, indeed, LMA is a key variable in plant ecological strategies (e.g., Grime et al. 1997; Westoby et al. 2002; Reich et al. 2003; Poorter et al. 2009).

In this chapter, we first describe correlations among key leaf traits, both on a mass- and area-basis across species, using data compiled by Wright et al. (2004) and Maire et al. (2015). These data were collected from mature leaves from outer canopies. The strength and direction of correlations between area-based traits were often different from those between mass-based traits (Reich et al. 1998a; Wright et al. 2004), suggesting that LMA covaries with these properties. In particular, the ratio of A to N, called photosynthetic N use efficiency (PNUE), systematically declined with increasing LMA across species, which is a key tradeoff in the leaf economics spectrum. Therefore, secondly, we focus in more detail on physiological and structural properties underlying variation in PNUE, and discuss how these underlying components underpin the leaf economics spectrum (Onoda et al. 2017).

II. Leaf Economics Spectrum

A. Mass-Based Traits

In general, species with higher leaf mass per area (LMA) had lower nutrient concentrations and lower metabolic activities per unit mass: the mass-based traits photosynthetic rate (A_{mass}), dark respiration rate (R_{mass}), N concentration (N_{mass}), and P concentration (P_{mass}) each declined with increasing LMA across species (Fig. 16.1a–d). In contrast, higher LMA species had a longer revenue stream from investments of nutrients and dry mass in leaves: leaf lifespan showed a strong positive relationship with LMA (Fig. 16.1e). Herbaceous species tended to have higher A_{mass} and nutrient concentration and shorter leaf lifespan than woody species. Among woody species, deciduous species generally had higher A_{mass} and nutrient concentrations and shorter leaf lifespan than evergreen species. However, there was substantial overlap among functional groups for each of these leaf traits.

Reasonably tight (and always highly significant) correlations were found among metabolic traits and nutrient concentrations (Fig. 16.2). Fifty-five per cent of variation in both A_{mass} and R_{mass} was explained by N_{mass}, supporting the fact that N is an essential element for proteins that operate in photosynthetic and other metabolic activities. Leaf N and P concentrations were tightly correlated ($R^2 = 0.72$), with a log-log "scaling" slope of 1.5 (Standardized major axis slope, Warton et al. 2006) indicating that high nutrient leaves tend to have lower N:P ratios (Wright

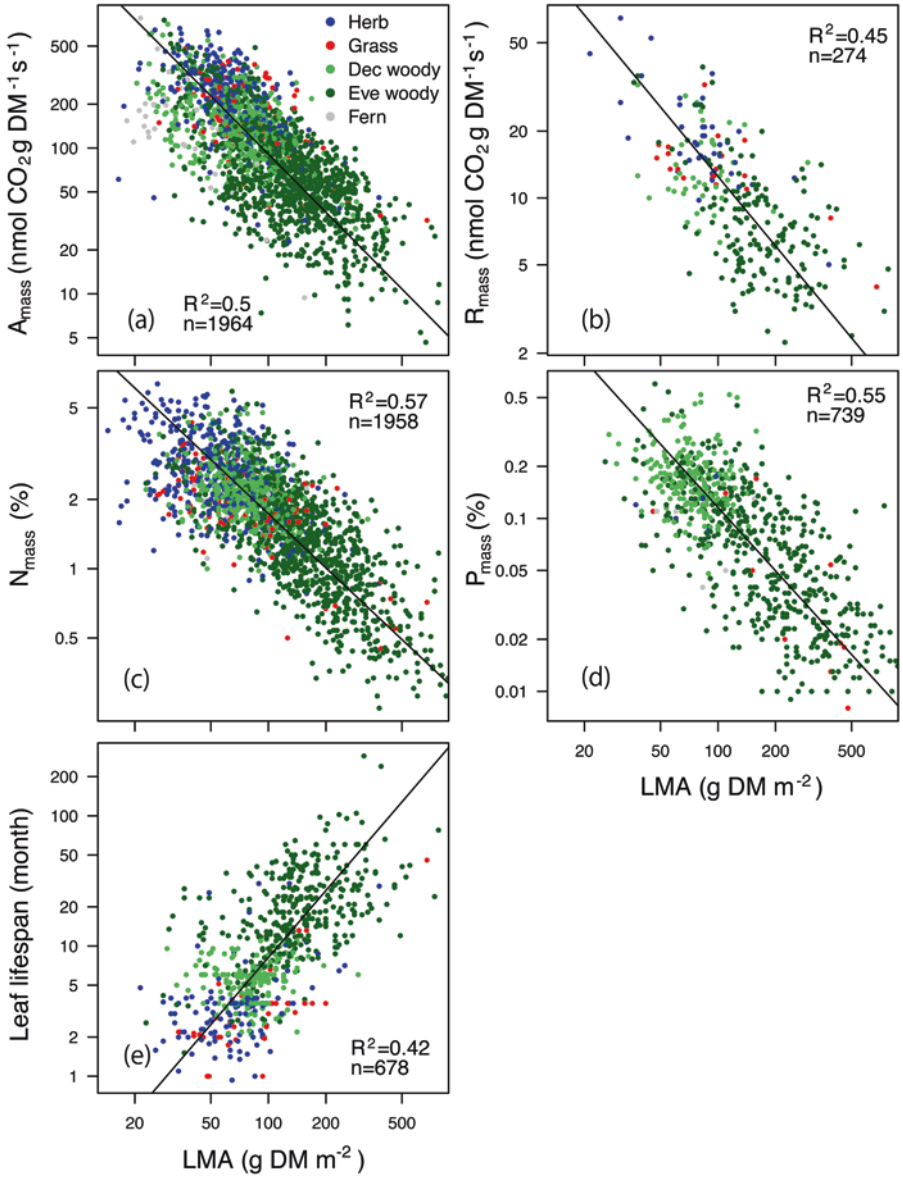

Fig. 16.1. Relationships between mass-based leaf physiological traits and leaf dry mass per area (LMA). (**a**) Light-saturated photosynthetic rate per unit leaf dry-mass (A_{mass}); (**b**) Dark respiration rate per unit leaf dry-mass (R_{mass}); (**c**) Leaf nitrogen concentration (N_{mass}); (**d**) Leaf phosphorous concentration (P_{mass}); and (**e**) Leaf lifespan. Correlations are all significant ($P < 0.001$). Standardized major axis (SMA) slopes (Warton et al. 2006): (**a**) $y = 40{,}340x^{-1.32}$, (**b**) $y = 1501x^{-1.04}$, (**c**) $y = 63.5x^{-0.782}$, (**d**) $y = 31.4x^{-1.22}$, (**e**) $y = 0.0031x^{1.71}$. (Data from Maire et al. (2015) for (**a**) and Wright et al. (2004) for (**b**–**e**)). *DM* dry mass, *Dec* deciduous, *Eve* evergreen

et al. 2004; Niklas et al. 2007). A_{mass} was strongly negatively correlated with leaf lifespan ($R^2 = 0.68$), which may be surprising because there was seemingly no direct physiological link between these two variables. The negative correlation suggests that leaves with slow carbon return for a given leaf investment required a longer photosynthetic period to pay-back the initial construction cost and get surplus of carbon for growth

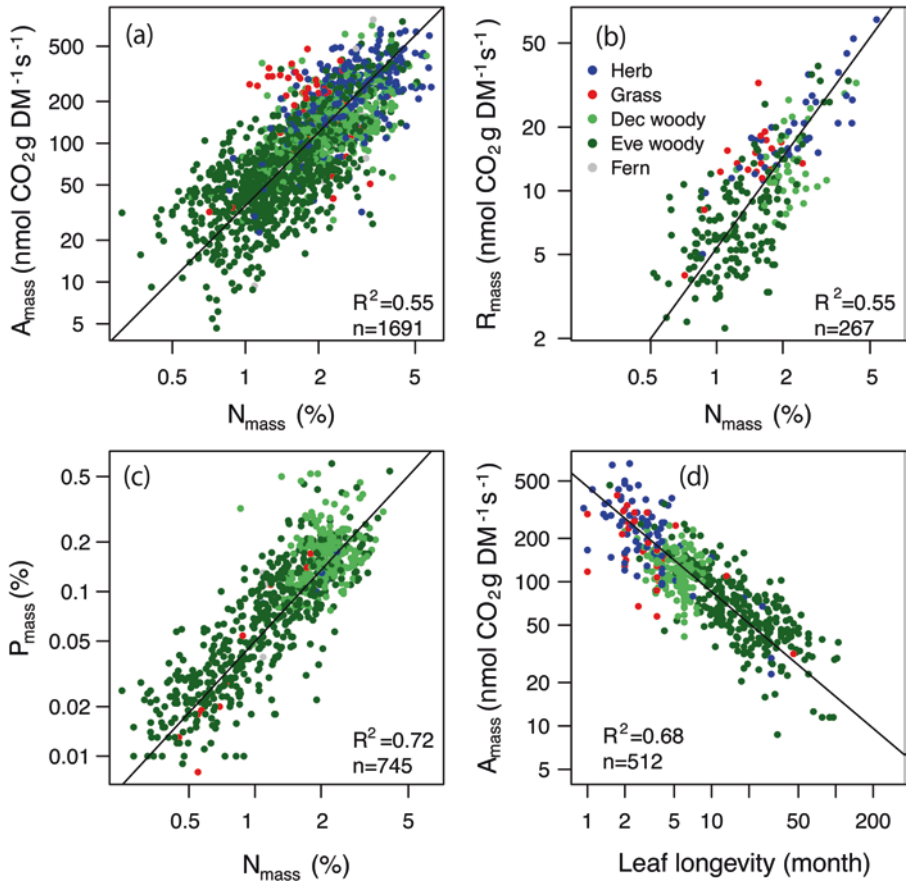

Fig. 16.2. Relationships between mass-based leaf physiological traits. (**a**) Light-saturated photosynthetic rate per unit leaf dry-mass (A_{mass}), (**b**) dark respiration rate per unit leaf dry-mass (R_{mass}), and (**c**) leaf phosphorous concentration (P_{mass}) plotted against leaf nitrogen concentration (N_{mass}). (**d**) A_{mass} plotted against leaf lifespan. Correlations are all significant ($P < 0.001$). SMA slopes (Warton et al. 2006): (**a**) $y = 35.4x^{1.76}$, (**b**) $y = 5.29x^{1.43}$, (**c**) $y = 0.0551x^{1.51}$, (**d**) $y = 457x^{-0.729}$. (Data from Maire et al. (2015) for (**a**) and Wright et al. (2004) for (**b**–**d**)). *DM* dry mass, *Dec* deciduous, *Eve* evergreen

(Chabot and Hicks 1982; Kikuzawa 1991; Ackerly 1999; Givnish 2002; Westoby et al. 2002).

Wright et al. (2004) showed that approximately three-quarters of variation in these six traits were accounted for by the first axis in a principal components analysis, which they called the "leaf economic spectrum" (LES). They described the spectrum as running from "quick-return" to "slow-return" species. Quick-return leaves are characterized by high photosynthetic capacity per unit mass, high nitrogen and phosphorus concentrations, but a short leaf lifespan, meaning that quick returns are not sustained long. Species at the other end of the spectrum have the opposite set of traits, meaning that they are likely to have a longer income stream from investments in leaf tissue, compensating for their higher construction costs per unit leaf area (Chabot and Hicks 1982; Field and Mooney 1986; Reich et al. 1991, 1997; Wright et al. 2004; Reich 2014). This spectrum was largely independent from biomes and functional groups (e.g., woody species, herbaceous plants) such that a similar pattern of inter-trait correlations was observed within different climates and functional

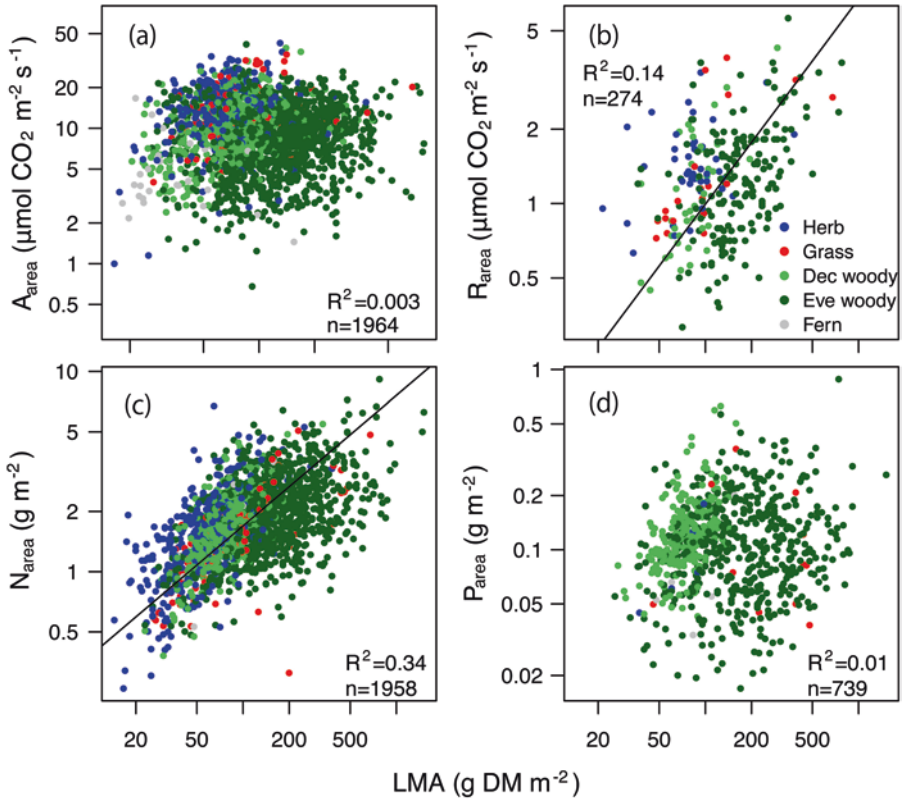

Fig. 16.3. Relationships between area-based leaf physiological traits and leaf mass per area (LMA). (**a**) Light-saturated photosynthetic rate per unit leaf area (A_{area}), (**b**) dark respiration rate per unit leaf area (R_{area}), (**c**) leaf nitrogen content per unit leaf area (N_{area}), and (**d**) leaf phosphorous content per unit leaf area (P_{area}). SMA slopes (Warton et al. 2006) were drawn when correlations were significant ($P < 0.01$): (**a**) no correlation, (**b**) $y = 0.0218x^{0.833}$, (**c**) $y = 0.0844x^{0.651}$, (**d**) no correlation. (Data from Maire et al. (2015) for (**a**) and Wright et al. (2004) for (**b**–**d**)). *DM* dry mass, *Dec* deciduous, *Eve* evergreen

groups (Wright et al. 2004). Different functional groups were located somewhat predictably along the LES: evergreen woody species tended to have slow-return strategies, while many herbaceous species exhibited fast-return strategies. Still, as in individual traits there was substantial overlap among the groups in their leaf economic strategies.

B. Area-Based Traits

When we consider area-based traits (Figs. 16.3 and 16.4), correlations among them were often weak and the direction of correlations was in some cases opposite compared to that between mass-based traits and LMA (i.e., N_{area} and R_{area} both increased with LMA, while their mass-based counterparts decreased with LMA: Fig. 16.3b, c). Of special interest, A_{area} was almost completely unrelated to LMA across 1964 species ($R^2 < 0.01$). This contrasts to what is often found *within* species, where A_{area} is often higher in high LMA leaves, especially when LMA variation relates to growth under different light conditions (e.g., Terashima et al. 2001, 2006). Leaf N content was on average higher in high-LMA leaves, but this trend was not found in leaf P content. Overall, while high-LMA leaves had lower nutrient concentrations per unit mass (Fig. 16.1), they had higher nutrient (at least N) content per unit area due to their higher dry mass per area.

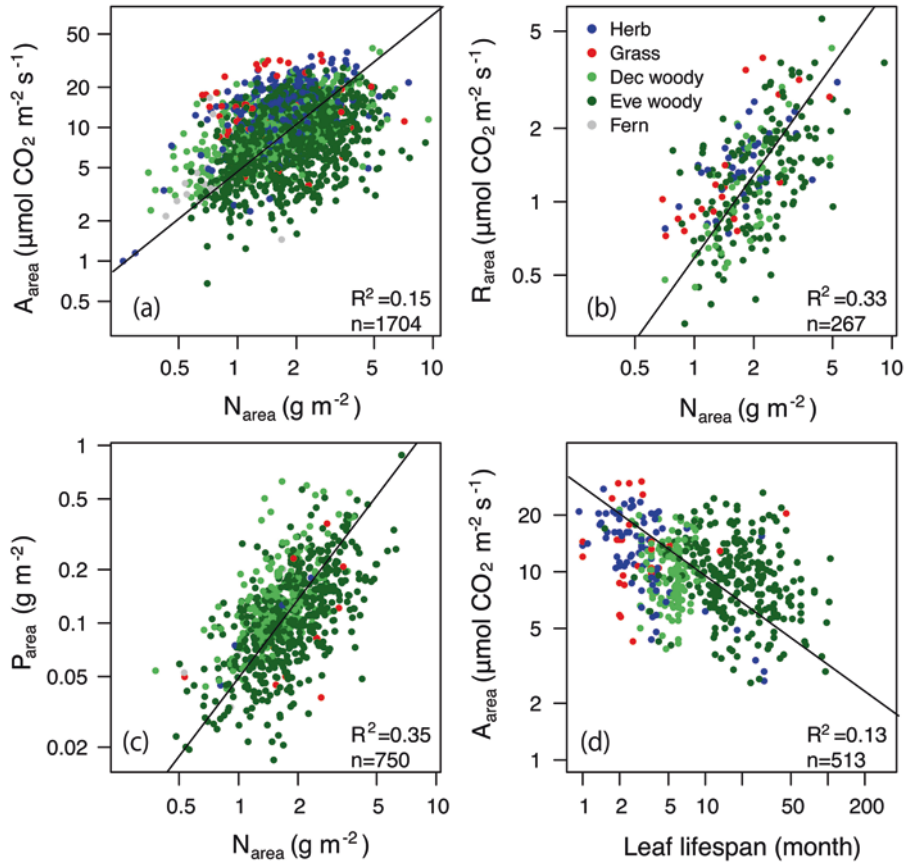

Fig. 16.4. Relationships between area-based leaf physiological traits. (**a**) Light-saturated rate of photosynthetic rate per unit leaf area (A_{area}), (**b**) dark respiration rate per unit leaf area (R_{area}), and (**c**) leaf phosphorous content per unit leaf area (P_{area}) plotted against leaf nitrogen content per unit leaf area (N_{area}). (**d**) A_{area} is plotted against leaf lifespan. SMA slopes (Warton et al. 2006) were drawn when correlations were significant ($P < 0.01$): (**a**) $y = 4.81x^{1.17}$, (**b**) $y = 0.589x^{1.13}$, (**c**) $y = 0.0491x^{1.47}$, (**d**) $y = 27.7x^{-0.472}$. (Data from Maire et al. (2015) for (**a**) and Wright et al. (2004) for (**b**–**d**)). Dec = deciduous; Eve = evergreen

Correlations among metabolic traits and nutrient concentrations were broadly similar irrespective of whether mass-based or area-based traits were used, though correlation strength was typically weaker for area-basis relationships (Figs. 16.2 and 16.4). For example, R^2 was 0.55 for the A_{mass}–N_{mass} relationship while 0.15 for the A_{area}–N_{area} relationship. Because area-based traits are the product of mass-based traits and LMA (i.e., $A_{area} = A_{mass} \times LMA$), the reductions in correlations between area-based traits are logically due to covariation in LMA. As already well understood (Reich et al. 1998a; Peterson 1999; Wright et al. 2004), the LMA-independent relationship of A_{mass} or A_{area} with leaf N can be quantified using partial regression coefficients in multiple regression analysis where variation in LMA is statistically controlled. Such analyses clearly show that both leaf structure (LMA) and leaf N have independent effects on photosynthetic rates (Reich et al. 1998a) and on dark respiration rates (Reich et al. 1998b; Wright et al. 2004). Recently, Osnas et al. (2013) rediscovered this fact, applying more advanced statistical techniques to the issue. In their view, covariation of leaf economic traits with LMA is a statistical nuisance that needs to be controlled for before one can then quantify the

multivariate correlation structure using principal components analysis. By contrast, in our view trait covariation with LMA is an absolutely fundamental feature of leaf economics, and it needs to be better understood. Indeed, that is the over-arching theme of most subsequent sections in this chapter.

C. *Photosynthetic N Use Efficiency*

The relationship between photosynthetic rates and leaf N has been analyzed in many studies (e.g., Field and Mooney 1986; Evans 1989; Hikosaka et al. 1998; Poorter and Evans 1998; Reich et al. 1998a; Wright et al. 2004, 2005). This is because a large fraction of N is allocated for photosynthetic proteins, in particular Rubisco (Evans and Seemann 1989; Harrison et al. 2009), and also because N is often a limiting resource for plant growth – and therefore efficient use of N should presumably be important (Chapin 1980; Aerts and Chapin 2000). Photosynthetic N use efficiency (PNUE) is the ratio of photosynthetic rate to N calculated on either a mass- or area-basis (the units end up being the same). PNUE varies far more widely across than within species (Evans 1989; Poorter and Evans 1998). In the data compilation of Maire et al. (2015) (2409 species), 90% quantile range of PNUE values fall within a 7.5-fold range, between 1.78 and 13.3 μmol [C] g $[N]^{-1}$ s^{-1}. Of special interest here is the fact that PNUE varies systematically with LMA: on average, higher LMA species show considerably lower PNUE (Fig. 16.5; Reich et al. 1998a; Hikosaka 2004; Wright et al. 2005). In order to better understand why that is, and also to understand the basis for the lack of a broad-scale relationship between A_{area} and LMA, it is necessary to tease apart the interacting physiological and structural underpinnings of the leaf economic spectrum.

III. Physiological and Structural Basis Underlying LES

Mechanisms underlying the LES have been explored by several studies from various perspectives (Grubb 2016) such as the cell wall (Hikosaka 2004; Shipley et al. 2006; Onoda et al. 2017), venation networks (Blonder et al. 2011, 2013, 2015; Sack et al. 2013), and phylogeny (Mason et al. 2016). Here we frame our approach as to explain how traits required for long leaf lifespan reduce the efficiency of photosynthesis.

As shown in the conceptual diagrams (Figs. 16.6 and 16.7), high LMA is an essential component for long leaf lifespan (Reich et al. 1992; Wright and Westoby 2002), LMA contributes positively to N_{area} but negatively to PNUE, and these properties together determine A_{area}. The question is, why do high LMA-leaves have low PNUE? It can be expected that some traits required for long leaf lifespan (i.e., high LMA, and underlying anatomical and physiological traits) are associated with lower PNUE.

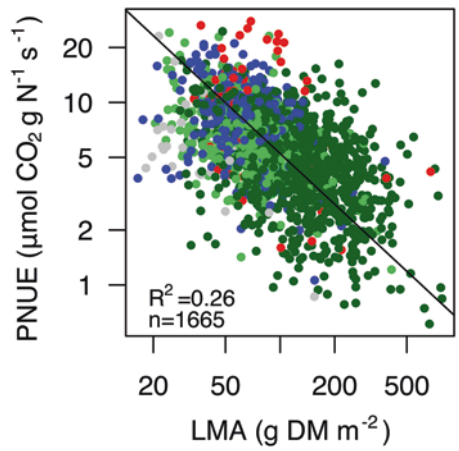

Fig. 16.5. Relationship between photosynthetic N use efficiency (PNUE) and leaf dry mass per area (LMA). Symbols are the same as in Fig. 16.1. (Data from Maire et al. (2015)). SMA slope; $y = 369x^{-0.924}$. *DM* dry mass

Variation in PNUE among species can be attributed to variation in several properties (Field and Mooney 1986; Hikosaka et al. 1998; Poorter and Evans 1998): (1) stomatal conductance (g_s), which affects intercellular CO_2 concentration (C_i); (2) mesophyll diffusional conductance (g_m), which affects CO_2 concentration at the sites of carboxylation within the chloroplasts (C_c); (3) the proportion of N allocated to photosynthetic versus non-photosynthetic functions; (4) N allocation among photosynthetic proteins such as light harvesting complexes, electron transport, and CO_2 fixation; (5) the activation state or specific activity of Rubisco; (6) respiration rate in the light; and (7) the amount of light absorbed. As our concern is to understand why long-lived leaves have low PNUE, it is necessary to clarify how these underlying properties are linked with traits required for long leaf lifespan.

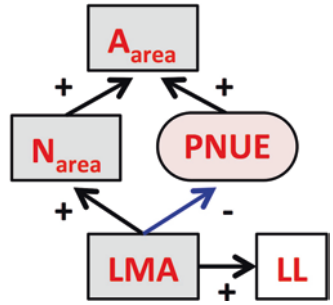

Fig. 16.6. A conceptual diagram for the associations between major leaf economic traits. Abbreviations: A_{area} light-saturated rate of photosynthetic rate per unit leaf area, N_{area} leaf nitrogen content per unit leaf area, *PNUE* photosynthetic N use efficiency, *LMA* leaf mass per unit leaf area, *LL* leaf lifespan. Traits in grey boxes are expressed per unit leaf area, and the components determining PNUE are described in Fig. 16.7b. Black and blue arrows indicate positive and negative associations, respectively (Colour figure online)

A conceptual framework for the physiological and structural mechanisms underlying the variations in PNUE and other major leaf economics traits is shown in Fig. 16.7 (also see Onoda et al. 2017). In this framework, cell wall is assumed as a major trait that drives variation in both leaf lifespan and PNUE. High cell wall content per unit area or unit mass is important for resisting abiotic and biotic stresses such as rainfall, drought,

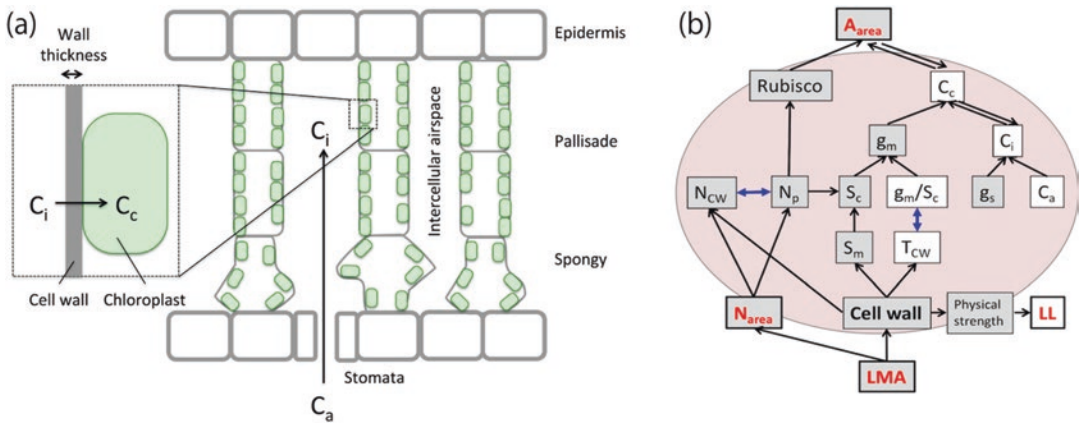

Fig. 16.7. (**a**) A diagram of leaf anatomy and (**b**) the conceptual framework for the physiological/anatomical associations among the major leaf economic traits. The diagram of leaf anatomy shows how CO_2 diffuses from the ambient air to the chloroplast stroma. The conceptual framework (**b**) shows how the major traits in leaf economics spectrum (in red letters) are linked via the underlying physiological/anatomical traits. These underlying physiological/anatomical traits as a whole determine PNUE (see Fig. 16.6; these components are summarized into PNUE). Traits in grey boxes are expressed per unit leaf area and double-headed arrows indicate hypothetical tradeoffs (see text for detail). Abbreviations: C_a ambient CO_2 concentration, C_c chloroplast CO_2 concentration, C_i intercellular CO_2 concentration, g_m mesophyll conductance, g_s stomatal conductance, *LL* leaf lifespan, N_{CW} cell wall-N, N_p photosynthetic-N, S_c chloroplast surface area per unit leaf area, S_m mesophyll surface area per unit leaf area, T_{CW} thickness of mesophyll cell wall. (Reproduced from Onoda et al. (2017)) (Colour figure online)

and herbivory, and therefore contributes to longer leaf lifespans. On the other hand, high cell wall mass is hypothesized to decrease PNUE in two chief ways: (1) lower N allocation to photosynthetic proteins due to higher N allocation to cell walls (Onoda et al. 2004; Takashima et al. 2004); and (2) lower mesophyll diffusion conductance due to thicker cell walls (Terashima et al. 2006, 2011; Tosens et al. 2012). These mechanisms are described in more detail below.

Since the light-saturated rate of photosynthesis per unit area (A_{area}) can be expressed according to Fick's law, i.e., $A_{area} = g_m(C_i - C_c)$, PNUE can be expressed as follows;

$$PNUE = \frac{g_m}{N_{area}}\left(C_i - C_c\right) \qquad (16.1)$$

In this study, we largely focus on g_m rather than g_s because (1) g_m is more tightly associated with leaf anatomy (Tosens et al. 2012) and (2) g_m and g_s are often correlated when stomata are open (Flexas et al. 2008; Warren 2008). Equation 16.1 can be further decomposed by considering N allocation to photosynthetic proteins and the CO_2 exchange surface, that is, the mesophyll cell surface where the chloroplasts are attached expressed per unit leaf area, S_c (Oguchi et al. 2003; Terashima et al. 2006);

$$PNUE = \frac{g_m}{S_c}\frac{S_c}{N_p}\frac{N_p}{N_{area}}\left(C_i - C_c\right) \qquad (16.2)$$

where S_c is chloroplast surface area per unit leaf area and N_p is the amount of N associated with photosynthesis per unit leaf area. This equation shows that PNUE can be expressed as the product of (1) mesophyll conductance per chloroplast surface area (g_m/S_c), (2) the ratio of chloroplast surface area to photosynthetic proteins (S_c/N_p), (3) the fraction of N allocated to photosynthetic proteins (N_p/N_{area}), and (4) CO_2 drawdown from intercellular spaces to chloroplasts ($C_i - C_c$). The first and third terms relate to the two hypotheses mentioned above. Other possible determinants of PNUE as described earlier, i.e., stomatal conductance, N allocation among photosynthetic proteins, the amount of light absorbed under saturating light, and the specific activity of Rubisco, are not included in this analytical framework though in principle they could be. Stomatal conductance can influence the photosynthetic rate via altering C_i, but the variation of C_i is often small across species ($C_i/C_a = 0.6$–0.8; Wong et al. 1979) because of a coordination between stomatal conductance and A_{area}. N allocation among photosynthetic proteins differs across species or growth conditions, but there is some stoichiometry among the photosynthetic proteins (Hikosaka and Terashima 1995), implying the magnitude of the variation may be smaller than that of g_m/S_c or N_p/N_{area}. The variation in light absorption rate is generally limited because the absorption rate of photosynthetically active radiation is typically more than 80% (Moss and Loomis 1952). Variation in the specific activity of Rubisco in relation to LMA is not well known, but a couple of studies suggested the specific activity may be lower in high LMA leaves (Poorter and Evans 1998; Hikosaka and Shigeno 2009). In the following section, we investigate variation among species in leaf cell wall fractions, and the extent to which this variation is associated with PNUE via leaf N allocation and mesophyll diffusional conductance.

A. Cell Walls

Plant cell walls are complex macromolecules consisting of pectin, hemicellulose, cellulose, lignin, and structural proteins (Carpita and McCann 2000). Cell walls provide structural support and protection from various abiotic and biotic stresses (Merino et al. 1984; Read and Sanson 2003). Cell walls can be chemically isolated by various methods such as alcohol extraction, detergent extraction, and organic solvent extraction from ground samples at cold or heated temperatures (Fry 1988). Currently available data for cell wall

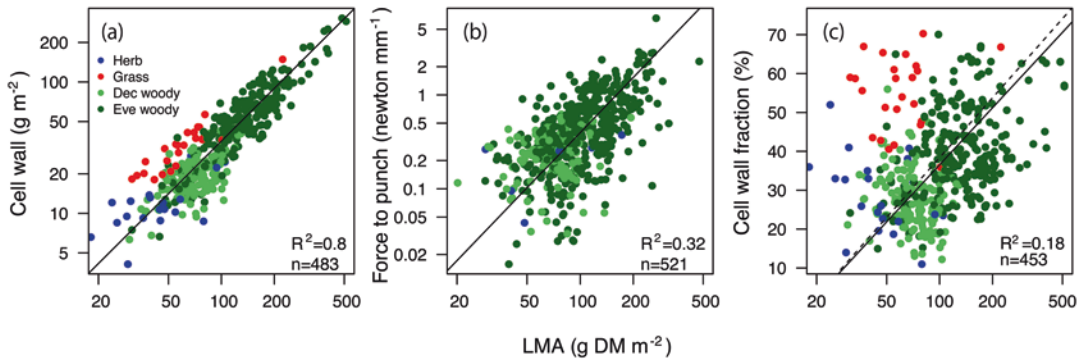

Fig. 16.8. Relationships between leaf structural traits and leaf dry mass per area (LMA). (**a**) Cell wall mass per unit leaf area; (**b**) Maximum force required for a punch rod to penetrate a leaf lamina expressed per unit circumference of the punch rod; and (**c**) Fraction of leaf mass in cell walls. Correlations are all significant ($P < 0.001$). SMA slopes: (**a**) $y = 0.0764x^{1.33}$; (**b**) $y = 2.78e\text{-}05x^{2.07}$; (**c**) $y = 2.07x^{0.611}$ for data without grass species (black line) and $y = 2.04x^{0.624}$ for all data (dashed line). (Redrawn from Onoda et al. (2017)). *DM* dry mass, *Dec* deciduous, *Eve* evergreen

mass in plant ecological studies were often extracted by neutral detergent (see van Soest 1994 for the protocol), and cell wall mass per unit leaf area (CW_{area}) was tightly correlated with LMA across 483 species (Fig. 16.8; Onoda et al. 2017). High-LMA leaves had higher physical strength, i.e., higher 'force to punch' as measured with a penetrometer (Fig. 16.8b), which reflects the level of leaf protection against herbivory and other mechanical stresses and contribute to longer leaf lifespan (Read and Sanson 2003; Onoda et al. 2011). The fraction of leaf mass in cell walls (CW_{mass}) ranged from 19 to 66% (90% quantile range, hereafter 90%QR) with an average of 40%. High LMA-leaves tended to have higher CW_{mass}; evergreen woody species typically had higher CW_{mass} (42 ± 12%) than herbaceous and deciduous woody species (29 ± 10% and 28 ± 8%, respectively). Evergreen conifer species were not significantly different from evergreen broad-leaved species in CW_{mass} while they tend to have higher LMA. Grasses are an exception for herbaceous species; they had notably high CW_{mass} (59 ± 10%) despite not generally having high LMA. Presumably, high CW_{mass} in grasses is partly due to the fact that many grass leaf blades are relatively self-supporting (i.e., no petiole and long leaf blade).

B. Leaf N Allocation

In leaves, a large fraction of N is allocated to chloroplasts; as much as 70–80% in some vegetable and crop species (Evans and Terashima 1987; Makino and Osmond 1991). Rubisco, a key enzyme of photosynthesis, accounts for 30–40% of chloroplast N. However, these numbers cannot automatically be applied to wild plants. N allocation to Rubisco varies substantially among species and tends to be lower in woody than in herbaceous plant types (Evans 1989; Hikosaka and Shigeno 2009). This suggests that N allocation to non-photosynthetic components may be important for understanding leaf N economy (Lambers and Poorter 1992). As mentioned above, cell walls account for a large fraction of leaf mass (19–66%) and primary cell walls contain 2–10% proteins (ca. 0.3–1.5%[N] in cell walls) (Lamport 1965; Carpita and McCann 2000; Held et al. 2015). Cell wall proteins function in defense, growth, development, signaling, intercellular communication, and environmental sensing (Showalter 1993; Kieliszewski et al. 2010). If cell walls contain substantial N, one might expect a tradeoff between N allocation to cell walls and to photosynthetic proteins.

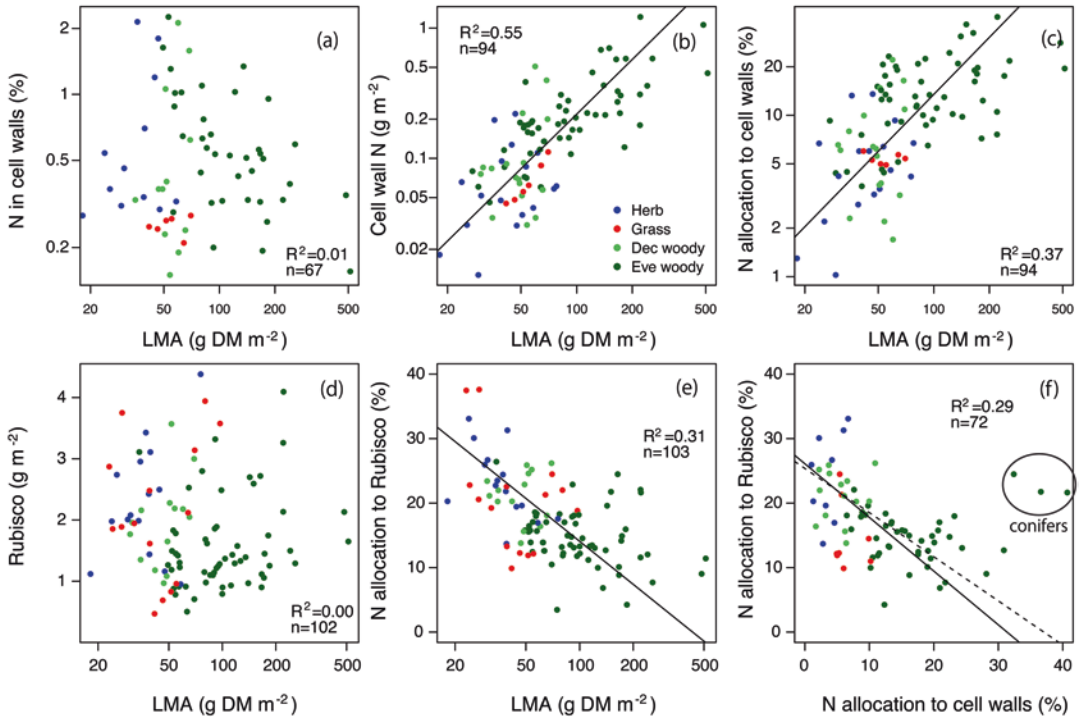

Fig. 16.9. Leaf N allocation traits and leaf mass per area (LMA). (**a**) N concentration in cell walls, (**b**) cell wall-N per unit leaf area, (**c**) cell wall N per unit leaf nitrogen, and (**d**) Rubisco mass per unit leaf area, (**e**) Rubisco N per unit leaf nitrogen plotted against leaf mass per area. (**f**) Relationship between the fractions of N allocated to cell walls and Rubisco. SMA slopes were fitted when correlations were significant ($P < 0.01$): (**a**) no correlation; (**b**) $y = 0.000355x^{1.39}$; (**c**) $y = 0.0603x^{1.17}$; (**d**) no correlation; (**e**) $y = -22.1 \log(x) + 58.3$; (**f**) $y = -0.685x + 25.4$ for all data and $y = -0.832x + 26.1$ excluding data for three conifers. (Redrawn from Onoda et al. (2017)). *DM* dry mass, *Dec* deciduous, *Eve* evergreen

Using data on cell wall N uncontaminated by N from cytosolic proteins, N concentration in cell walls ranged from 0.2 to 1.75% of cell wall mass (90%QR) and was unrelated to LMA (Fig. 16.9a; Onoda et al. 2017). Cell wall N content per unit leaf area increased tightly with LMA ($R^2 = 0.55$; Fig. 16.9b) due to the higher CW_{area} in high LMA leaves. Leaf N allocation to cell walls ranged from 2.3 to 25% (90%QR) with an average of 11.2% (Fig. 16.9c). There was a positive correlation between LMA and the fraction of N allocated to cell walls ($R^2 = 0.37$; Fig. 16.9c), suggesting that high-LMA leaves tend to have higher fraction of N in cell walls.

Rubisco content per unit leaf area, which is usually measured by electrophoresis (e.g., Makino et al. 1986), was not significantly correlated with LMA in the available dataset (138 species, Fig. 16.9d). N allocation to Rubisco varied from 9.2 to 26.6% (90%QR) with an average of 17.2%, and was significantly lower in high LMA leaves ($R^2 = 0.27$; Fig. 16.9e). On average, herbaceous plants allocated a slightly larger fraction of leaf N to Rubisco (23.2 ± 5.5%) than did woody deciduous or woody evergreen species (21.1 ± 4.0% and 14.6 ± 4.5%, respectively). There was a significant negative correlation between N allocation to cell walls and Rubisco ($R^2 = 0.10$; Fig. 16.9f), suggesting that N allocation to cell walls trades off with N allocation to Rubisco. Three evergreen conifers (these are all the conifers in this dataset) were notable outliers in this relationship; they had both relatively high N alloca-

tion to cell walls as well as Rubisco. If conifers were excluded from this analysis, the negative correlation was much stronger ($R^2 = 0.29$).

Assuming that Rubisco constitutes about half of photosynthesis-related leaf N (Evans and Seemann 1989), the summed N allocation to photosynthetic proteins and cell walls was on average 46% (90%QR = 30–70%). This means that not only photosynthetic proteins but also cell walls are major players in the leaf N economy, and it would not be surprising that there is a tradeoff in N allocation between these two components. On the other hand, this also means that about 50% of leaf N consists of materials that are neither photosynthetic proteins nor cell walls, and there is a rather large variation in the summed N allocation to the two components across species. These results suggest that there is broad flexibility in N allocation across species and may be a reason why previous studies across fewer species did not find a negative relationship between N allocation to Rubisco and to cell walls (Harrison et al. 2009; Hikosaka and Shigeno 2009; Funk et al. 2013). Nitrogen is allocated to several other pools besides proteins associated with photosynthesis and cell walls, such as nucleic acids, amino acids, numerous proteins in the cytosol, and mitochondria (Chapin and Kedrowski 1983). Some plants also contain nitrogenous osmolytes such as glycine betaine or proline (Ashraf and Foolad 2007; Erskine et al. 1996), N-rich defensive compounds such as alkaloids and cyanogenic glycoside (Miller and Woodrow 2008), and nitrate (particularly herbaceous species growing in fertile soils; Sage and Pearcy 1987; Evans and Poorter 2001). N allocation to these components is not adequately quantified in term of leaf N economy.

C. *Mesophyll Diffusion Conductance*

Atmospheric CO_2 diffuses through stomata into intercellular airspaces, dissolves in apoplastic water layers, and subsequently diffuses through a number of cellular components, including cell walls, membranes, cytosol, chloroplast envelopes, and chloroplast stroma (Fig. 16.7a; Evans et al. 1994; Syvertsen et al. 1995; Terashima et al. 2006; Evans et al. 2009; Chaps. 5 and 7). The total mesophyll conductance (g_m) is therefore determined by a series of conductances, and roughly divided into the gas-phase conductance and the liquid phase conductance (Niinemets and Reichstein 2003; Evans et al. 2009; Tosens et al. 2012). In most plants the chief anatomical limitations to CO_2 diffusion are in the liquid phase, particularly in cell walls (Warren 2008; Terashima et al. 2011; Tosens et al. 2016). Mesophyll conductance was variously measured via a combined chlorophyll fluorescence/gas exchange method (Harley et al. 1992), carbon isotope discrimination (Evans et al. 1986), and/or curve fitting to A_{area}–C_i relationships (Ethier and Livingston 2004).

A higher mass of cell wall per leaf area could reflect (1) thicker (and denser) mesophyll cell walls, which would restrict CO_2 diffusion to chloroplasts; (2) a larger total surface area of mesophyll cells per unit leaf area, which in theory would increase CO_2 diffusion to chloroplasts; or (3) more structural tissues. The thickness of mesophyll cell walls ranges from 0.1 to 0.5 μm in angiosperms, generally with lower values reported for herbaceous plants and higher values for woody species, especially evergreen trees and shrubs (Evans et al. 2009; Terashima et al. 2011; Tosens et al. 2012). Some rather high values are also known for ferns and fern allies (0.1–0.8 μm; Tosens et al. 2016). All other things being equal, thicker mesophyll cell walls increase resistance to CO_2 diffusion, thereby reducing mesophyll conductance and the CO_2 concentration at carboxylation sites (Nobel et al. 1975; Terashima et al. 2006; Evans et al. 2009; Niinemets et al. 2009; Tosens et al. 2012). On the other hand, thicker leaves with higher LMA can have a greater total mesophyll surface area (S_m) and therefore accommodate a

greater chloroplast surface area facing the intercellular airspaces (S_c). Higher S_c in conjunction with more S_m enhances mesophyll conductance (Björkman 1981). Thus, the nature of the relationship between LMA and mesophyll conductance may be subject to the balance between these opposing effects.

Currently available data shows that both stomatal conductance and mesophyll conductance for CO_2 diffusion were negatively associated with LMA, but the correlations were weak (R^2 = 0.07 and 0.11; Fig. 16.10). Furthermore, stomatal conductance and mesophyll conductance were significantly positively correlated as had been shown previously (Fig. 16.10c; Flexas et al. 2008; Warren 2008).

LMA was significantly positively correlated with both mesophyll cell wall thickness, T_{CW} (R^2 = 0.41; Fig. 16.11a), and mesophyll surface area per unit area, S_m (R^2 = 0.28; Fig. 16.11b). Across 15-fold variation in LMA, T_{CW} varied fivefold and S_m varied fourfold. Yet, the greater S_m in high LMA leaves did not directly translate into greater chloroplast surface area per unit leaf area (S_c); S_c was not correlated with LMA (Fig. 16.11c). The proportion of S_m covered by chloroplasts (= S_c/S_m) varied widely but was significantly lower in high LMA species (R^2 = 0.11; Fig. 16.11d). The overall correlation between S_c and N_{area} was not significant across species such that some evergreen species with high N_{area} did not have high S_c (Fig. 16.11e), suggesting that N allocation to chloroplasts in these evergreen species was not as high as in some herbaceous species. Mesophyll cell wall thickness was tightly and negatively correlated with mesophyll conductance per unit S_c (R^2 = 0.50; Fig. 16.4h), supporting the second hypothesis, that mesophyll cell wall thickness trades-off with CO_2 diffusion conductance. The log-log scaling slope between g_m/S_c and T_{CW} was −2.41, indicating that g_m/S_c decreased disproportionately more with increases in cell wall thickness. This may suggest that thicker cell walls have lower cell wall porosity (Evans et al. 2009; Tosens et al. 2012).

One may wonder why thicker mesophyll cell wall is a feature of high-LMA leaves, when it presumably comes at the expense of a reduced photosynthetic rate. That is, presumably thinner mesophyll cell walls should always be better for photosynthesis and water use efficiency (Flexas et al. 2013). Why is it that thicker epidermal cell walls and cuticles are not sufficient protection for leaves? Onoda et al. (2017) proposed at least two advantages for thicker mesophyll cell walls. First, long-lived leaves are more likely to experience strong drought stress at some time during their life, therefore rigid mesophyll structures as well as rigid outer struc-

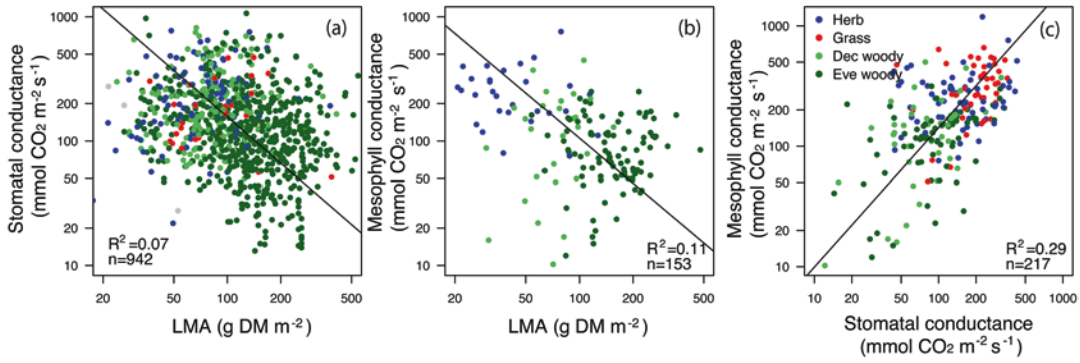

Fig. 16.10. (**a**) Stomatal conductance and (**b**) mesophyll conductance (g_m) plotted against leaf dry mass per area (LMA). (**c**) Relationship between stomatal and mesophyll conductance. Correlations are all significant ($P < 0.001$). SMA slopes; (**a**) $y = 47{,}046x^{-1.24}$; (**b**) $y = 27{,}005x^{-1.2}$ and (**c**) $y = 0.692x^{1.16}$. (Data from Onoda et al. (2017)). *DM* dry mass, *Dec* deciduous, *Eve* evergreen

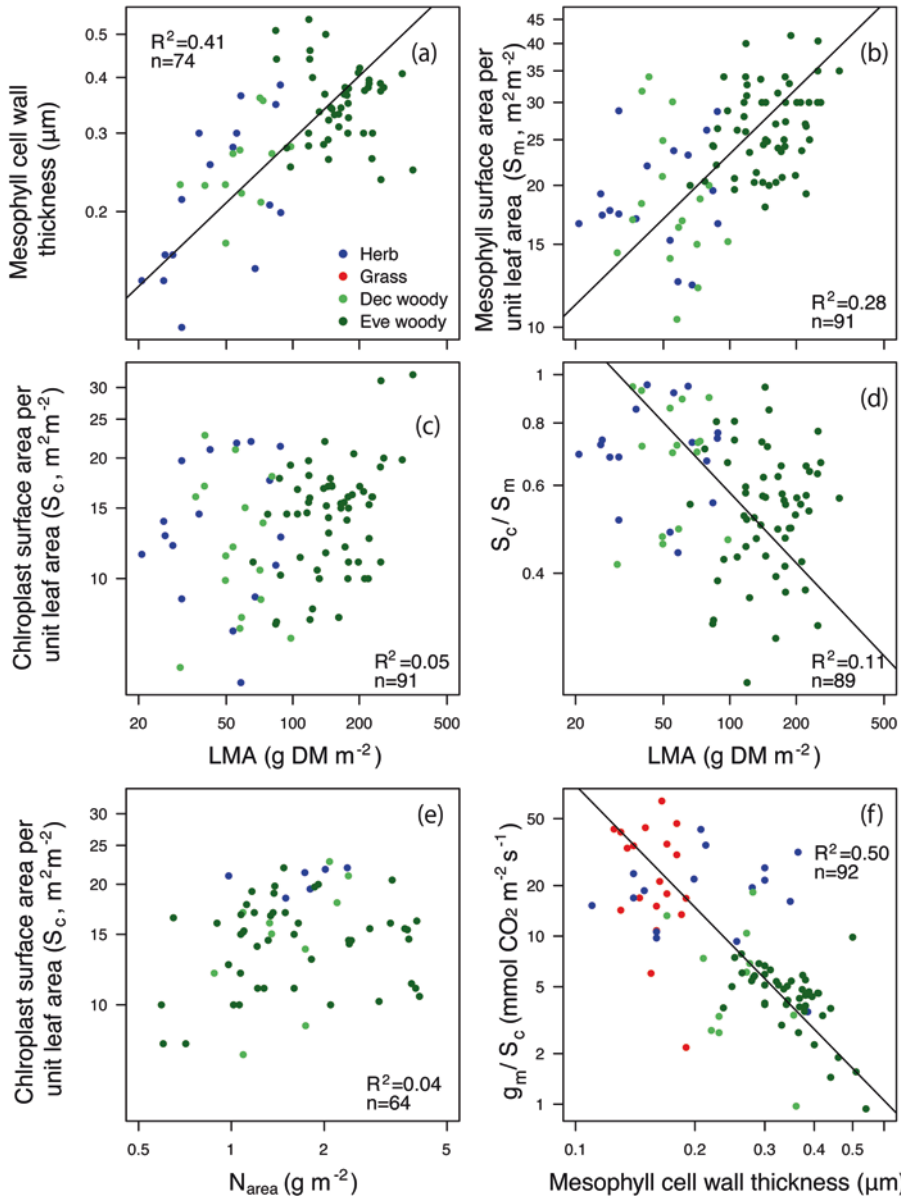

Fig. 16.11. Relationships between leaf dry mass per area (LMA) and (**a**) mesophyll cell wall thickness, (**b**) mesophyll surface area exposed to intercellular airspace per unit leaf area (S_m), (**c**) chloroplast surface exposed to intercellular airspace area per unit leaf area (S_c), and (**d**) the ratio of S_c to S_m. (**e**) S_c is plotted against leaf N content per unit area (N_{area}). (**f**) Mesophyll conductance per chloroplast surface area (g_m/S_c) is plotted against mesophyll cell wall thickness. SMA slopes were fitted when correlations were significant ($P < 0.01$); (**a**) $y = 0.033x^{0.472}$; (**b**) $y = 2.83x^{0.458}$; (**c**) no correlation, n = 91; (**d**) $y = 5.0x^{-0.468}$; (**e**) no correlation, n = 64; (**f**) $y = 0.307x^{-2.41}$. (Redrawn from Onoda et al. (2017)). *DM* dry mass, *Dec* deciduous, *Eve* evergreen

ture may be required to maintain the mesophyll anatomy and consequently the photosynthetic activity over a long period. This notion may be supported by the evidence that high-LMA leaves had higher bulk elastic modulus measured by pressure chamber techniques (Niinemets 2001). Second, thicker mesophyll cell walls may be important in defense against herbivores, especially leaf miners. Leaf miners preferentially feed on mesophyll tissues, which cannot be prevented by stronger epidermal tissues once leaf miners get into the inner leaf lamina tissue (Kimmerer and Potter 1987; Sinclair and Hughes 2010). Therefore long-lived leaves may in some sense require thicker mesophyll cell walls as well as tougher outer structure.

D. Relative Influence

We compiled evidence suggesting that lower PNUE in high LMA leaves is due to both (1) a leaf N allocation tradeoff between structural and photosynthetic pools and (2) CO_2 diffusional limitations. But, which factor is more influential in this regard? Since there is no study that has examined these two factors on the same set of species, a strict test to quantify their relative importance on the LES is not yet possible. Still, we can explore the relative importance of factors identified in Eq. 16.2 by comparing their scaling slopes in relation to PNUE. Equation 16.2 can be expressed as follows after log-transformation:

$$\log\left(PNUE\right) = \log\left(\frac{g_m}{S_c}\right) + \log\left(\frac{S_c}{N_p}\right) + \log\left(\frac{N_p}{N_{area}}\right) + \log\left(C_i - C_c\right) \tag{16.3}$$

This equation assumes that relative changes in each term have additive effects on log(PNUE). In particular, we can compare the scaling slopes of g_m/S_c−PNUE and that of N_{Rub}/N_{area}−PNUE (assuming Rubisco content and photosynthetic protein contents are linearly correlated).

Fig. 16.12 shows the scaling slopes between PNUE and its underlying components except for S_c/N_p, for which we have few available data. The scaling slope of N allocation to the Rubisco – PNUE relationship was +0.60 and that of the g_m/S_c – PNUE relationship was +0.79, suggesting that the gas diffusion limitation may contribute somewhat more to the lowering of PNUE. C_i−C_c, on the other hand, was negatively correlated to PNUE, with a scaling slope of −0.48, which partly counteracted the effects of N allocation to Rubisco and g_m/S_c on PNUE. The sum of these scaling coefficients was 0.91 (= 0.6 + 0.79–0.48), which was close to 1, suggesting that S_c/N_p (the remaining component) may not be strongly associated with PNUE. Similar analyses can be made when PNUE and its components are analyzed in relation to LMA. In this case, there were more data available because LMA was measured more frequently than PNUE, yet our conclusion is similar: gas diffusion limitations may contribute more to the lowering of PNUE than N allocation (Onoda et al. 2017). Larger C_i−C_c should be associated with higher carboxylation capacity (i.e., Rubisco content; Wright et al. 2003) and/or lower g_m (Niinemets et al. 2009). Therefore, the relative importance of N allocation to Rubisco and g_m/S_c may depend on the strength of inter-correlations among these components, requiring careful consideration. Yet, the magnitude of change in C_i−C_c was smaller than those in N allocation to Rubisco and g_m/S_c, suggesting that N allocation to Rubisco and g_m/S_c are both key components contributing to the negative PNUE – LMA relationship even when variation in CO_2 drawdown (i.e., C_i−C_c) is taken into account.

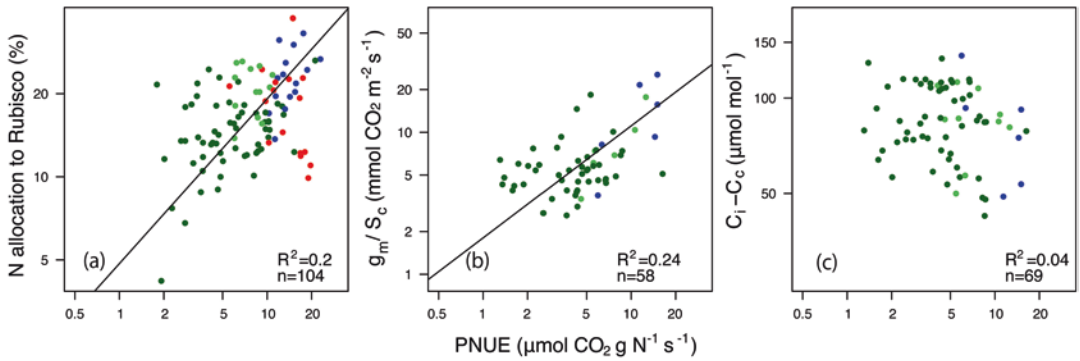

Fig. 16.12. The underlying components of PNUE in relation to PNUE. (**a**) Percentage of leaf N allocated to Rubisco, (**b**) mesophyll conductance per unit chloroplast area (g_m/S_c), and (**c**) difference in CO_2 concentration between intercellular airspace and the sites of carboxylation (C_i-C_c). SMA slopes: (**a**) $y = 4.91x^{0.597}$; (**b**) $y = 1.8x^{0.789}$; (**c**) $y = 175x^{-0.477}$. (Data from Onoda et al. (2017)). *Dec* deciduous, *Eve* evergreen

IV. Conclusions

Leaf functional trait data have been gathered in the last few decades in many locations worldwide, and we now can identify major correlations among leaf traits at a global scale. Leaf traits can be expressed per leaf area or per leaf dry mass. Each basis of expression has its merits; a leaf-area basis reflects fluxes in relation to surfaces while a leaf-mass basis reflects leaf economics, i.e., revenues and expenditures per unit investment of biomass. The "Leaf economic spectrum" is underpinned by a positive relationship between leaf lifespan and LMA, meaning that the duration of the photosynthetic revenue stream is generally longer in high LMA species. Leaf N and P concentrations, and photosynthetic and respiration rates – all considered on a mass basis – are negatively correlated with leaf lifespan and with LMA. Relationships among area-based traits are less consistent and less understood in relation to leaf economy; hence, this was a key focus in this chapter. LMA is, by definition, a converter between area- and mass-based traits, but it is far more than a conversion factor: structural, chemical, and physiological properties of leaves vary systematically with LMA. In this chapter, we described relationships among key area-based traits. LMA (and leaf lifespan) was tightly positively correlated with N_{area} but mostly independent from A_{area}. Given that N is an essential element in photosynthetic proteins and thus photosynthesis, understanding why PNUE (photosynthetic N use efficiency, A_{area}/N_{area}) decreases with LMA is a major concern. We showed that a higher fraction of mass allocated to cell wall in high LMA leaves seemingly reduced the efficiency of photosynthesis in two ways, via (1) a lower fraction of N allocated to photosynthetic proteins and (2) via lower mesophyll CO_2 diffusion. We note, however, that there are other factors – such as the specific activity of Rubisco – whose significance in interspecific variation of photosynthetic efficiency is still poorly known (Poorter and Evans 1998).

Regardless of the causes, the lower efficiency of photosynthesis in long-lived leaves is seemingly compensated for by higher N_{area}, which almost equalizes A_{area} across LMA (Fig. 16.3). It could be argued that the potential A_{area} in sunlit leaves is roughly invariant among species that differ in leaf lifespan because the maximum solar energy, which determines the potential for photosynthetic rate, is independent from disturbance regimes (note: A_{area} was somewhat negatively correlated with LL, but its correlation was much weaker than A_{mass}—LL, Fig. 16.4d).

Leaf lifespan, on the other hand, varies extensively with disturbance regimes such that short-lived species are favored in frequently disturbed environments while long-lived species are favored in stable environments (Reich et al. 1992). These tendencies may result in larger interspecific variation in leaf lifespan (22.5-fold for the 90% QR of leaf lifespan, n = 749; Wright et al. 2004) than in A_{area} (7.8-fold for the 90% QR of A_{area}, n = 2400; Maire et al. 2015). The large variation in leaf structure and function seen among the world's plant species surely results from adaptation to a range of environmental factors, yet there are fundamental physiological and structural constraints underlying this diversity.

Acknowledgments

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Chapter 17

Leaf Photosynthesis Integrated over Time

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Summary

We review a series of papers based on Kikuzawa's (1991) cost-benefit model for leaf longevity, including its extension to whole plants and entire communities in seasonal environments. This simple model of net carbon gain over the life of a leaf can explain relationships among key foliar traits such as the positive correlation between leaf longevity (L) and leaf mass per area (LMA) and the negative correlations between photosynthetic rate (A) and both L and LMA. The extension of the model to seasonal environments can explain and reproduce various biogeographical trends including bimodality in the distribution of evergreen species across latitude, increase and decrease in L of evergreen and deciduous species with shortening of the period favorable for photosynthesis (f), modulation of L-LMA relationships with f, and decrease in functional type richness in terms of phenology patterns towards higher latitudes and altitudes. Finally, the model suggests the possibility that the lifetime carbon gain by a single leaf can be extended by analogy to predict the productivity of forest ecosystems.

I. Introduction

Leaf longevity (L) is recognized as a central element (Shipley et al. 2006) leading to general covariations among leaf mass per leaf area (LMA), photosynthetic rate (A), and foliar nitrogen content (N) in the worldwide leaf economics spectrum (LES; Reich et al. 1997; Wright et al. 2004). The tradeoffs among foliar traits comprising the LES arise in a fundamental evolutionary tradeoff between instantaneous photosynthetic rate and leaf longevity. Species fall along a functional gradient from those with high photosynthetic capacity and short-lived leaves to those with longer-lived but less productive leaves, and the distribution and abundance of species across resource regimes depends in part on their position along the LES (Reich 2014). For a given species, the inverse of L also defines the rate of leaf turnover as new leaves are produced at the periphery of a mature plant canopy and older leaves that become increasingly shaded in the canopy are shed (Kikuzawa et al. 2009). A dynamic equilibrium between leaf production and leaf shedding is widely observed in both deciduous and evergreen plant canopies, within a very short period of growing season in herbaceous and deciduous plants, and over a number of growing seasons in evergreen plants (Kikuzawa and Lechowicz 2011).

In this chapter, we review theory that stems from Kikuzawa's (1991) model predicting leaf longevity as a function of photosynthetic rate, the construction cost of leaves, and the rate of decline in photosynthetic capacity with leaf age. The theory helps make sense of the empirical patterns in the LES and can be extended to seasonal environments to account for biogeographical trends in leaf longevity, the distribution of evergreen and deciduous species, and patterns of species richness. By rescaling the theory from single leaves to plant populations, communities, and ecosystems we show that the aggregate net productivity of a community of plants can be predicted from the life-time carbon gain of single leaves of the species comprising the community.

II. Leaf Longevity – Optimizing Model for Carbon Gain

The carbon gain (G) by a leaf is given as:

$$G = \int_0^t p_n(t)\,dt - C \tag{17.1}$$

where $p_n(t)$ is the net photosynthetic rate per unit leaf area at time t and C is the aggregate cost of constructing the leaf and its supporting organs including any investments in leaf defense against disease and herbivores (Kikuzawa 1991). The parameter $p_n(t)$ can be approximated by a linear decreasing function as

$$p_n(t) = a\left(1 - \frac{t}{b}\right) \tag{17.2}$$

where a is the daily photosynthetic rate per unit leaf area at the moment the developing leaf becomes functionally mature and b is the potential leaf longevity taken as the time elapsed until the photosynthetic capacity of the aging leaf declines to zero. A model suggests that the optimum timing of leaf shedding (t_{opt}) to maximize carbon gain of the plant is given by the time that maximizes the marginal carbon gain (g) at the leaf level:

$$g = \frac{1}{t}G \tag{17.3}$$

Substitution of Eqs. (17.1) and (17.2) into (17.3) gives the following analytic result:

$$t_{opt} = \left(\frac{2bC}{a}\right)^{\frac{1}{2}} \tag{17.4}$$

A. Parameter a and Mean Labor Time

The parameter a can be decomposed into the instantaneous photosynthetic rate (A_{area}) and a measure of mean labor time (m) attributable to leaf function:

$$a = mA_{area} \tag{17.5}$$

Mean labor time is defined as the ratio of the mean daily photosynthetic rate of a leaf to the mean value of potential photosynthetic rate of the leaf assuming that the leaf could work 24 h at maximum photosynthetic rate (Kikuzawa et al. 2004). The dimension of m is seconds per day, but for convenience m is usually expressed as hours per day. The concept of mean labor time allows for the fact that various factors such as changes in photoperiod, solar angle, clouds, shading by other leaves or by other plants, water deficit, and midday depression in photosynthesis can all reduce the hypothetical maximum photosynthetic rate to a realized rate below 24 h (Kikuzawa et al. 2004). Mean labor time calculated by considering these factors for *Alnus sieboldiana* was 5.5 h per day (Kikuzawa et al. 2004). Assuming that actual leaf longevity is t_{opt} in Eq. (17.4), the mean labor time can also be estimated using Eqs. (17.4) and (17.5) as

$$m = \left(\frac{2bC}{A_{area}(0)L^2}\right). \tag{17.6}$$

Kikuzawa and Lechowicz (2006) reported mean labor times ranging from 1 to 6 h (average around 3 h day^{-1}), although Oikawa et al. (2006) reported more than 10 h day^{-1} in an herbaceous annual plant.

B. Instantaneous Photosynthetic Rate per Unit Leaf Area, A_{area}

Kikuzawa's (1991) theory for leaf longevity, which is framed with reference to photosynthetic gains measured as A_{area} (cf. Eq. 17.1), leads to an expectation that L should be negatively correlated with A_{area} (cf. Eq. 17.4). In reality, this bivariate correlation can be modulated through the interaction between A_{area} and LMA; A_{area} can be expressed as a product of LMA and A_{mass} (photosynthetic rate per unit leaf mass), which provides a link between areal gains of photosynthate and the mass-based carbon cost (C) of constructing a leaf. In part because of this interaction with LMA, A_{area} does not differ as much among

leaves differing in longevity as does A_{mass}. For example, in a comparison among five tree species, Gower et al. (1993) found A_{mass} varied fivefold across species and was inversely correlated with L, but A_{area} varied less than twofold across species and did not correlate with L. Similarly, the A_{mass} in the LES database (Wright et al. 2004) varied 150-fold across species (n = 770) and A_{area} only 40-fold (n = 825). Wright et al. (2004) reported no correlation between A_{area} and L of well-lit leaves in the LES data while LMA and N_{area} are positively correlated (Wright et al. 2004; Onoda et al. 2017). There is essentially a tradeoff between photosynthetic capacity expressed as A_{area} which is accomplished by N_{area}, and persistence expressed as leaf longevity, but the relationship is modulated by the variation in leaf structure expressed as LMA. Onoda et al. (2017) argued that two opposing effects could largely cancel out: (1) higher LMA is correlated with higher A_{area}, because greater leaf thickness is attributable to thicker mesophyll layers and (2) leaves with higher LMA have greater cell wall density that reduces photosynthetic rates as a result of lower CO_2 diffusion. Chabot and Hicks (1982) were the first to consider that the lower photosynthetic rates in evergreen compared to deciduous species may be a consequence of dilution of photosynthetic tissue by non-photosynthetic tissue, in particular vascular tissue rich in cell-wall material. To persist longer a leaf must invest in defense against herbivory and disease as well as structural support against mechanical damage. Hence the investment in photosynthetic machinery will be diluted by allocation to these ancillary aspects of leaf function and the photosynthetic rate per unit leaf mass will be reduced. In addition to the dilution theory, the greater LMA in evergreen leaves can be attributed to other factors that reduce the photosynthetic rates of evergreens such as lower conductance of CO_2 or lower penetration of light because of the higher tissue density in evergreen leaves (Lusk et al. 2008; Wyka and Oleksyn 2014; see also Chap. 16 of this book).

C. Potential Leaf Longevity or Parameter b

Parameter b is the potential leaf longevity, or the time required for the photosynthetic rate of the aging leaf to decline to zero. Since it is difficult to measure the rate at the instant when the rate just becomes zero, b is best estimated from the slope of repeated measurements of the same leaf over time (Koyama and Kikuzawa 2010; Kikuzawa et al. 2013a). Alternatively, the rate of decline can be estimated by the measurement of leaves at different positions on shoots or on differently-aged leaves (Kitajima et al. 1997, 2002), assuming that basal leaves are oldest and distal youngest and the ages can be estimated by the bud-scars remaining on the shoots (i.e., the chronosequence method; Osada et al. 2015). The chronosequence approach is particularly useful for species with long-lived leaves that maintain a wide range of leaf ages on individual shoots and is less laborious than repeated measurements on single leaves (Osada et al. 2015).

Kikuzawa's (1991) cost-benefit model for leaf longevity predicts that daily carbon gain should be positive at the optimum time of leaf shedding and that potential leaf longevity (b) should be longer than the optimum timing (t_{opt}) if the total number of leaves per plant is limited. But the model also predicts that the photosynthetic rate at t_{opt} should be zero with t_{opt} coinciding with b if there is no limitation to the total number of leaves per plant. In many cases, potential leaf longevity is longer than realized longevity. Kikuzawa et al. (2013a) found that potential leaf longevity (b) is around twice the realized leaf longevity. Reich et al. (2009) found that carbon balance was positive when leaves died in 10 woody Australian plant species. Ackerly (1999) also reported A_{mass} greater than zero at the time of shedding for three tropical pioneer tree species, although not so great as expected from Eq. (17.4).

By a simple simulation, Osada et al. (2015) found that, even if individual leaves are shed when daily carbon gain becomes zero, the cohort mean carbon gain for surviving leaves is positive at the mean L. The chronosequence estimate of relationship between leaf age and photosynthetic rate inevitably depends on the "surviving" leaves; hence researchers might falsely infer that all leaves are shed when their carbon balance is positive (Osada et al. 2015). Even if repeated measurements were adopted, estimated photosynthetic rate at leaf fall will be biased when average leaf longevity is used to evaluate the relationship between age and photosynthetic capacity of surviving leaves. Only repeated measurements on individual leaves can provide definitive estimates of photosynthetic rate at leaf fall.

D. Construction Costs and Parameter C

Chabot and Hicks (1982) first presented an equation for the carbon economy of a single leaf that included expenditure for defense of the leaf against herbivores and disease, defense against environmental stress, and so forth. For simplicity Kikuzawa (1991, 1995) included these terms in a single construction cost (C) for a leaf. Kikuzawa and Ackerly (1999) also suggested incorporating a cost of non-photosynthetic organs such as branch and petiole to mechanically support leaves and conducting tissues to transport water and nutrients through root, stem, and branch.

$$C = C_l + C_s \tag{17.7}$$

Where C_l is the costs of construction of a leaf and C_s is the costs to construct supporting organs for the leaf. The significance of costs for supporting organs is illustrated by the shorter leaf longevity of seedlings (Seiwa and Kikuzawa 1991) compared to adult trees (Kikuzawa 1983); both support and conducting systems are physically near seedling leaves, hence leaf longevity is less than in older trees where support and transport involve greater distances (Kikuzawa and Ackerly 1999). But later, Kitajima and Poorter (2010) reported that tissue density and toughness, the two correlates of leaf longevity, increase from saplings to adults in tropical trees. However, this may be caused by the difference in light condition on saplings and adults (Russo and Kitajima 2016). Comparison of leaf longevities among plants of different life forms also suggests the importance of C_s (Kikuzawa and Ackerly 1999). Reich et al. (2009) further suggested that leaf level carbon balance should still be above zero at the leaf age of the typical leaf life span because leaves must support not only their own carbon costs but also those of other plant parts (branch, stem, or roots) that are required to sustain the canopy.

Defense necessarily has some material basis, therefore any investment into foliar defense such as a thicker cuticle, thicker epidermis, higher values of vein per unit leaf area or more concentrated chemical defenses must result in an increase in leaf mass. The aggregate investment in constituents of a leaf such as protein, chlorophyll, total nonstructural carbon (TNC), defensive chemical, and so forth (cf. Poorter et al. 2009) can be expressed simply as:

$$\mathrm{LMA} = \sum_j M_j / A \tag{17.8}$$

where M_j is the mass of the j^{th} compound and A is leaf area (Poorter et al. 2009). This simple equation could be expanded to include all individual constituents in each tissue of a leaf as:

$$\mathrm{LMA} = \sum_i \sum_j M_{ij} / A \tag{17.9}$$

where M_{ij} is the mass of the j^{th} compound in the i^{th} tissue in a leaf. The proportion of the total amount of the corresponding leaf attributable to the construction and defense of the

leaf is C_l. The total investment for defense and construction then can be described as

$$C_l = c\,\mathrm{LMA}, \tag{17.10}$$

where c is a proportionality constant.

In 1980s, the construction cost or energy to convert glucose to leaf tissue was considered to vary substantially among species and hence might explain the high interspecific variation in leaf longevity. In fact, there is less than twofold variation in the construction cost among species (Williams et al. 1989; Villar and Merino 2001), although Wyka and Oleksyn (2014) reported slightly greater construction cost in evergreen (1.55 g glucose g^{-1}) compared to deciduous (1.46 g glucose g^{-1}) species. Hence, the differences in parameter C among species are attributable in large part to the differences in LMA. Since measuring defensive material is not easy, in many cases LMA is taken as a surrogate for C.

E. Leaf Mass per Area (LMA)

Actual leaf longevity is positively correlated with LMA, which is consistent with the idea that leaf longevity depends in part on the defense material invested in the leaf (C_l). A positive, significant relationship was reported between leaf longevity and LMA in 19 tropical saplings (Kitajima and Poorter 2010). Similar positive trends were reported in global data sets (Reich et al. 1992; Wright et al. 2004; Donovan et al. 2011). Greater LMA is associated with thicker cuticle, thicker epidermis, and denser leaf veins, etc. (Blonder et al. 2011; Kitajima et al. 2013; Onoda et al. 2015). For example, Blonder et al. (2011) incorporated three venation parameters (distance, density, and loopiness) into a model predicting four leaf traits: A_{mass}, L, N_{mass} (nitrogen content per unit leaf mass), and LMA. Blonder et al. (2011) argued that the leaf economic spectrum relationships among the four traits were well reproduced by their model (Blonder et al. 2011, 2013). Sack et al. (2013), however, rejected Blonder's analysis, arguing instead that vein length per leaf area contributes to the LES via leaf hydraulic conductance and thereby leaf stomatal conductance and photosynthetic rate. Venation networks augmented by investments in epidermis can also extend leaf longevity by strengthening the sandwich structure that confers a degree of structural support and mechanical defense for leaves (Onoda et al. 2015).

Leaf mass per leaf area (LMA) can be further decomposed into laminar density and laminar thickness (Castro-Diez et al. 2000; Kitajima and Poorter 2010).

$$\mathrm{LMA} = \mathrm{lamD} \times \mathrm{lamT} \tag{17.11}$$

Where lamD is laminar density or leaf mass per unit leaf volume (g dry weight m^{-3}) and lamT is laminar thickness (m; Kitajima and Poorter 2010). Castro-Diez et al. (2000) compared 52 European woody plant species and found that LMA was correlated with laminar density but not with laminar thickness. A comparison of 19 tropical tree species revealed that leaf longevity did not correlate with leaf thickness, but instead with leaf density (Kitajima and Poorter 2010). Dense, thicker leaves are usually tough and long-lived but this reduces maximum photosynthetic rate due to the slow diffusion of CO_2 within the leaf as a result of thicker cell walls (Onoda et al. 2017). On the contrary, thick but low density leaves enable good CO_2 diffusion within the leaf. Investments in photosynthetic machinery can also act to increase LMA. The LMA for a given species basically reflects a balance struck between making tough leaves that confer greater L versus leaves better suited to photosynthesis that have lower L (Lusk et al. 2008; Reich 2014).

There are both plastic and evolutionary responses of quantitative traits to environmental gradients. For example, L is usually longer in shaded individuals than in those

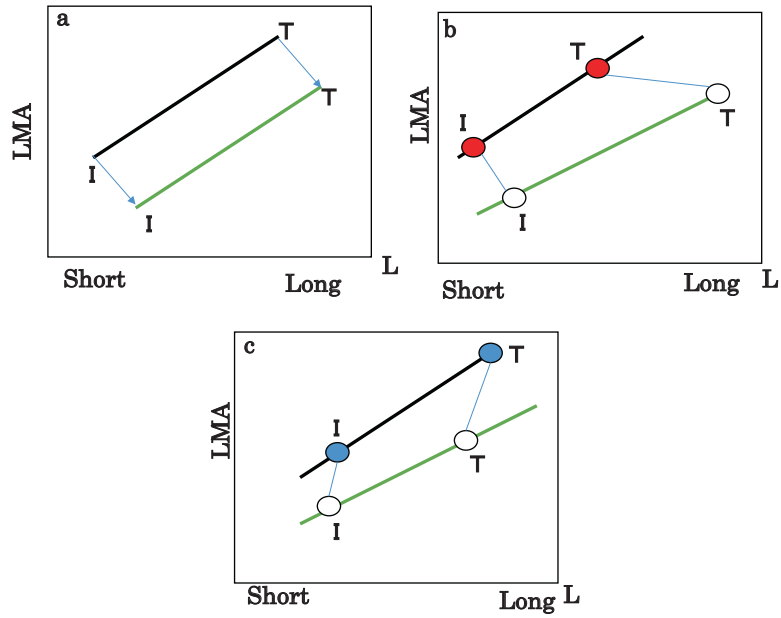

Fig. 17.1. Schematic representation of counter gradient (**a**, **b**) and co-gradient (**c**) variation of LMA with L. **Panel a**: The effect of changes in habitat light condition on the LMA-L relationship (plastic response having negative gradient) which differs from the LMA-L relationship among species (evolved relationship with positive gradient). The thick black line represents the LMA-L relationship in a sunny environment and the thick green line in a shady environment; thin arrows indicate the plastic changes in shade tolerant (T) and intolerant (I) species. (Redrawn from Lusk et al. 2008). **Panel b:** Red circles and thick black lines represent the L-LMA relationship in a sunny environment and white circles and thick green lines in a shady environment; thin lines represent plastic responses. Response in L is greater in the species with longer L, while response in LMA is relatively greater in the species with shorter L. **Panel c**: Co-gradient variation in the LMA-L relationship in the case of soil fertility. Closed blue circle represents less fertile soil and white circle fertile soil. Responses in LMA are greater than L in all species. (Panels **b** and **c** are redrawn after Russo and Kitajima (2016)) (Colour figure online)

grown in brighter sites (plastic response) and also longer in shade tolerant species adapted to late-successional habitats than in light demanding species (evolutional response). Lusk et al. (2008) distinguish co-gradient variation in leaf traits (i.e. similar directions of plastic response and evolutional trends) and counter-gradient variation (i.e. the direction of plastic response differs from the evolutional response). For example, there is indeed a marked divergence between the plastic and evolutionary responses of LMA to shade. Within species, individuals grown in shaded habitats have lower LMA than those grown in sunnier habitats. But in interspecific comparisons, shade tolerant species tend to have higher LMA than light demanding species (Lusk et al. 2008; Fig. 17.1a).

Russo and Kitajima (2016) proposed a similar conceptual model to that by Lusk et al. (2008) that more explicitly predicts the degree of plastic responses of L and LMA to experimentally standardized light conditions in different species adapted to sun and shade conditions (Fig. 17.1b, c). They predicted that species having longer L will show greater plasticity to changing light in L than in LMA because of the existence of an upper limit in LMA, but species having shorter L will show less plasticity in L because of the existence of a lower limitation in L. Their prediction was supported by an experiment on 41 Panamanian tree species. In the case of responses of L and LMA to soil nutrient conditions, directions of responses among species and among leaves within species are

predicted to be similar (i.e., co-gradient variation Fig. 17.1c; Russo and Kitajima 2016).

F. Leaf Economic Spectrum (LES)

As shown in the previous chapter of this book, the pattern of correlations among leaf traits referred to as the leaf economic spectrum (Wright et al. 2004) reflects contrasting strategies for productivity (Reich 2014) that are strikingly consistent among biomes (Reich 2014; Reich et al. 1997, 1999). One end of the spectrum represents slow-growing species that produce long-lived, structurally expensive leaves with low photosynthetic rate. The other end represents fast-growing species that produce short-lived leaves with low LMA and high photosynthetic rate. Of the various leaf traits, three (A_{mass}, L, and LMA) or four (A_{mass}, L, LMA, and Nitrogen content) can explain a large part of the variation observed among plant species (Shipley et al. 2006; Donovan et al. 2011). Important correlations among traits include the negative correlations between A_{mass} and L, which is largely determined by A_{mass} and LMA (Osnas et al. 2013), the positive correlation between L and LMA, and the positive correlation between A_{mass} and N_{mass}.

Parameters in Eq. (17.4) can be interpreted to be leaf traits in LES and many correlations among leaf traits can be reproduced by Eq. (17.4). Although there is a report that real values of L for three pioneer tree species were from 24 to 60% greater than model predictions (Ackerly 1999), we can predict L from the calculated t_{opt} values. The daily photosynthetic rate (parameter a) can be interpreted as an instantaneous rate (A_{mass}) mediated by mean labor time m. The effects of construction cost C in Eq. (17.4) can be referenced against LMA by Eq. (17.9). Hence, many relationships between leaf traits in the LES can be linked to leaf parameters in Eq. (17.4). For example, L and A_{mass} are negatively correlated. L and LMA are positively correlated, although the correlation between N and A_{mass} is not explicitly shown in Eq. (17.4). In conclusion, many leaf traits and correlations between traits are expressed in the single Eq. (17.4).

III. Extension of the Model to Seasonal Environments

A. Favorable Period (f)

Equation (17.1) is a model of leaf carbon gain in an ideal stable environment where plants can perform photosynthesis every day throughout a year. For example, one may consider the conditions in the equatorial wet tropics where temperature and water supply do not limit plant growth. In regions outside the equatorial tropics, however, photoperiod, solar angle, air temperature, precipitation amounts, and other environmental factors that influence photosynthetic activity all change seasonally. In general, when air temperature falls below about 5 °C photosynthesis diminishes rapidly (Luo et al. 2002). Many plants shed leaves during the winter season when air temperature drops below freezing (deciduous habit), although some retain leaves during winter (evergreen habit). In warm temperate regions, some plants perform photosynthesis in winter on warmer days. Since insolation is better on the forest floor of deciduous forests in winter, evergreen plants in the understory can be more productive during winter (Miyazawa and Kikuzawa 2006). But in cool temperate and boreal regions low winter temperatures and heavy snow depth preclude photosynthetic activity. Even in tropical regions where temperature is usually high throughout a year, a dry season with monthly rainfall less than about 25 mm can limit photosynthetic activity (Eamus and Prior 2001). Similarly, a dry summer in temperate regions is not suitable for photosynthesis (Manzoni et al. 2015). In these sorts of unfavorable periods for photosynthesis, some plants shed all their leaves (drought deciduous) and some retain dormant leaves (summer evergreen), with many intermediate types (Eamus and Prior 2001; Manzoni et al. 2015).

Kikuzawa (1991) allowed for these effects by adapting his basic model to seasonal environments where favorable and unfavorable periods alternate within a year. In favorable periods, all plants photosynthesize but in unfavorable periods some plants shed leaves and show a deciduous habit, while other plants retain leaves in a dormant state (evergreen). The consequent carbon gains per unit time can be written as,

$$g = \frac{1}{t}\left(\int_0^f p_g(t)\,dt + \int_1^{1+f} p_g(t)\,dt + \cdots + \int_{[t]}^{t} p_g(t)\,dt - \int_0^t r(t)\,dt - C \right) \tag{17.12}$$

where $p_g(t)$ is gross photosynthetic rate, r(t) is the respiration rate of a unit leaf area ($p_n = p_g - r$) and [ ] indicates Gaussian notation. Note that photosynthesis is carried out only during f in each year but maintenance respiration persists throughout all seasons.

Kikuzawa's (1991) model implicitly assumed that leaves appeared at the start of the favorable period, but Seki et al. (2015) explicitly showed there are, in fact, three alternative strategies for seasonal timing of leaf expansion: (1) immediately after shedding of an old leaf, (2) only at the beginning of favorable season, and (3) a combination of (1) and (2): immediately after shedding of an old leaf if the shedding occurs during f or otherwise at the beginning of favorable season. Their new model clarified that the combined strategy will usually yield the highest carbon gain.

B. Functional Leaf Longevity (L_f)

From the functional point of view, leaf longevity must be changed by considering periods within the year when that function is precluded. For example, for photosynthesis, functional leaf longevity is defined as the time during the year that a leaf actually carries out photosynthesis (Kikuzawa and Lechowicz 2006). For evergreen leaves in seasonal environments, we can assume that leaves are dormant during an unfavorable period and thus L_f is essentially L minus the unfavorable period. In the case of plants in the wet tropics or deciduous plants in temperate regions, L_f is essentially the same as L.

We can extend the concept of functional leaf longevity to a consideration of forest productivity. The ratio of total leaf biomass in the canopy and leaf longevity, which suggests the leaf production rate, is greater in aseasonal forests (wet tropics) than in seasonal forests (seasonal tropics, temperate, boreal, and subarctic regions; Fig. 17.2a). If we use functional leaf longevity instead of leaf longevity, however, this apparent difference between seasonal and aseasonal forests disappears. Both types of forests can be regressed by a common single line (Fig. 17.2b), suggesting similar leaf production rates prevail in the favorable periods across latitudes (Kikuzawa and Lechowicz 2006). The main difference between tropical and non-tropical forests is not the mean annual temperature itself, but the temperature-mediated variation in favorable period length.

C. Leaf Lifetime Performance

We can define lifetime carbon gain (P_L; g dry weight g^{-1} dry weight) as the product of average daily carbon gain and functional leaf longevity (Kikuzawa and Lechowicz 2006). The former in turn is defined as the product of mean labor time (s day^{-1}) and average instantaneous photosynthetic rate ($\bar{A}_{mass}$, as g dry weight g^{-1} dry weight s^{-1}). Finally, P_L is given as,

$$P_L = mL_f\bar{A}_{mass.} \tag{17.13}$$

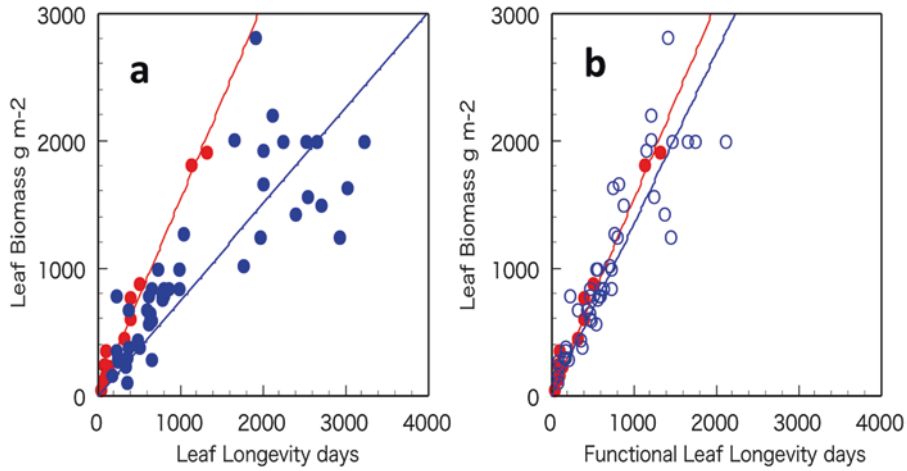

Fig. 17.2. The relationships between leaf biomass and both (**a**) leaf longevity and (**b**) functional leaf longevity. (Redrawn after Kikuzawa and Lechowicz 2006). Blue closed circles in (**a**) and open circles in (**b**) indicate forests in seasonal environments and red closed circles those in non-seasonal environments. The slope of lines in (**a**) indicates average daily leaf production rate, which in non-seasonal forests (red line, ~1.5 g dry weight m^{-2} day^{-1}) is around twice that of seasonal forests (blue line, ~0.75 g dry weight m^{-2} day^{-1}). (**b**) Blue open circles indicate leaf production in seasonal forests, which is not much different from non-seasonal forests (red closed circle) (Colour figure online)

Surplus production (gross primary production minus leaf respiration; Monsi 1960) of a forest stand can be defined by the following.

$$P = B\partial\bar{A}_{mass.} \tag{17.14}$$

where B is leaf biomass of the stand, ∂ is the cumulative duration of favorable time for photosynthesis, the product of daily (m) and seasonal (f) favorable period (∂ = mf). Equation (17.14) can be extended by incorporating $L_f/L_f = 1$.

$$P = \frac{B}{L_f} fmL_f\bar{A}_{mass.} \tag{17.15}$$

where B/L_f represents daily leaf production (see Fig. 17.2) and thus $(B/L_f)f$ represents annual leaf production. The product of the last three terms in Eq. (17.15) is life time gain (P_L) of a leaf. Surplus production then should be easily obtained as the product of annual leaf production, which can be estimated by annual leaf fall using litter-traps, and the life-time gain of a single leaf (Kikuzawa and Lechowicz 2006).

To demonstrate this possibility, 55 leaves were selected in an artificial beech stand and their photosynthetic rate was periodically measured. Parameter b was estimated from the linear decline of A_{mass} (Fig. 17.3a), and parameter m using Eq. (17.5). Finally, P_L was estimated to range from 1.5 to 6 g dry weight g^{-1} dry weight, an average of 3.0 g dry weight g^{-1} dry weight (Fig. 17.3b). In short, 1 g dry weight of beech leaf produced on average 3 g dry weight of biomass, which can be used for the production of leaves, and production and maintenance of stems, roots and so forth.

The concept of lifetime performance can be extended to other aspects of leaf performance such as lifetime respiration or lifetime transpiration. For example, Suzuki et al. (2013) applied the concept to herbivore damage to leaves in forests on Mt. Kinabalu, Malaysia. Fallen leaves were collected by litter traps set on the forest floor, and the leaf area lost to herbivorous insect larvae (i.e. the

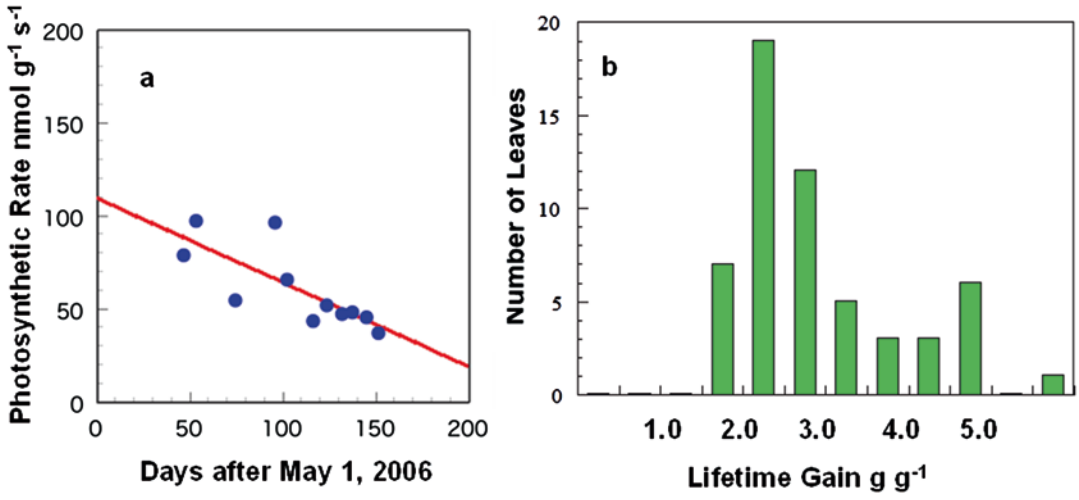

Fig. 17.3. Lifetime photosynthetic gain by beech (*Fagus crenata*) leaves. (**a**) Decline of photosynthetic rate with time for a beech leaf. (A_{mass} = −0.45 day + 110.) (**b**) Histogram of lifetime photosynthetic biomass gain (g dry weight biomass gain/g dry weight leaf) by single leaves. Average was 3.0 g dry weight g^{-1} dry weight

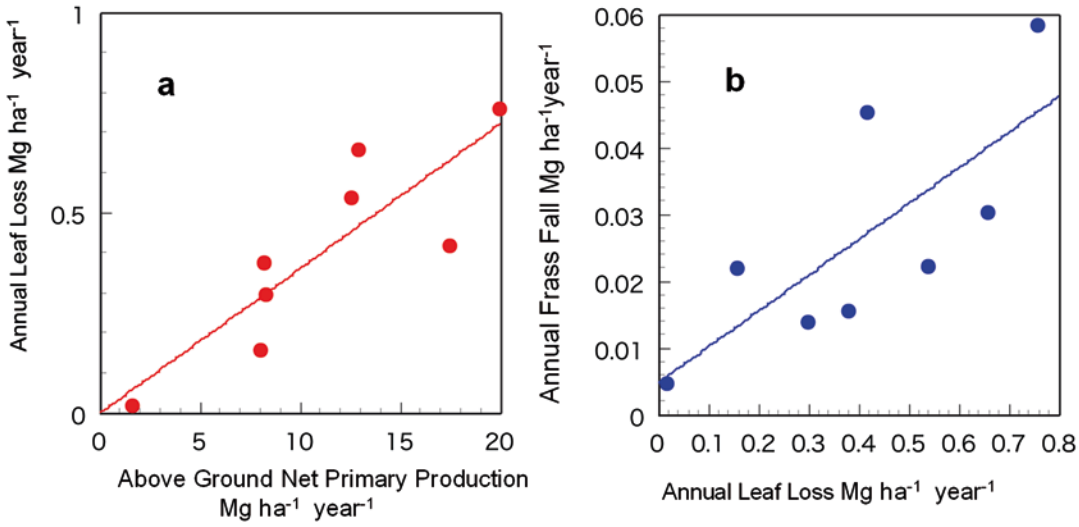

Fig. 17.4. (**a**) Annual leaf loss estimated from lifetime leaf loss against aboveground net primary production in tropical montane forests at different altitudes (700 m~3100 m above sea level) on Mt. Kinabalu. (Redrawn after Suzuki et al. 2013). Annual leaf losses were around 5% of aboveground net primary production (ANPP; Leaf Loss = 0.036 ANPP+0.0017; $r^2 = 0.73$). (**b**) Annual frass fall against annual leaf loss. Frass fall is less than 10% of leaf loss. (Frass Fall = 0.054 Leaf Loss+0.0049; $r^2 = 0.57$)

lifetime leaf loss) estimated to be 0.02~0.76 mg dry weight ha^{-1} $year^{-1}$. This estimate correlated well to the above ground net production (Fig. 17.4a) of forests at different altitudes on Mt. Kinabalu, and was also positively correlated with frass fall collected in the litter traps (Fig. 17.4b; Kikuzawa et al. 2002).

IV. Plant Size, Plant Performance and L

Leaf longevity is related to a broad array of traits associated with variation in plant life history (Reich 2014). The shorter L the more rapid is the acquisition of resources, juvenile growth, reproductive maturation, and the shorter the plant lifespan. For example, Reich et al. (1992) reported a negative relationship between the relative growth rate (RGR) of individual plants and L. Similarly, Seiwa and Kikuzawa (2011) showed a negative trend between RGR of seedlings and L.

A. Normalization Constant of Allometry

West et al. (1997) proposed a general metabolic scaling equation to show the relationship between plant performance (Q) and plant mass (M).

$$Q = Q_o M^{\theta} \tag{17.16}$$

where Q is any value relating to some aspect of metabolism such as leaf mass, photosynthesis, or respiration, θ is a scaling exponent which takes a value usually less than unity, and M is plant mass. Q_o is a normalization constant that adjusts the general relationship (M^{θ}) across environments and species.

Although Eqs. (17.14) and (17.15) are for production of forest stands, they are equally applicable to the production of individual plants if we consider parameter B as the leaf biomass of an individual plant. Instantaneous photosynthetic rate declines with time as daily rate does in Eq. (17.2).

$$A_{mass} = A_{mass}(0)\left(1 - \frac{L}{b}\right) \tag{17.17}$$

Average photosynthetic rate, $\bar{A}_{mass}$ is given as

$$\bar{A}_{mass} = A_{mass}(0)\left(1 - \frac{L}{2b}\right) \tag{17.18}$$

On the other hand, applying Eq. (17.16) to the leaf biomass-plant biomass allometry, we obtain the following:

$$B = \beta M^{\theta} \tag{17.19}$$

where β is a normalization constant. Substitution of Eq. (17.6), which is written using A_{mass} as

$$m = \left(\frac{2bC}{A_{mass}(0)LMA\,L^2}\right). \tag{17.20}$$

Substitution of this equation and Eqs. (17.18) and (17.19) into (17.15) gives an individual plant's surplus production as

$$P = \frac{fC}{LMA\,L}\left(\frac{2b}{L} - 1\right)\beta M^{\theta} \tag{17.21}$$

It is noteworthy that productivity is independent of photosynthetic rate (A_{mass}) but is affected by leaf longevity (L). In the bracket of Eq. (17.21), b/L represents the ratio of potential to actual leaf longevity. Usually, potential leaf longevity is longer than realized L and thus this equation expresses surplus production. Kikuzawa et al. (2013a) examined the ratio of potential to realized L for 34 species-year-site combinations and found the ratio to be approximately 2.0.

B. Relative Growth Rate

If we assume that a fixed ratio (γ) of production is allocated to an increment of plant mass then dM/dt = γP. Relative growth rate (RGR) is (1/M)dM/dt. If we set b/L = 2, then the following is easily derived from Eq. (17.21).

$$\mathrm{RGR} = 3\gamma \frac{\mathrm{fC}}{\mathrm{LMA\,L}} \beta \mathrm{M}^{\theta-1} \qquad (17.22)$$

If first-year seedlings are compared, γ will be invariant among individual plants of the same species, since plant size at the end of the first growing season is determined by seed reserves and current year production and seed size is relatively invariant within the same species (Westoby and Rice 1982; Westoby et al. 1992). The effect of θ will also disappear in the case of seedlings, since θ takes a value near unity for small plants (Reich 2001). Considering these simplifications, Eq. (17.21) can explain the negative relationship between RGR and L (Reich et al. 1992; Seiwa and Kikuzawa 2011; Kikuzawa et al. 2013a). This relationship can be extended to the comparison of RGR across species when the seedling size is far greater than the supply from seed reserve, or where differences in seed size among species are not so great.

The negative relationship between RGR and L as indicated in Eq. (17.22) could also be derived from traditional growth analysis (Poorter et al. 2009)

$$\mathrm{RGR} = \frac{1}{\mathrm{w}}\frac{\mathrm{dw}}{\mathrm{dt}} = \frac{1}{\mathrm{A}}\frac{\mathrm{dw}}{\mathrm{dt}}\frac{\mathrm{A}}{\mathrm{w}} = \frac{1}{\mathrm{A}}\frac{\mathrm{dw}}{\mathrm{dt}}\frac{\frac{\mathrm{w_L}}{\mathrm{w}}}{\frac{\mathrm{w_L}}{\mathrm{A}}}. \qquad (17.23)$$

Where 1/w(dw/dt) is RGR, 1/A(dw/dt) is net assimilation rate (NAR), A/w is leaf area ratio and w_L is leaf weight. w_L/A in the denominator of the fourth term is nothing but the LMA and is positively correlated with L. Thus, RGR is predicted to be negatively correlated with L from the growth analysis.

V. Ecosystems

A. Productivity of a Stand

Resource acquisition is asymptotic with the investment for resource capture. For example, total photosynthesis by an individual plant is best expressed as a quadratic equation against total leaf mass, not by a straight line (Koyama and Kikuzawa 2009). Similarly, the growth equation for an individual plant is expressed by a logistic equation (Shinozaki and Kira 1956), which allows for the relationship between total plant biomass per unit land area (y) against plant number per unit land area (n) as

$$\mathrm{y} = \mathrm{n}/\left(\mathrm{dn} + \mathrm{e}\right) \qquad (17.24)$$

where d and e are parameters of the equation (Kikuzawa and Lechowicz 2016). Under completely one-sided competition, this inevitably leads to the cumulative mass versus cumulative number relationship:

$$\mathrm{Y} = \mathrm{N}/\left(\mathrm{DN} + \mathrm{E}\right) \qquad (17.25)$$

where Y is cumulative mass from the largest tree in a stand, N is cumulative number of trees in the stand also from the largest tree, and D and E are parameters that ultimately correspond to d and e, respectively (Kikuzawa 1999; Kobayashi and Kikuzawa 2000; Kikuzawa and Lechowicz 2016). Eq. (17.24) in turn leads to the distribution density function (ϕ(M)) for tree mass (M) (Hozumi et al. 1968) as

$$\phi\left(\mathrm{M}\right) = \frac{\sqrt{\mathrm{E}}}{2\mathrm{D}}\mathrm{M}^{-3/2} \qquad (17.26)$$

Multiplying Eqs. (17.26) and (17.21) and integration of the individual plant production in relation to plant mass from the largest to smallest individuals gives the production of a stand:

$$P_T = \frac{3\sqrt{E}}{D(2\theta-1)}\frac{fC}{LMA\,L}\beta M_{max}^{\theta-\frac{1}{2}} \quad (17.27)$$

where P_T is the stand production of a pure stand which is composed of a single species; the term M_{min} (the smallest plant mass in the stand) is omitted as it is too small to matter compared to M_{max} (mass of the largest individual). In case of the total production in a mixed species stand, which is composed of multiple species, P_T will be given by the summation of production P_t of each species present, which can be a daunting task.

Here, we will propose an alternative, more feasible method to estimate productivity in a mixed species stand. From Eq. (17.15), surplus production of species i (P_i) is expressed as

$$P_i = \frac{B_i}{L_{fi}} f_i m_i L_{fi} \bar{A}_{massi} \quad (17.28)$$

where the suffix i expresses each species. This equation can be simplified as,

$$P_i = F_i P_{Li} \quad (17.29)$$

The leaf production per species i (F_i) can be obtained using litter traps set on the forest floor. Repeated measurements of photosynthesis (cf. Fig. 17.3a) and monitoring of leaf numbers will give the average lifetime gain by a leaf (P_{Li}). The total surplus production of the forest ecosystem is then given by

$$P_T = \sum_i F_i P_{Li} \quad (17.30)$$

B. Longevity of Fallen Leaves in Ecosystems

Since leaf fall is an important path connecting the production processes in an ecosystem to the decomposition processes, the characteristics of fallen leaves can influence ecosystem function (Kikuzawa 2004). Various traits affecting decomposition processes vary with leaf longevity, which differs substantially among herbaceous and woody plant life forms (Kikuzawa and Ackerly 1999). Other leaf traits such as LMA, nitrogen content, and photosynthesis also vary among life forms, for example among annual and perennial forbs, grasses, deciduous trees, evergreen trees, and needle-leaf conifers (Niinemets et al. 2015). Some chemical defense materials can remain in fallen leaves, which together with mechanical traits such as high LMA act against consumption and decomposition by soil animals and microorganisms on the forest floor (Cornelissen and Thompson 1997; Cornelissen et al. 1999; Thomas and Sadras 2001). Santiago (2007) showed that the decomposability of leaf tissue for 35 plant species in a tropical forest was related to LES characteristics. Thin or less dense leaves with high nutrient concentrations from fast-growing species were easily decomposable whereas thick and tougher leaves from slow-growing species were not readily decomposable. These differences in decomposability in fallen leaves affect the nature of soils, micro-and meso-organisms in forest soils, and thereby ultimately affect the nutrient circulation in forest ecosystems.

Similarly, tree leaves occasionally fall in streams where differences in the decomposition rate are observed among species. For example, the rate of leaf-area loss in the stream was greater in alder (*Alnus glutinosa*) than in oak (*Quercus petraea*) leaves. Aquatic insect larvae (shredders) are responsible to the leaf area-loss, which in turn was affected by the presence of predacious insect-larvae such as dragonflies (Jabiol et al. 2014).

C. Comparison of Ecosystems

In the leaf economics spectrum (LES), Reich (2014) recognized two extreme strategies of plants: slow and fast. The slow strategy is characterized by a low rate of photosynthesis but longer leaf longevity and high LMA, the fast strategy by high but rapidly declining A_{mass}, short L, and low LMA. Analogous to slow and fast leaf traits, ecosystems can be similarly classified as slow and fast.

Terrestrial forest ecosystems are typically slow ecosystems where forests are characterized by high levels of plant mass stored in the woody biomass of trees with long lifespans. Among forest ecosystems, some are relatively fast and some are relatively slow. Fast forest ecosystems at high latitudes in the northern hemisphere, for example, are dominated by early successional species such as *Alnus*, *Betula*, *Populus*, and *Mallotus* with short L (Kikuzawa 1983). Slow forest ecosystems in these regions are dominated by tree species such as *Fagus* and *Quercus* with leaves of longer L that are not easily decomposable and therefore accumulate as litter layers in the soil and with higher biomass. In contrast, aquatic ecosystems can be characterized as very fast with primary producers such as phytoplankton, aquatic algae, and aquatic herbaceous plants that have short longevity and much lower biomass than the trees that dominate forest terrestrial ecosystems.

By analogy to trade-offs among leaf-traits, we can expect some relationships among ecosystem-traits. Keystone traits characterizing ecosystems are the longevity of photosynthetic organs and their supporting systems. In forest ecosystems, leaves are supported by woody roots, stems, and branches. A major portion of the carbon that the plants in these ecosystems accumulate is invested in these woody organs. This results in great C_s in Eq. (17.7) and thus great C in Eq. (17.4) and finally entails long L. On the other hand, in aquatic ecosystems, photosynthetic organs can float in water by buoyancy and thus minimize C_s (Kikuzawa and Ackerly 1999). Differences in the arrangement of leaves in different environments entails different turnover of leaves. Additionally, energy flow through herbivores in terrestrial ecosystems is smaller (Fig. 17.4) than in aquatic ecosystems (Cyr and Pace 1993). Terrestrial plants are hard, tough, and not readily digestible, and herbivores are more limited by predators (Polis 1999; Jabiol et al. 2014).

VI. Biogeographical Patterns

Several biogeographical patterns that map onto LES traits have been recognized such as latitudinal trends in LMA, leaf longevity, and the relative proportion of species with evergreen versus deciduous habits. The functional basis of such patterns arises in adaptations to the onset of an unfavorable season for productivity. In broad terms, plants follow one of two alternative strategies that comprise the contrasting foliar habits. Deciduous trees shed all their leaves and resume photosynthesis at the next favorable period. Evergreen species retain leaves through the unfavorable period, paying a maintenance cost but with the advantage that photosynthesis resumes quickly at the start of the next favorable period using leaves that were retained during the winter and/or during a period of low water availability. In evolutionary terms, the relative advantages of the two foliar habits is decided by a combination of environmental factors and foliar traits that together define alternative adaptations to maximize carbon gain. We illustrate the nature of these complex interactions in a series of examples.

(a) Broadleaf evergreen trees dominate in tropical, subtropical, and warm temperate forests, deciduous trees in temperate forests, and needle-leaf or small-leaved evergreen trees in boreal and subarctic forests at high latitudes. Chabot and Hicks (1982) found this to

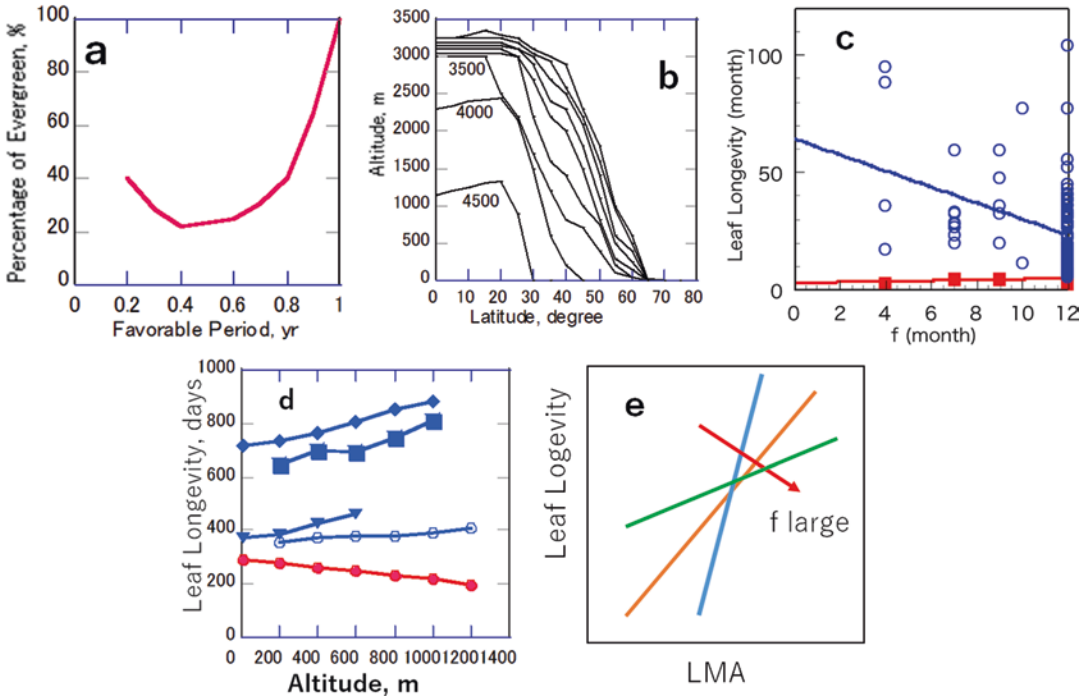

Fig. 17.5. Biogeographical patterns at both global and local scales reproduced by Eq. (17.12). (**a**) A schematic representation of global pattern of percentage of evergreen species. Two peaks were observed at lower and higher f. Redrawn after Kikuzawa (1991). (**b**) A schematic representation of plant species richness in monsoon Asia. The number of species is highest at low latitude and altitude and decreases toward higher latitude and altitude. Numerals affixed to the curves are number of species. (Redrawn after Kikuzawa (1996)). (**c**) Different global patterns of leaf longevity against f. L of deciduous species increases while that of evergreen species decreases with increasing f. (Redrawn after Kikuzawa et al. (2013b)). (**d**) Different altitudinal patterns of evergreen (green symbol) and deciduous (red symbol) species on Yakushima Island. *Sm Stewartia monadelpha, Qs Quercus salicina*, *Cc Castanopsis cuspidata*, *La Litsea acuminate*, *Dr Distylium racemosum*. (Redrawn after Fujita et al. (2012)). (**e**) L-LMA relationships modulated by **f**. In short **f**, the gradient of the L-LMA relationship is steep, but it becomes gentle with greater **f**. (Redrawn after Kikuzawa et al. (2013b))

be a puzzling bimodal pattern for evergreen species, but Kikuzawa (1991) was able to explain the bimodal pattern by extending Eq. (17.12) (cf. Fig. 17.5a).

(b) The number of tree species is richest in tropical rain forest and decreases towards higher latitudes and higher altitudes. Several models have been proposed to explain this pattern but none reproduce the pattern successfully (Pianka 1966; Iwasa et al. 1993). Kikuzawa (1996) was able to reproduce the pattern by considering different parameter values of a, b, C, and r in simulations under given f values in Eq. (17.12). Each combination of parameter values a, b, C, and r was considered to represent one species, and the parameter space was explored to count the number of species that achieved positive carbon balance under a given f. The number of evergreen species with positive carbon balance was highest at low latitude and low altitude and decreased toward higher altitudes and latitudes (Fig. 17.5b).

(c) Kudo (1991, 1992) tried to clarify the effect of the length of the snow-free period (f) on the phenology of alpine plants in a limited geographical area in northern Japan where the timing of snow disappearance differs from site to site providing different f with other factors being equal (Kudo 1992; Kikuzawa and Kudo 1995). Leaf longevity (L) of two evergreen plants decreased with

increasing f, while L of a deciduous plant increased with f. Wright et al. (2005) reported a similar contrast in the relationship between L and mean annual temperature (MAT) for evergreen and deciduous species. Leaf longevity of evergreen trees decreased with increasing MAT but L of deciduous species increased. Decreases in L with increasing MAT have also been reported at a global scale for evergreen conifers (Reich et al. 2014) and at a local scale in China (Zhang et al. 2010). These contrasting trends for L against MAT can be interpreted as a corollary of the relationship between L and favorable period length (f; Kikuzawa et al. 2013b) (Fig. 17.5c). Mean annual temperature is correlated with f (Enquist 2011; Kikuzawa et al. 2013b), especially when data for tropical mountains are excluded. Equation (17.11) suggests that the divergent trends in evergreen and deciduous plants can be interpreted as the outcome of adaptive behavior of plants to maximize carbon gain. With decreasing f, the model predict that evergreen species need to prolong their leaf longevity to compensate for the shorter photosynthetic period within a year. Thus, in evergreen species L is negatively correlated with f. Deciduous species could behave similarly, but in doing so, by definition, they would no longer be deciduous (Kikuzawa et al. 2013b).

(d) On temperate mountains, MAT and f decrease with altitude; changes in temperature and f are a simple analog of latitudinal change. Hence it is not surprising that the L for deciduous *Stewartia monadelpha* (Theaceae) on a temperate mountain in Japan decreased with elevation, while that of four evergreen species increased (Fig. 17.5d; Fujita et al. 2012). Similar altitudinal trends were also found on a mountain slope in central Japan (Takahashi and Miyajima 2008). However, although MAT decreases with altitude on tropical mountains, f is unchanged (Kikuzawa 1996). In this case, only MAT affects L in the condition of f = 1.0, and Eq. (17.3) can predict the altitudinal change in L. Parameter a will decrease with altitude in eq. (17.3) and thus L is predicted to increase with altitude.

(e) As shown in Sect. 2.5 (Fig. 17.1), there are positive correlations between L and LMA. In a global analysis, Wright et al. (2005) found that the slope of L-LMA changed systematically with MAT. Similar changes were also found when Kikuzawa et al. (2013b) examined the L-LMA relationships with respect to f. The actual L-LMA relationship was steeper in shorter f than that in longer f. (Fig. 17.5d). These changes in the slope of the L-LMA relationships are simulated using Eq. (17.12), indicating that the change in the slope of the L-LMA relationship is caused by adaptation to different f. Evergreen leaves in shorter f need a longer L for a given LMA to pay back their construction cost, while deciduous leaves in a short f have shorter L for a given LMA due to the limited length of the growing season, which results in a steeper slope in shorter f.

VII. Conclusions

Plant productivity is often viewed simply through the lens of net photosynthetic activity, whether assessed by gas exchange measurements at the level of single leaves or eddy covariance measurements at the level of entire plant canopies. In this review of work derived from Kikuzawa's (1991) theory for leaf longevity, we have tried to make the case for the value and utility of an alternative perspective that gives a certain primacy to leaf longevity. On the one hand, leaf longevity can be considered simply as a part of the leaf economic spectrum (Wright et al. 2004; Shipley et al. 2006), a complex of foliar traits that also includes the leaf's net photosynthetic rate, its dark respiration rate, its nitrogen and phosphorus concentrations, and the ratio of its mass and areal surface. On the other hand, leaf longevity stands apart as the only one of the LES traits that integrates the influence of all the others over the leaf lifespan – in other words, leaf longevity has the character of an emergent foliar trait. In this

sense, leaf longevity is perhaps the LES trait that best links function at the level of single leaves to function at the level of the plant canopy, and even perhaps to the production of plant communities (Kikuzawa and Lechowicz 2016).

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Chapter 18

Photosynthetic Modulation in Response to Plant Activity and Environment

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Summary

Modulation of foliar photosynthesis rate during short-term changes in abiotic environment as well as longer-term acclimation of photosynthetic capacity in response to abiotic factors or other organisms are summarized. Acclimation is informed by the balance between the rate at which photosynthate is produced and moved into the phloem and out of the leaf (source activity) and the rate at which photosynthate is removed from the phloem and utilized or stored in sinks (sink activity). We first review various experimental manipulative approaches utilized to perturb source to sink ratio within a plant and evaluate the impact of such perturbations on photosynthesis. In general, alterations that markedly increase the whole plant ratio of source to sink activity results in photosynthetic downregulation, whereas decreases in source to sink ratio often lead to photosynthetic upregulation. We then examine the impact of various environmental factors on plant activity and development, photosynthesis, dissipation of excess energy by the xanthophylls zeaxanthin and antheraxanthin, source-sink balance, photosynthate transport, and foliar carbohydrate levels. Environmental factors considered include fire, light, temperature, salinity, availability of water and essential nutrients, pollution, and herbicides. The response of foliar photosynthesis varies depending on species (growth habit) and the environmental condition(s) to which the plant is exposed. In some circumstances, photosynthesis is upregulated in concert with enhanced foliar mesophyll, vascular infrastructure, and photosynthate transport and utilization. In yet others, photosynthesis is downregulated in concert with decreased growth and photosynthate transport as well as increased accumulation of carbohydrates and employment of sustained zeaxanthin-associated energy dissipation as a form of photoinhibition. As source to sink ratio increases, surplus photosynthate may be used to produce compatible solutes, phenolics (including anthocyanins as well as other flavonoids), structural components of cells and the cuticle, and volatile organic compounds released into the atmosphere. In addition to serving as enhanced sinks for photosynthate, all of these products can also serve in defense against abiotic and biotic stresses. Biotic factors impacting photosynthesis include competition, herbivory, pathogens (bacteria, fungi, and viruses), xylem- and phloem-tapping parasitic plants, and symbiotic and mutualistic fungi and bacteria. Many biotic factors can also trigger either upregulation or downregulation of photosynthesis, depending on the nature of the interaction. For instance, in some cases herbivory can result in a decreased ratio of plant source to sink activity and photosynthetic upregulation, while in other cases an increased ratio of source to sink activity and photosynthetic downregulation is seen. Competition for water by xylem-tapping insects or parasitic plants can lead to photosynthetic downregulation, while phloem-tapping insects and parasitic plants, as additional sinks for photosynthate, can induce photosynthetic upregulation. Some pathogens block photosynthate export from leaves, leading to foliar carbohydrate accumulation and feedback inhibition of photosynthesis, whereas other pathogens induce production of additional tissue that acts as a sink for photosynthates and stimulates photosynthetic upregulation. Symbiotic fungi and bacteria (e.g., mycorrhizal fungi, nitrogen-fixing bacteria, endophytes) and mutualistic soil microbes generally promote plant growth and act as additional sinks themselves, thereby stimulating photosynthetic upregulation. Across the spectrum of abiotic and biotic influences, foliar photosynthesis responds to the demand for its products through acclimation either within the constraints of the existing foliar infrastructure in mature leaves or through production of new leaves compatible with the altered level of need. These connections are placed into the context of global climate change and its impact on plant abiotic and biotic interactions.

I. Introduction

The rate of photosynthesis a leaf can exhibit is highly flexible, both over the course of a day as light availability and other environmental factors vary as well as on longer time-scales through acclimatory adjustments. Enzymatic activation/deactivation and upregulation or downregulation of the products of photosynthetic genes occurs in response to available resources and the activity of the plant's sinks in consuming photosynthate. Any photosynthetically competent and photosynthate-exporting plant part, chiefly the mature leaf (see Chap. 3), is a source organ, while photosynthate-importing parts of the plant are sinks. Tissues that grow and differentiate, including developing leaves, are major sinks for photosynthate. A leaf's intrinsic photosynthetic capacity is regulated in response to the overall balance of sink activity (photosynthate removed from the phloem by sink tissues) relative to the amount of photosynthate loaded into the phloem by source leaves or storage tissues mobilizing their stores. In plants with low levels of sink activity due to slow or arrested growth, foliar photosynthetic rates are typically relatively low. In contrast, photosynthesis rates are typically high in source leaves of plants with high photosynthate requirements from growing and storing tissues. While there is ongoing discussion of the relative roles of photosynthesis and sink activity in plant productivity (Körner 2013; Poorter et al. 2013; DeLucia et al. 2014; Fatichi et al. 2014; Patrick and Colyvas 2014; Long et al. 2015a; Ort et al. 2015; Burnett et al. 2016; Niinemets et al. 2017), there is a strong relationship between photosynthesis and plant growth (Kruger and Volin 2006). Moreover, other than in some agricultural contexts, plants rarely operate without environmental limitations imposed on their metabolism, growth, or development, and consequently on photosynthetic capacity, by abiotic factors and other organisms (Bugbee and Monje 1992; Körner 2012; Sinclair and Rufty 2012; Slattery et al. 2013; Suzuki et al. 2014). When the sum of all sink activities is low compared to source activity, feedback inhibition of photosynthesis via repression of photosynthetic genes brings sink and source activity back into balance (Sheen 1994).

Plant growth is orchestrated by multiple signaling networks with inputs from the plant's genetic potential and abiotic and biotic factors in its environment. The extent of phenotypic adjustment to prevailing abiotic and biotic conditions reflects a plant population's evolutionary history (Lambers and Poorter 1992). For instance, evergreen or perennial plants repeatedly persist through periods of environmental conditions precluding rapid growth. As part of their adaptation to such limitations, evergreens or perennials typically exhibit relatively slow growth even during environmentally permissive periods and arrest growth completely in seasons with freezing temperatures, excessively high temperatures, reduced water availability, etc. Plants that are not evergreen actively disassemble and mobilize essential components for future use and commit to senescence of remaining structures, such as leaves, branches, entire above-ground portions, or root systems. Such slow-growing, long-lived species typically have low maximal photosynthesis rates (Fig. 18.1a; Demmig-Adams et al. 2017). Other species evolved to grow rapidly, supported by high maximal photosynthesis rates (Fig. 18.1b; Demmig-Adams et al. 2017). Exemplars for the latter approach are herbaceous biennials, summer annuals, winter annuals, and opportunistic annuals. Opportunistic annuals include desert ephemerals that germinate and rapidly complete their life cycle following a major precipitation event (the occurrence of which may be years apart), and species in temperate regions that germinate and set seed during the moderate conditions of autumn and spring, thereby avoiding subfreezing winters and excessively hot, dry summers. Such species may exhibit growth throughout the winter in geographic areas

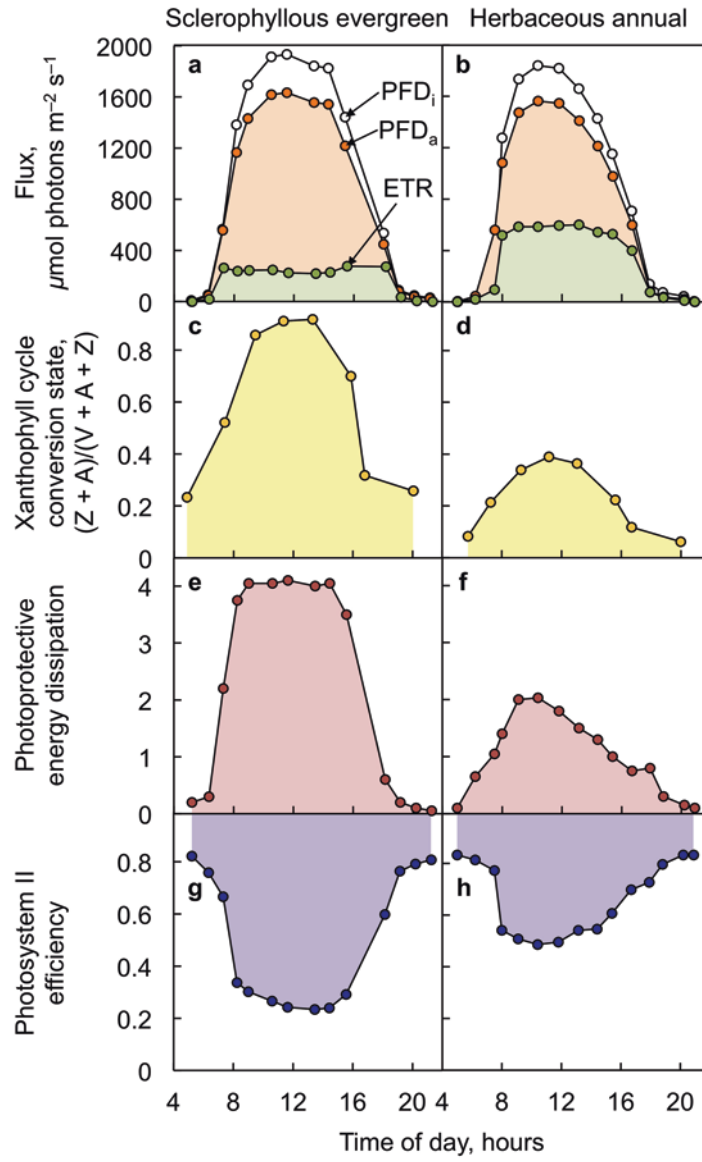

Fig. 18.1. Diurnal characterization of (**a**, **b**) sunlight incident upon (photon flux density [PFD_i], open circles), absorbed by (PFD_a, orange circles), and utilized through photosynthetic electron transport (ETR, green circles), (**c**, **d**) the conversion state of the xanthophyll cycle carotenoids (zeaxanthin [Z] and antheraxanthin [A] as a fraction of the total pool of the cycle, including the precursor [violaxanthin = V] of the active forms Z and A, yellow), (**e**, **f**) the engagement of photoprotective energy dissipation as assessed through nonphotochemical quenching of chlorophyll fluorescence (red), and (**g**, **h**) photosystem II efficiency (purple) for leaves of a sclerophyllous evergreen (*Vinca major*) and an herbaceous annual (*Helianthus annuus*). (Data from Demmig-Adams et al. (1996))

without strong freezes. Among plant species, there is thus a range of potential growth and photosynthetic rates that respond to growth conditions, but is limited by inherent genetic constraints.

Plant growth is governed by cell cycle advancement leading to cell division, followed by cell expansion (Inagaki and Umeda 2011; Müller and Leyser 2011; De Vos et al. 2012; Komaki and Sugimoto 2012; Nishihama

and Kohchi 2013; Danisman 2016; Gutierrez 2016). In the case of leaves (Blomme et al. 2013), signals originating in the chloroplasts play a role (Pedroza-Garcia et al. 2016; Van Dingenen et al. 2016). Coordination of growth and development involves transcription factors, hormones, and reactive oxygen species (McAtee et al. 2013; Fambrini and Publiesi 2013; Foyer and Noctor 2009, 2013; Lu et al. 2014a; Tank et al. 2014; Pacifici et al. 2015; Schmidt and Schippers 2015; Wachsman et al. 2015; Schmidt et al. 2016; Smet and De Rybel 2016; Campbell and Turner 2017; Tognetti et al. 2017).

Both progression through the cell cycle and cell expansion are influenced by

1. light (Okello et al. 2016; Casal and Qüesta 2017; Wang et al. 2018)
   (a) daily total acting through photomorphogenic photoreceptors as well as generation of chemical energy and building blocks necessary to support growth
   (b) quality (especially the red to far-red ratio that decreases from canopy tops to foliage-filtered shade, as well as blue light) acting through photomorphogenic photoreceptors
   (c) intensity, through generation of chemical energy and building blocks necessary to support growth; in addition, glucose and sucrose products of photosynthesis contribute to multiple signaling networks (Rolland et al. 2006; Ruan 2014), including modulation of cell cycle progression (Sheen 2014)
2. temperature (Casal and Qüesta 2017), with optimal growth in a "moderate range" that varies from species to species depending on evolutionary history
3. water availability
   (a) sufficient to support the positive pressure potential necessary for cell expansion
   (b) but not causing water logging and low oxygen supply to roots and shoots (to which aquatic and semi-aquatic plants have special adaptations; see Chap. 11)
4. soil conditions (see Chap. 13) including available rooting volume, soil compactness, soil composition (water- and nutrient-holding capacity, nutrient availability, excess levels of deleterious elements and compounds), etc.
5. mechanical disturbance by wind, matter carried by wind (dust, sand, rain and its frozen counterparts, insects, debris, etc.), neighboring plants blown by wind, flowing water and snow, shifting or heaving substrate (as seen in moist soil subjected to freeze-thaw cycles), etc.
6. other organisms, including pathogens, symbionts, competitors, parasites, epiphytes and hemi-epiphytes, gall-forming insects, various herbivores, etc.

As a plant grows and responds to abiotic and biotic factors, the relative investment into roots (acquisition of water and nutrients) versus shoots (light harvesting and generation of chemical energy, loss of water via transpiration) is continually adjusted via hormone, nitrogen, sugar, mRNA, transcription factor, reactive oxygen, small peptide signals, and small RNAs to keep all parts of the plant adequately supplied and in balance (Hilbert and Reynolds 1991; Shashidhar et al. 1996; Jackson 1997; Bangerth et al. 2000; Hsiao 2000; Grechi et al. 2007; Brewer et al. 2013; Van den Ende 2013; Lastdrager et al. 2014; Ruan 2014; Long et al. 2015b; Schmidt and Schippers 2015; Schippers et al. 2016; Wang and Ruan 2016). In concert with continual adjustments in plant activity, photosynthesis is highly responsive over both short and long time scales, as will be explored in the following.

II. Photosynthesis in the Context of Whole Plant Source and Sink Strength

Alteration of the balance between source and sink activity through a variety of experimental manipulative approaches has demonstrated that photosynthetic capacity decreases when photosynthate supply exceeds utilization, and increases when utilization exceeds

photosynthate production and distribution (Paul and Foyer 2001; Kasai 2008; Adams et al. 2014a). Although many studies have demonstrated such responsiveness to altered source to sink balance within a plant, a few exceptions have been noted when those manipulations were imposed on plants exposed to a normal diurnal pattern of solar radiation in greenhouses or in the field (Lin et al. 1994; Macedo et al. 2005; Matsuda et al. 2011), which might be associated with diel changes in temperature (Haque et al. 2015; Ikkonen et al. 2015). Signal transmission of any imbalance between source and sink activity within the plant is often via the phloem through which the sugars or sugar alcohols are transported between the two (Lemoine et al. 2013).

A. Alteration of Source Strength

1. Partial Shading, Defoliation, or Girdling

Shading part of a plant canopy results in an overall decrease in plant source strength, and partial compensation by increased photosynthetic capacity of leaves that remain unshaded (Kasai 2008; McCormick et al. 2008a, b). A simple, albeit more destructive, way to decrease plant source strength is removal of a fraction of mature source leaves. Following such pruning, the photosynthesis rate of remaining leaves is greater, presumably to meet the demand of sink tissues (Hofäcker 1978; von Caemmerer and Farquhar 1983; Layne and Flore 1993, 1995; Iacono et al. 1995; Suwignyo et al. 1995; Pinkard and Beadle 1998; Bruening and Egli 1999; Vanderklein and Reich 1999; Iglesias et al. 2002; Pinkard 2003; Zhou and Quebedeaux 2003; Macedo et al. 2006a; Medhurst et al. 2006; Pinkard et al. 2007; Turnbull et al. 2007; Kasai 2008; Eyles et al. 2011, 2013; Lee et al. 2011a; Barry and Pinkard 2013; Guo et al. 2015). Partial defoliation of actively growing red oak seedlings resulted in photosynthetic upregulation and depletion of carbohydrate stores; in contrast, partial defoliation of dormant seedlings had no impact (Woolery and Jacobs 2011). Removal of source-leaf buds also resulted in higher photosynthesis rates of pre-existing foliage in young conifer saplings (Ozaki et al. 2004; Lavigne et al. 2001; see also Eyles et al. 2013), and removal of all fir needles from previous years likewise led to higher photosynthesis rates in current-year foliage (Little et al. 2003). Girdling of branches was used to reduce the number of leaves supplying photosynthate to fruits, resulting in higher photosynthesis rates in the remaining leaves (Ben Minoun et al. 1996; Famiani et al. 2000; Urban et al. 2004; Urban and Léchaudel 2005). In other words, photosyntheis rates were higher in leaves on trees with a low ratio of source leaves to fruits compared to a high ratio of leaves to fruits. In addition, the rooting of detached single sweet potato leaves and development of tuberous sinks over 50 days was accompanied by decreases in the initial high levels of foliar carbohydrates and increases in photosynthesis rates (Sawada et al. 2003). If defoliation occurs in low light (where light may already be limiting to photosynthesis), no compensatory photosynthetic upregulation is observed (Anten and Ackerly 2001). If defoliation is too extensive, as in small plants with limited stored resources for investment into new growth, compensatory upregulation of photosynthesis may likewise not occur (Zhu et al. 2014). On the other hand, in some species defoliation resulted in no adjustment of photosynthetic rate among remaining leaves, but did stimulate production of additional, larger leaves (Man et al. 2013).

2. Continuous Light

Several approaches have been utilized to increase source to sink ratio by increasing source strength, which resulted in accumulation of carbohydrates and decreased photosynthetic rates. Growth under continuous light, or transfer from a day/night photoperiod regime to continuous light, resulted in

production of more photosynthate than could be utilized and subsequent photosynthetic downregulation (Layne and Flore 1993, 1995; Murage et al. 1996; Roswell et al. 1999; Sawada et al. 1999; Van Gestel et al. 2005; Equiza et al. 2006a, b; Matsuda et al. 2014). Such downregulation in response to continuous light can involve disassembly of the photosynthetic apparatus and, ultimately, leaf chlorosis of preexisting leaves (Murage et al. 1996; Velez-Ramirez et al. 2017). Nevertheless, overall carbon gain and productivity of the plant can be elevated in continuous light despite reductions in foliar photosyntheis rates (Rowell et al. 1999; Xiao et al. 2007; Haque et al. 2015). Such effects are a more pronounced version of transient regulatory declines in photosynthesis rate seen at the end of a photoperiod with constant photon flux density in a growth chamber, which represent an increased source to sink ratio as carbohydrates accumulate at the end of the day (Foyer 1988; Layne and Flore 1995; Greer 1998).

3. *Sugar Feeding*

Direct feeding of sugars to plants or leaves is another experimental approach that demonstrated the impact of an increased source to sink ratio, with decreased rates of source leaf photosynthesis compared to control plants or leaves (Krapp et al. 1991; Morcuende et al. 1997; Logan et al. 1999; de la Viña et al. 1999; Le et al. 2001; Iglesias et al. 2002; Franck et al. 2006; Araya et al. 2006, 2010). Such overloading of the leaf with products of photosynthesis results in decreased foliar levels of chlorophyll and photosynthetic enzymes (Krapp et al. 1991, 1993; Krapp and Stitt 1994; Van Oosten and Besford 1994; Jones et al. 1996; Smeekens 2000; Roland et al. 2002; Lobo et al. 2015). In some special cases, such as rapidly-growing herbaceous summer annuals under relatively low light intensity, sugar feeding did not negatively impact photosynthesis (Furbank et al. 1997; Le et al. 2001). These results are consistent with the absence of an effect of partial defoliation in low light (Anten and Ackerly 2001). On the other hand, Kilb et al. (1996) observed significant reductions in photosynthesis, chlorophyll, and photosynthetic proteins when leaves of an herbaceous winter annual were fed glucose in low light.

4. *Elevated Level of Carbon Dioxide*

Plant response to CO_2 varies depending on source to sink ratios. Increased photosynthesis and productivity is a common response of plants to increased CO_2 levels (Drennan and Nobel 2000; Ainsworth et al. 2003; Naumburg et al. 2003; Ainsworth and Long 2005; Springer and Thomas 2007; Logan et al. 2009; Bader et al. 2010; Lee et al. 2011b; Ellsworth et al. 2012; Ruhil et al. 2015; DaMatta et al. 2016; Kimball 2016; van der Kooi et al. 2016; Bourgault et al. 2017a; Thompson et al. 2017; however, see Bunce 2016) as long as plants are not sink limited by restricted rooting volume (e.g., growth in pots), insufficient supply of nutrients, limiting water, etc. (Arp 1991; Thomas and Strain 1991; Ainsworth et al. 2002; Ellsworth et al. 2004; Sans-Sáez et al. 2010; Tissue et al. 2010; Schaz et al. 2014; Gray et al. 2016; Asif et al. 2017; Bourgalt et al. 2017b; Dong et al. 2017; Ruiz-Vera et al. 2017; Yilmaz et al. 2017). Across conditions, the enhancement of photosynthesis by elevated CO_2 is proportional to the level of sink activity (Greer et al. 2000). In addition, enhancement of photosynthesis in response to high CO_2 can diminish with lessening sink activity as a plant progresses through its life cycle (Adachi et al. 2014). Dwarf varieties of crops (with lesser overall sink strength) may exhibit little responsiveness to greater CO_2 availability (Sicher et al. 2010). Furthermore, Ainsworth et al. (2004) found that lines of soybean with more limited sink activity exhibited photosynthetic downregulation in response to high CO_2, whereas soybean with stronger sink activity did not (see also Ruiz-Vera et al. 2017). In addition, overexpression

of sedoheptulose-1,7-bisphosphatase (functions in regeneration of the acceptor for CO_2 in photosynthesis) yielded plants with higher rates of photosynthesis when grown under elevated CO_2 (Rosenthal et al. 2011).

Even though photosynthesis rates can initially be greater at elevated versus ambient CO_2 concentration, longer-term acclimation of leaves to higher CO_2 often involves a return to the lower rate accompanied by decreased investment into photosynthetic enzymes (Nie et al. 1995; Van Oosten and Besford 1996; Moore et al. 1999; Drake et al. 1997; Aranjuelo et al. 2005; Bernacchi et al. 2005; see also von Caemmerer and Farquhar 1983). It can be deduced that the initial, lower rate matched the demand for sugar from the plant's sinks, and that sink strength could not be increased. Accordingly, growing plants under elevated CO_2 resulted in downregulation of photosynthesis in a number of studies (Ceulemans and Mousseau 1994; Makino and Mae 1999; Roden et al. 1999; Le et al. 2001; Eguchi et al. 2008; Goicoechea et al. 2014; Gao et al. 2015; Koike et al. 2015; Salazar-Parra et al. 2015; Choi et al. 2017; Porras et al. 2017; Wang et al. 2017). These findings are also consistent with the following observations. When photosynthesis is repressed in response to high CO_2, such repression can be reversed by foliar pruning, resulting in photosynthetic upregulation in the remaining source leaves (Bryant et al. 1998). For long-lived plants, an initial CO_2-stimulated enhancement of photosynthesis is often lost during continued exposure to high CO_2 over multiple years (Körner 2006; Warren et al. 2015). Downregulation of photosynthesis in response to high levels of CO_2 can be exacerbated by additional stresses that decrease sink activity, including low temperature, high temperature, water stress, and nutrient limitation (Roden and Ball 1996; Hymus et al. 1999, 2001; Adachi et al. 2014; Ruiz-Vera et al. 2015, 2017). On the other hand, elevated CO_2 levels can help to alleviate the negative impacts of high temperature (Bauweraerts et al. 2014; Sicher 2015; Cai et al. 2016; Xu et al. 2016) and water stress (Xu et al. 2013; Bussotti et al. 2014), but not among C_4 plants (Xu et al. 2013; Wang et al. 2014). All of these findings support the conclusion that the effect of elevated CO_2 on photosyntheis depends on the source to sink ratio.

5. *Decreased Light Harvesting*

Another approach to increasing plant source strength is to decrease foliar light-harvesting capacity, thereby decreasing the impact of self-shading and allowing additional light to penetrate through the upper leaves to the lower portion of the canopy and permitting greater investment of nitrogen in the biochemistry of photosynthesis (Song et al. 2017). A mutant of tobacco deficient in chlorophyll did accumulate more biomass than wild-type tobacco plants when grown in pots arrayed in close proximity to each other under greenhouse conditions (Kirst et al. 2017). However, chlorophyll-deficient mutants of soybean exhibited no improvement in yield (Slattery et al. 2017) and lower biomass accumulation (Sakoska et al. 2018) under field conditions. Any benefits of lower light-harvesting capacity may thus depend on differences among species, or may apply specifically to plants grown in pots (with limited rooting volume and sink activity – see the next section) under conditions of pronounced self-shading.

B. *Alteration of Sink Strength*

1. *Root Activity*

Various approaches to alter sink strength or carbon export have been used to evaluate the effect on foliar photosynthesis. One of the most straightforward approaches is to provide a small rooting volume that significantly limits root growth and the activity of the root system as a sink, leading to carbohydrate accumulation throughout the plant and lower rates of photosynthesis (Tu and Tan 1988; NeSmith 1992; Rieger and Marra 1994; Whitfield et al. 1996; Pezeshki and Santos 1998; Ferree et al. 2004; Ronchi et al. 2006; Prins et al. 2008; Poorter et al. 2012a;

Bourgault et al. 2017b; Campany et al. 2017a). Even compacted soils that restrict root growth compared to less compacted soils impact photosynthesis rates negatively (Conlin and van den Driessche 1996; Tubeileh et al. 2003; Alameda and Villar 2012; Cambi et al. 2017; but see Andrade et al. 1993). Likewise, reduced oxygen availability in the rooting zone limits the sink activity of roots and also results in lower rates of foliar photosynthesis (Pezeshki and Santos 1998; Bhattarai et al. 2004; Xiao et al. 2015). On the other hand, stimulation of root growth by increased soil temperature can prompt greater sink strength and photosynthetic upregulation (Greer et al. 2006).

A limited rooting volume can also significantly reduce total available leaf area (as seen in bonsai trees or shrubs) for photosynthesis (Thomas and Strain 1991; Dubik et al. 1992; NeSmith 1992, 1993a, b; Rieger and Marra 1994; Hsu et al. 1996; van Iersel 1997; Goto et al. 2001; Graham and Wheeler 2016; Sinclair et al. 2017). The model plant *Arabidopsis thaliana* responds to restricted rooting volume both morphologically and physiologically. Individual leaf size (Fig. 18.2), total leaf area per plant (Fig. 18.2), and total shoot biomass (Fig. 18.3a) are smaller in plants grown in small versus large pots, with more pronounced differences in plants grown under

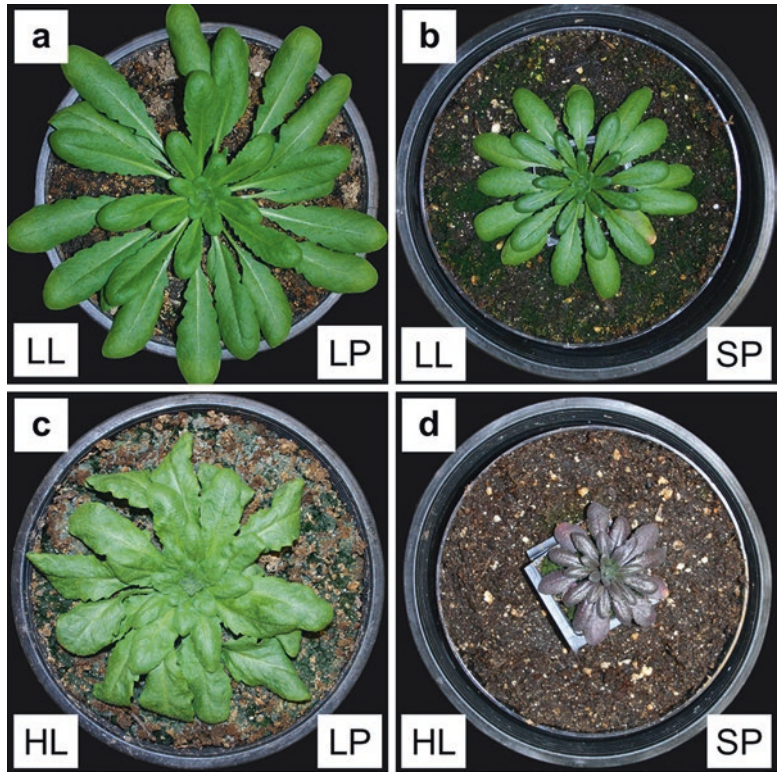

Fig. 18.2. *Arabidopsis thaliana* (Col-0) grown under a 9 h photoperiod of (**a**, **b**) low light (LL = 100 μmol photons m^{-2} s^{-1}) for approximately 8 weeks or (**c**, **d**) high light (HL = 1000 μmol photons m^{-2} s^{-1}) for approximately 6 weeks in (**a**, **c**) large pots (LP = 2900 cm^3 soil volume) or (**b**, **d**) small pots (SP = 50 cm^3 soil volume) at 20 °C/12 °C day/night leaf temperature. To provide comparable environments below the leaves, the small pots were mounted in large petri dishes (150 mm diameter) that had circumferences similar to those of the large pots that were filled with soil. These modified pots were suspended within their own large pots so that all plants in the study were grown at a similar height above the growth chamber floor. The perimeter of the small pots can be seen as a square in the center of the surrounding soil contained within the petri dishes (**b**, **d**)

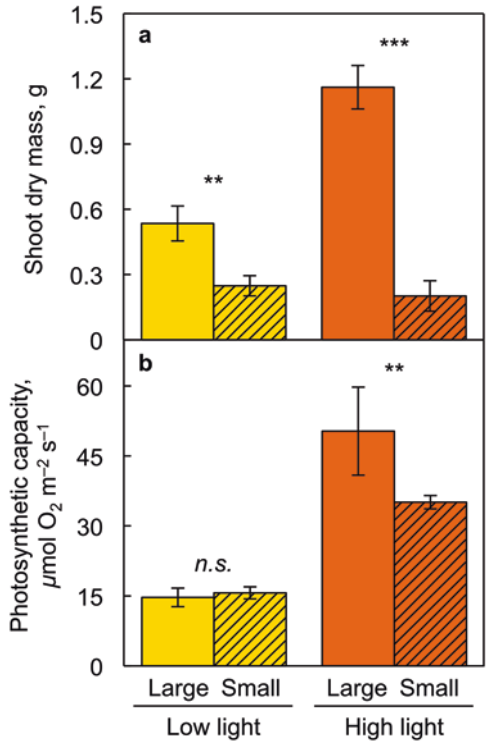

Fig. 18.3. (**a**) Shoot dry mass and (**b**) capacity for photosynthetic oxygen evolution determined under saturating light (2000 μmol photons m^{-2} s^{-1}) and CO_2 (50,000 ppm) in a water-saturated atmosphere at 25° C for plants or leaves of *Arabidopsis thaliana* (Col-0) grown in large or small (hatched columns) pots under low (light orange) or high (orange) light (see legend of Fig. 18.2 for additional details). Mean values ± standard deviation (n = 4) and significant differences (Student's t-test) indicated by asterisks – ** = $P < 0.01$, *** $P < 0.001$, *n.s.* = not significantly different

high versus low light intensity. Photosynthetic capacity per unit leaf area was significantly higher for plants grown in large versus small pots under high light, but was similar for plants grown in low light (Fig. 18.3b). This effect of growth light intensity is consistent with the results of other source-sink manipulations discussed above.

2. *Synthesis of Leaf-Localized Molecules*

Moreover, anthocyanin accumulation in the upper epidermis of leaves was only observed in plants grown in small pots under high light (Fig. 18.2). Elevated sugar levels stimulate foliar accumulation of anthocyanins (Jeannette et al. 2000; Lloyd and Zakhleniuk 2004; Teng et al. 2005; Loreti et al. 2008; Connor et al. 2012; Van den Ende and El-Esawe 2014), the synthesis of which (along with other flavonoid-pathway products) represents another sink for photosynthate that can be upregulated when the production of carbohydrates exceeds their export from leaves (Hernández and Van Breusegem 2010; see also Zhang et al. 2015a). For instance, leaves on shoots from which apples (as fruits constituting major carbohydrate sinks) had been removed accumulated sugars and anthocyanins, whereas leaves on apple-bearing shoots did not (Tartachnyk and Blanke 2004). Anthocyanins also accumulate in young, expanding leaves as long as the developing leaves import carbohydrates; once leaves mature, become photosynthetically competent, and begin to export photosynthate, anthocyanins are degraded (Hughes et al. 2007). Likewise, anthocyanins often appear in leaves of plants that experience reduced sink activity in response to environmental stresses (Close and Beadle 2003; Gould 2004; Boldt et al. 2014; Van den Ende and El-Esawe 2014; Landi et al. 2015; see also Hoque et al. 2017), including declining temperatures in autumn and winter (Hughes et al. 2005; Hughes and Smith 2007; Murakami et al. 2008; Hughes 2011) that reduce sink activity as well as slow nocturnal export of viscous sugar-laden phloem sap (Paul et al. 1991; Miao et al. 2007; Murakami et al. 2008). Accumulation of anthocyanins in the foliar epidermis may also provide the added benefit of screening excessive light to protect the photosynthetic apparatus (Gould et al. 2002a; Steyn et al. 2002; Neill and Gould 2003; Hughes et al. 2012; Carpenter et al. 2014; Logan et al. 2015; Nichelmann and Bilger 2017), serving as antioxidants (Gould et al. 2002b; Nagata et al. 2003; Neill and Gould 2003; van den Berg and Perkins 2007), contributing to foliar osmotic adjustment (Hughes et al.

2013), and other potentially beneficial activities (Gould 2004; Boldt et al. 2014; Landi et al. 2015).

In addition to phenolic compounds, synthesis of other molecules can serve as sinks for the photosynthate accumulating in leaves when production exceeds export capacity. Even as some provide a signal for photosynthetic downregulation (or not; see Strand et al. 1997; Sugiura et al. 2015, 2017), foliar accumulation of hexoses, disaccharides, starch, raffinose family oligosaccharides, fructans, sugar alcohols, and cellulose (and the other components comprising the cell walls such as hemi-celluloses and lignin), as well as nitrogen-containing compounds occurs in various species (Pontis 1989; Rare 1989; Paul and Foyer 1991; Pollock and Cairns 1991; Goldschmidt and Huber 1992; Van Oene et al. 1992; Wang and Stutte 1992; Hendry 1993; Moore et al. 1997; Gilbert et al. 1998; Sawada et al. 2003; Streeter et al. 2001; Iglesias et al. 2002; Klotke et al. 2004; Adams et al. 2005, 2014a; Lee et al. 2005; Merchant and Adams 2005; Equiza et al. 2006a, b; Kasai 2008; Sanchez et al. 2008; Valluru and Van den Ende 2008; Azevedo Neto et al. 2009; Fulda et al. 2011; Matsuda et al. 2011; Nebauer et al. 2011; Warren et al. 2011; Llanes et al. 2012; Egert et al. 2013, 2015; Angelcheva et al. 2014; Barchet et al. 2014; Findling et al. 2015; Hamanishi et al. 2015; Kurepin et al. 2015; Meguro-Maoka and Yoshida 2016; Van den Ende et al. 2016). Excluding cell wall components, all of these accumulate intracellularly, and some can accumulate extracellularly (Livingston and Henson 1998). With the exception of the glucose polymers starch and cellulose (and the rest of the cell wall components), the smaller molecules also serve other important roles under sink-limited conditions (see references above), including as compatible (with proteins and membranes, i.e., they preserve their integrity) solute osmoregulants and cryoprotectants, membrane stabilizers, and antioxidants (Smirnoff and Cumbes 1989; Wanner and Junttila 1999; Williamson et al. 2002; Uemura and Steponkus 2003; Livingston et al. 2009; Lee et al. 2012; Keunen et al. 2013; Peshev et al. 2013; Van den Ende 2013; Puniran-Harley et al. 2014; Matros et al. 2015; Sun et al. 2015). The waxes, flavonoids, and other compounds of the cuticle are another leaf-localized destination for photosynthates that is enhanced in response to higher source to sink activity (North et al. 1995; He and Zhang 2003; Bacelar et al. 2004; Liu et al. 2004; Issarakraisila et al. 2007; Koleva et al. 2010; Gratani et al. 2011; Pierantozzi et al. 2013; Zhang et al. 2015b). While partitioning of photosynthetically generated photosynthate to volatile organic compounds increases with increasing light intensity (Niinemets and Sun 2015; Jardine et al. 2016), such emissions may (Sharkey and Loreto 1993; Niinemets and Sun 2015) or may not (Centritto et al. 2004; Pegoraro et al. 2007) be enhanced under conditions of higher source to sink activity. Consistent with the former findings, emission of volatile isoprene can be stimulated by shifts in environmental conditions (Wiberley et al. 2008) that may result in transiently elevated ratios of source to sink activity.

3. *Sink Removal*

Experimental removal of distant sinks invariably resulted in lower rates of foliar photosynthesis. For instance, removal of a plant's developing sinks resulted in decreased rates of foliar photosynthesis (Nösberger and Humphries 1965; Hofäcker 1978; Mayoral et al. 1991; Suwignyo et al. 1995; Wibbe and Blanke 1995; Bhatt and Srinivasa Rao 1997; Basu et al. 1999; Iglesias et al. 2002; Veberic et al. 2002; Li et al. 2005, 2007; Duan et al. 2008; Kasai 2008; Wu et al. 2008a; Blanke 2009; Cheng et al. 2009; Yan et al. 2013a; Jorquera-Fontena et al. 2016; Pan et al. 2017) involving downregulation of numerous photosynthetic genes (Duan et al. 2016). As a mechanism to partially compensate for the loss of such sinks, plants may increase secretion of carbohydrates by the root system

(Kläring et al. 2014). The negative impact of sink removal on photosynthesis was exacerbated by insufficient nutrients, further lowering sink activity (Pan et al. 2017). Leaves of mandarin trees that naturally bore few fruit accumulated significantly higher levels of starch compared to leaves of trees bearing lots of fruit, with increasing starch accumulation correlating with decreasing leaf chlorophyll level (Stander et al. 2017). Consistent with this, leaves on the branches of willow that were not flowering had lower rates of photosynthesis than leaves on branches that were producing catkins (Dawson and Bliss 1993; see also Tozawa et al. 2009), and female plants of the dioecious plant *Silene latifolia* on which fruit were developing had higher rates of photosynthesis than non-fruiting plants (Laporte and Delph 1996; see also Said et al. 2013). Removal of developing sinks coupled with branch girdling (inhibiting the export of photosynthate) also led to reduced photosynthesis (Meyers et al. 1999; Urban et al. 2004; Equiza et al. 2006b; Jorquera-Fontena et al. 2016).

4. *Shoot, Branch, or Petiole Girdling*

Shoot or branch girdling (physical removal of phloem) prevents transfer of photosynthates from source leaves to sink tissues. Girdling resulted in foliar accumulation of carbohydrates and decreased rates of photosynthesis (Hofäcker 1978; Mayoral et al. 1991; Bruening and Egli 1999; Meyers et al. 1999; Iglesias et al. 2002; Zhou and Quebedeaux 2003; Franck et al. 2006; Urban and Alphonsout 2007; Cheng et al. 2008; Fan et al. 2010; Yan et al. 2011; Qian et al. 2012; Vemmos et al. 2012; Quentin et al. 2013; López et al. 2015; Tang et al. 2016; Moscatello et al. 2017). Cold- or heat-girdling of petioles or grass leaves impaired phloem transport and resulted in foliar carbohydrate accumulation, decreased transcript levels of photosynthetic enzymes, and photosynthetic downregulation (Goldschmidt and Huber 1992; Krapp et al. 1993; Krapp and Stitt 1995; Jeannette et al. 2000; McCormick et al. 2008c) as well as upregulation of sink activity (flavonoid synthesis) and consumption of carbohydrates (increased respiration) in leaves (De Schepper et al. 2011; Zhang et al. 2015a). Moreover, girdling of branches on which fruit remained exhibited less or no downregulation of photosynthesis (Schaper and Chacko Csiro 1993; Di Vaio et al. 2001; Urban et al. 2004; Wu et al. 2008a; Nebauer et al. 2011).

5. *Genetic Engineering Approaches*

In addition, use of transgenic plants has demonstrated the impact of reduced foliar export of sugars on photosynthesis. Plants engineered to accumulate invertase (that cleaves sucrose into fructose and glucose) in the cell wall exhibit impaired loading of sucrose into foliar phloem. This impairment greatly reduced sucrose translocation and resulted in carbohydrate accumulation, decreased expression of photosynthetic proteins, and photosynthetic downregulation (von Schaewen et al. 1990; Sonnewald et al. 1991; Stitt et al. 1991; Krapp et al. 1993). Other transgenic approaches that reduced foliar sucrose export likewise yielded leaves with higher carbohydrate levels and lower rates of photosynthesis (Geigenberger et al. 1996; Waclawovsky et al. 2006). For example, downregulation of the sucrose-H^+ co-transporter responsible for phloem loading in potato leaves resulted in strong carbohydrate accumulation, chlorosis, and reduced photosynthesis rates (Riesmeier et al. 1994). Similarly, engineering of plants that results in reduced sink activity resulted lower rates of photosynthesis. For instance, trees engineered for impaired lignin synthesis accumulated higher levels of leaf nonstructural carbohydrates and exhibited reduced rates of CO_2 uptake compared to control trees (Coleman et al. 2008). In another example, plants whose mitochondria were engineered to have reduced levels of citrate and malate and elevated levels of acetyl coenzyme A

exhibited reduced levels of sink activity and feedback downregulation of photosynthesis (Bender-Machado et al. 2004). On the other hand, engineering of plants with increased sink activity via alterations of mitochondria that stimulated reproduction (Weraduwage et al. 2016), alteration of brassinosteroid levels that stimulated greater tillering and production of more numerous and larger seeds (Wu et al. 2008b), stimulation of more rapid growth and greater seed production (Zhang et al. 2012), and stimulation of more rapid growth and a greater number of tubers (Zhang et al. 2014) resulted in leaves with higher rates of photosynthesis.

Another process with significance for source to sink balance is photorespiration. Concern has been raised that photorespiration represents a cost to plants when oxygen rather than carbon dioxide is fixed by ribulose bisphosphate carboxylase oxygenase (Rubisco) of the Calvin-Benson cycle (Hagemann and Bauwe 2016; Walker et al. 2016; Sharwood 2017). However, overexpression of various photorespiratory enzymes resulted in higher rates of not only photorespiration, but also photosynthesis and growth (Timm et al. 2012, 2015; Wu et al. 2015; Han et al. 2017; see also Eisenhut et al. 2017). Busch et al. (2018) recently provided a possible explanation in that nitrogen incorporation into amino acids is enhanced by photorespiration. It seems reasonable to assume that this increased availability of organic nitrogen for building proteins would facilitate higher rates of growth leading to elevated levels of photosynthesis (see discussion of the impact of nutrient availability on sink activity in the following section).

6. Pruning and Fire

Pruning of leaves and shoots was discussed above as an approach to decrease source strength. However, pruning can also trigger proliferation of new shoots, which results in increased sink activity and higher photosynthesis rates (Tschaplinski and Blake 1995; Calatayud et al. 2008; see also Roiloa and Retuerto 2005). Blueberry bushes exhibited significantly higher rates of photosynthesis following pruning caused by burning, which was attributed to increased sink strength from vigorous growth of shoots that arose from belowground buds following the burn (Hicklenton et al. 2000). Species in various ecosystems evolved with fire as a regular component in their life cycles, with rapid growth from buds that survive under the bark and/or underground coupled with elevated rates of photosynthesis once the source to sink ratio is greatly diminished following exposure to such conflagrations (Feldman et al. 2004; Clemente et al. 2005; Huang et al. 2007; Turnbull et al. 2014; Parra and Moreno 2017). Fire is but one of many abiotic factors to which plants may respond, as will be explored in the following section.

III. Adjustment of Photosynthesis in Response to the Abiotic Environment

As sessile organisms subject to frequent changes in the abiotic environment, plants are, perhaps not surprisingly, highly plastic in physiological adjustment over short periods of time and in both morphological and physiological acclimation over longer time frames. Moreover, exposure to moderate stress can elicit responses that are adaptive in allowing better performance as long as growth is possible, and increase survival when growth ceases (Kozlowski and Pallardy 2002). Across a broad range of environmental factors, deviation from optimal conditions can result in reduced growth and decreased rates of photosynthesis, often in response to an elevated ratio of source to sink activity within the plant. While many components of the photosynthetic process can be part of this adjustment, Rubisco is typically involved through either alteration of its activation state and/or the number of enzymes (Yamori et al. 2012; Galmés et al.

2013). In this section, responses of foliar photosynthesis to a variety of environmental factors will be reviewed, with attention to the ratio of source to sink activity (sink activity, phloem loading and transport, foliar photosynthate levels) wherever relevant information is available. We should also note the difference between photosynthesis rates that vary in response to changing environmental conditions and the maximal capacity of photosynthetic electron transport that a leaf can exhibit based upon the photosynthetic components present per unit leaf area. For instance, photosynthesis rate can vary with changes in light intensity until light saturation is reached, above which no further increases are possible. Likewise, photosynthesis rate varies with temperature, decreasing below and above an optimum. On the other hand, it is components of photosynthetic electron transport and the enzymes of the Calvin-Benson cycle (determining the maximal capacity for photosynthesis under light- and CO_2-saturating conditions) that are largely adjusted to meet the needs of the plant in coordination with the overall levels of source and sink activity within the whole plant.

A. Light

In the absence of clouds and under otherwise optimal conditions, the rate of photosynthetic electron transport and CO_2 fixation in fully sun-exposed leaves increases as the level of incident sunlight rises (Fig. 18.1a, b). Above photosynthetic light saturation, accumulation of protons in the thylakoid lumen prompts violaxanthin de-epoxidase to convert the carotenoid violaxanthin to antheraxanthin and zeaxanthin (Z+A; Fig. 18.1c, d) and protonates the PsbS protein. PsbS and the two xanthophylls carry out photoprotective dissipation of excess excitation energy (quantified from nonphotochemical quenching of chlorophyll fluorescence; Fig. 18.1e, f). This harmless dissipation of excitation energy lowers the fraction of absorbed light used in photosystem II, or photosystem II efficiency (quantified from the ratio of variable to maximal chlorophyll fluorescence; Fig. 18.1g, h). Figure 18.1g, h shows transient decreases in photosystem II efficiency over the midday hours and return to high efficiency as light levels drop and become limiting again to photosynthesis. Leaves of plants with lower rates of photosynthesis convert a larger fraction of their pool of violaxanthin into the dissipaters and/or facilitators of dissipation zeaxanthin and antheraxanthin, that, when engaged in photoprotection, result in a greater lowering of photosystem II efficiency (Fig. 18.1). This photoprotective process involving three carotenoids of the xanthophyll cycle, and its employment under various circumstances when light is excessive, has been reviewed extensively elsewhere (Adams et al. 2006, 2014b; Demmig-Adams and Adams 2006; Demmig-Adams et al. 2006, 2012, 2014; Adams and Demmig-Adams 2014).

The majority of a plant's leaves are self-shaded, and even the top leaves not subject to self-shading experience limiting light on continuously or intermittently cloudy days (Knapp and Smith 1988; Reinhardt and Smith 2008; Hughes et al. 2015). In the understory of a forest, leaves responded to sunflecks (transient periods of higher light intensity; Fig. 18.4a) with increases in photosynthetic electron transport (Fig. 18.4b), conversion of violaxanthin to zeaxanthin and antheraxanthin (Fig. 18.4c), and dissipation of excess energy (Fig. 18.4d) that lowers photosystem II efficiency (Fig. 18.4e). Although zeaxanthin and antherxanthin levels remained relatively high throughout the day (Fig. 18.4c), onset and disengagement of energy dissipation occurred quite rapidly (Fig 18.4d) and tracked the rapid changes in light intensity (Fig. 18.4a). Rapid engagement of energy disspation when leaves are suddenly exposed to excess light is necessary to provide high levels of photoprotection, since less than a quarter of the absorbed sunlight was utilized

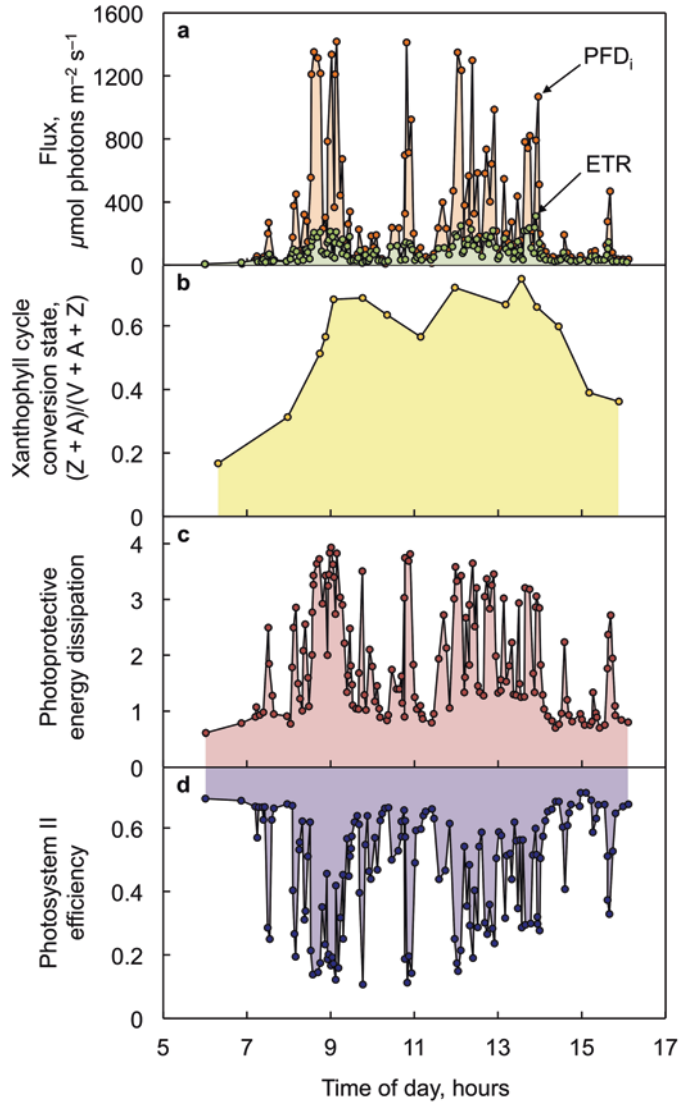

Fig. 18.4. Diurnal characterization of (**a**) sunlight incident upon (photon flux density [PFD_i], orange circles) and utilized through photosynthetic electron transport (ETR, green circles), (**b**) the conversion state of the xanthophyll cycle carotenoids (zeaxanthin [Z] and antheraxanthin [A] as a fraction of the total pool of the cycle, including the precursor [violaxanthin = V] of the active forms Z and A, yellow), (**c**) the engagement of photoprotective energy dissipation as assessed through nonphotochemical quenching of chlorophyll fluorescence (red), and (**d**) photosystem II efficiency (purple) for leaves of the vine *Stephania japonica* growing in a *Eucalyptus* forest understory punctuated by multiple sunflecks. (Data from Adams et al. (1999))

for photosynthesis (Fig. 18.4a, b). Rapid disengagement of energy dissipation is just as important to return to efficient collection and utilization of excitation energy in photosynthesis (Fig. 18.4e) when light intensity decreases below saturation for photosynthesis (Pearcy and Calkin 1983; Pearcy 1987; Logan et al. 1997; Valladares et al. 1997; Adams et al. 1999; Way and Pearcy 2012; Campany et al. 2017b).

As rapid as the disengagement of energy dissipation might be in some species, it was recently shown that further increasing the speed of that disengagement through a trans-

genic approach in tobacco resulted in enhanced photosynthetic carbon gain during the transition from high to lower light as well as greater biomass accumulation (Kromdijk et al. 2016; see also Armbruster et al. 2016). It has furthermore been suggested that a faster increase in the rate of photosynthesis during low to high light transitions might lead to greater crop productivity (Taylor and Long 2017). There is also natural variation in the speed of energy dissipation disengagement, such as between *Arabodopsis thaliana* populations from Sweden and Italy (unpublished results, J.J.S, W.W.A., and B.D.-A.) that also differ in other aspects of acclimation to the light environment (Stewart et al. 2016, 2017a).

With light being the source of energy for photosyntheis, plants growing in high light environments are typically not source limited, while plants grown in low light tend to be source limited, grow more slowly, and invest relatively less into most aspects of their leaves (Poorter et al. 2012b). Plants growing in shade produce thinner leaves with fewer and/or smaller cells and invest fewer resources in electron transport components and enzymes of the Calvin-Benson cycle resulting in lower rates of photosynthesis, while simultaneously investing relatively more in light collection (Anderson and Osmond 1987; Givnish 1988; Anderson et al. 1995; Demmig-Adams et al. 2017; but see Anderson et al. 2001). As a consequence, leaves of plants grown in high light under otherwise optimal conditions have higher photosynthetic capacities per leaf area than leaves of plants grown in lower light (Fig. 18.3 for plants grown in large pots) and a higher capacitiy to load and export sugars from their leaves (Adams et al. 2013a; Cohu et al. 2013a, b, 2014; Stewart et al. 2016, 2017a, b; Chap. 2). Plants that develop in the sun also have larger pools of xanthophyll cycle carotenoids (Demmig-Adams and Adams 1992, 1996; Logan et al. 1996) and higher capacities for photoprotective energy dissipation (Demmig-Adams and Adams 1994; Demmig-Adams et al. 1995). High light stimulates not only synthesis of xanthophyll cycle and other carotenoids, all of which are isoprenoids, but also synthesis of isoprene (a sink for photosynthate) among species that produce this volatile organic compound (Hanson and Sharkey 2001; see also Sect. 2.2 above).

Although plants growing in shade are source-limited, they limit growth to keep the root and shoot systems in balance; the level of nonstructural carbohydrates in both roots and shoots was the same in plants grown in low and higher light (Lambers and Posthumus 1980). This finding further underscores the tight regulation between source and sink activity in a steady state situation, where sun leaves efficiently move the greater volume of photosynthate they produce to their sink tissues. On the other hand, sudden shifts in the environment that alter source-sink balance will be met with gradual adjustment. Upon a shift to higher growth light, it takes time to upregulate sink activity to take advantage of the greater abundance of energy. Moreover, the foliar vasculature of existing leaves grown in low light may not have the necessary capacity to export increased levels of photosynthate. Consequently, carbohydrates can accumulate in leaves of plants transferred from low to high light (Amiard et al. 2005). Concomitantly, predawn photosystem II efficiency declines (Adams et al. 2007, 2014a) and photoprotective energy dissipation and elevated zeaxanthin are maintained (Demmig-Adams et al. 1998). The leaves of some species that utilize sucrose-H^+ symporters for phloem loading were able to upregulate photosynthetic capacity to the level found in high light-acclimated leaves over the course of a week upon transfer of low-light-grown leaves to high light (Adams et al. 2005; Amiard et al. 2005). However, species that exhibited higher vein density in leaves grown under high compared to low light failed to fully upregulate photosynthesis upon a similar transfer of mature leaves

from low to high light (Amiard et al. 2005; Adams et al. 2007). Failure to upregulate photosynthesis upon such a transfer may also be related to an inability to increase leaf thickness (see Chap. 5).

Increases in foliar carbohydrates accompanied by overnight retention of zeaxanthin-associated energy dissipation and decreases in photosystem II efficiency are a common phenomenon in plants that experience an increased source to sink ratio upon a shift to a growth environment with more light (Layne and Flora 1993; Adams et al. 2007; Velez-Ramirez et al. 2017). Such responses of leaves are also common in many other situations in which light becomes excessive due to an insufficient level of sink activity (see Adams et al. 2013b, 2014a for reviews). Excessive light levels experienced only transiently during peak light levels are associated with transient increases in zeaxanthin and antheraxanthin levels and energy disspation. In contrast, continuous imbalances between absorbed and utilized light are associated with overnight retention of high levels of zeaxanthin and antheraxanthin and photosystems primed for high levels of energy dissipation and low photochemical efficiency. This phenomenon, the photoinhibition of photosynthesis, is often seen in evergreen leaves with high source to sink ratios. For instance, in leaves of *Schefflera arboricola* grown under 0.5% of full sunlight (10 μmol photons m^{-2} s^{-1}) and exposed to high light levels for varying periods of time, the level of lasting zeaxanthin and antheraxanthin accumulation (Fig. 18.5a) and depression in photosystem II efficiency (Fig. 18.5b) was greatest following 24 h of exposure and least following 3 h of exposure to 1200 μmol photons m^{-2} s^{-1}. In addition, the kinetics of recovery from this excess light exposure upon return to low light was fastest in leaves exposed to high light for only 3 h (largely reversible over 24 h), intermediate for leaves exposed for 5 h, and slowest (not fully reversed after even 72 h) among leaves that were subjected to excess light for 24 h (Fig. 18.5a, b). In other words, the leaves that experienced the greatest imbalance between source and sink strength (during the sudden 24-h exposure to high light) responded with the most protracted engagement of xanthophyll-associated energy dissipation and photosystem II efficiency depression. Interpretations of such depressions have focused either on inactivation of photosystem II photochemistry (and inhibition of resynthesis of photosystem II core proteins) or on sustained xanthophyll-associated energy dissipation under sink limitation (Adams 2013b, 2014a). Both phenomena may go hand in hand in low-light-grown leaves suddenly transferred to high light. However, rapidly-growing species typically show different responses during transitions from low to high growth light; they quickly grow new leaves with higher photsynthetic capacities per leaf area and no apparent sign of photoinhition (Demmig-Adams et al. 1989a). This response suggests that rapidly growing annuals are able to quickly upregulate sink activity when light availability increases.

B. Temperature

Similar scenarios, and similar species-dependent differences, to those during transitions from low to high light are also seen during transition from warm to cold termperature. While temperature immediately affects the rate of photosynthetic reactions via its impact on enzyme activity (Berry and Björkman 1980), longer-term acclimation to growth temperature involves upregulation or downregulation of components of the photosynthetic apparatus (Berry and Björkman 1980) and varies depending on species and temperature (Steffen et al. 1995; Adams et al. 2001a, 2002; Hikosaka 2005; Yamori et al. 2005; Baurle et al. 2007; Cohu et al. 2014). In temperate regions, many evergreen species exhibit strong photosynthetic upregulation (Monson et al. 2002, 2005; Zarter et al. 2006a) and a single flush

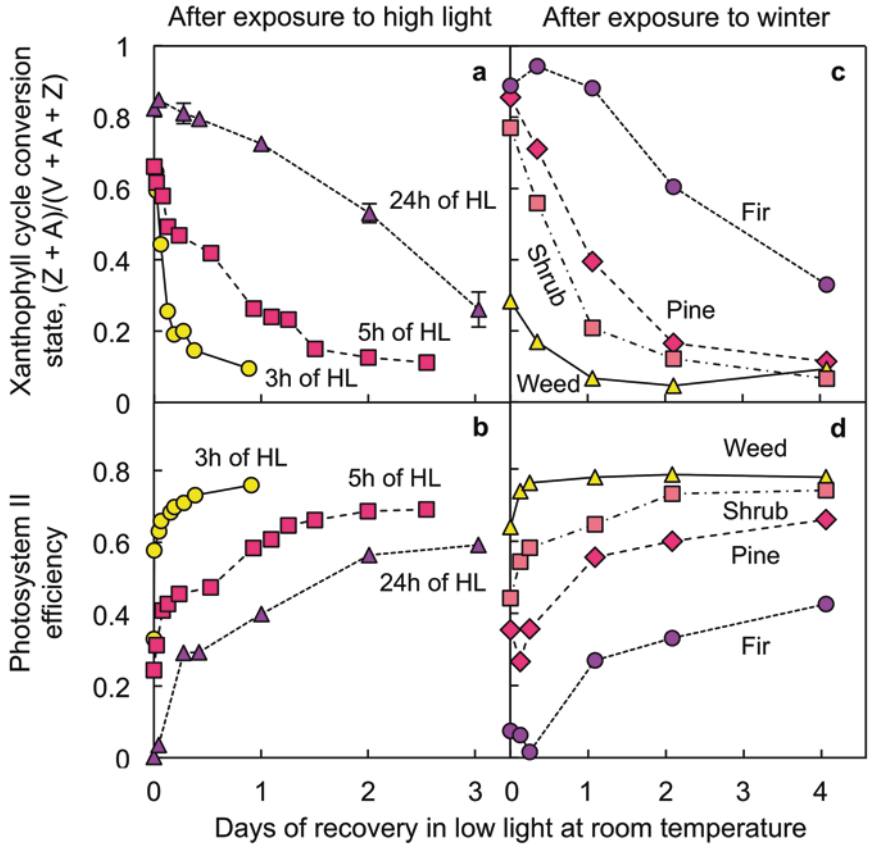

Fig. 18.5. Changes in (**a**, **c**) the xanthophyll cycle conversion state (the sum of zeaxanthin + antheraxanthin as a fraction of the total xanthophyll cycle pool) and (**b**, **d**) photosystem II efficiency during recovery from (**a**, **b**) exposure to high light (HL = 1200 μmol photons m^{-2} s^{-1}) or (**c**, **d**) winter conditions. (**a**, **b**) Shade leaves (grown under 10 μmol photons m^{-2} s^{-1}) of *Schefflera arboricola* were exposed to high light for three (yellow circles), 5 (red squares), or 24 (purple triangles) h and (**c**, **d**) leaves or needles of the herbaceous biennial weed *Malva neglecta* (yellow triangles), the woody shrub *Euonymus kiautschovicus* (pink squares), ponderosa pine (red diamonds), and Douglas fir (purple circles) were collected predawn in mid-winter from fully sun-exposed sites in Boulder, Colorado. Recovery consisted of low light (10 μmol photons m^{-2} s^{-1}) at room temperature. (Data for (**a**, **b**) from Demmig-Adams et al. (1998) and data for (**c**, **d**) from Verhoeven et al. (1996))

of new branch growth during the spring as the soil thaws and abundant soil water becomes available to permit stomatal opening, cell division, and support the positive pressure potentials necessary for cell expansion. High photosynthetic capacity during the spring can be followed by photosynthetic downregulation during the hot, dry summer followed by upregulation again during the cooler and moister autum (Fig. 18.6a) while predawn efficiency of photosystem II remains high during all three seasons (Fig. 18.6b). During the winter, however, plant growth is arrested and photosynthetic capacity of sun-exposed needles is strongly downregulated (Fig. 18.6a). This winter downregulation is coupled with high levels of zeaxanthin and antheraxanthin primed to facilitate photoprotective energy dissipation such that photosystem II remains in a state of low photochemical efficiency (for reviews, see Adams et al. 1995a, 2001a, 2002, 2006, 2014a; Demmig-Adams and Adams 2006; Fig. 18.6b).

The degree to which photosystem II efficiency remains depressed overnight depends

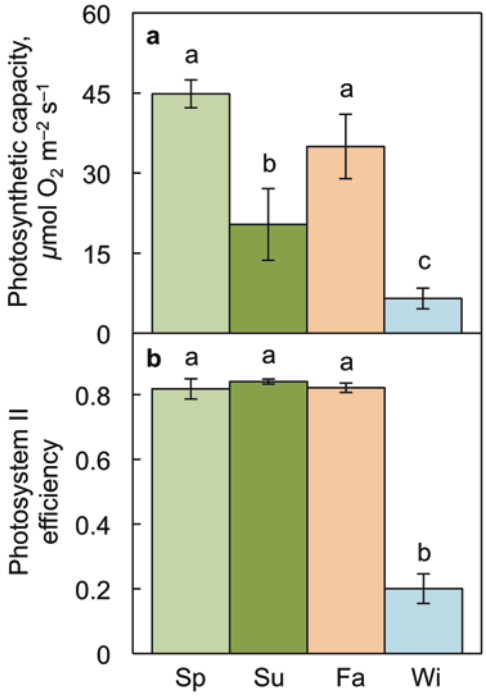

Fig. 18.6. (**a**) Light- and CO_2-saturated capacity for photosynthetic oxygen evolution determined at 25°C and (**b**) predawn photosystem II efficiency of fully sun-exposed needles of lodgepole pine (*Pinus contorta*) growing in a subalpine forest at 3000 m altitude in spring (Sp, light green), summer (Su, green), autumn (Fa, salmon), and winter (Wi, light blue). Mean values ± standard deviation, $n = 3$ trees. Significant differences indicated by different lower case letters (one-way analysis of variance coupled with a post-hoc Tukey-Kramer test for honestly significant differences). (Data from Zarter et al. (2006a))

on the diurnal intensity of light (Logan et al. 1998; Verhoeven et al. 1998; Adams et al. 2001a, 2002; Zarter et al. 2006b), the severity of the winter conditions (Adams and Demmig-Adams 1995; Zarter et al. 2006b, c), and the growth habit of different species. For instance, conifers exhibit strong winter downregulation of photosynthetic capacity (Verhoeven et al. 1999; Zarter et al. 2006a, c), substantial nocturnal retention of zeaxanthin and antheraxanthin (Fig. 18.5c), and strong predawn depressions in photosystem II efficiency (Fig. 18.5d). Broadleaved evergreen shrubs feature an intermediate level of winter downregulation of photosynthetic capacity (Adams and Demmig-Adams 1995; Logan et al. 1998; Zarter et al. 2006b), intermediate levels of nocturnally-retained zeaxanthin and antheraxanthin (Fig. 18.5c), and an intermediate level of nocturnal photosystem II efficiency depression (Fig. 18.5d). All of the aforementioned species, with sclerophytic leaves and needles, arrest growth (sink activity) during the winter.

In contrast to evergreens, biennials and winter annuals are herbaceous species that remain active and continue to grow and export carbohydrates from the leaves on milder winter days and nights (Adams et al. 2001b), despite some inhibition of growth (and respiration rates; Noguchi et al. 2015). It is noteworthy that these species accumulate compatible solutes that aid in acclimation to low and sub-freezing temperatures (Lee et al. 2012; Klopotek and Kläring 2014; Wingler 2015) without showing photosynthetic repression (Strand et al. 1997). Instead, overwintering annuals and biennials typically exhibit pronounced upregulation of photosynthetic capacity (Adams et al. 2001a, 2002; Demmig-Adams et al. 2017). These species also exhibit relatively little nocturnal retention of zeaxanthin and antheraxanthin (Fig. 18.5c) and predawn depression of photosystem II efficiency (Fig. 18.5d), both of which are rapidly reversible upon warming of the leaves under low light (Fig. 18.5c, d). In contrast, disengagement of photoprotective energy dissipation (Fig. 18.5d) and removal of zeaxanthin and antheraxanthin (Fig. 18.5c) upon warming from winter conditions proceeds more slowly in broadleaved evergreen species and even slower among conifers.

Once again, the different kinetics of disengagement of energy dissipation among different plant groups is related to their photosynthetic responses to the low temperatures of winter. In contrast to the strong, intermediate, or no winter downregulation of photosynthesis in evergreen conifers and broadleaved species mentioned above, mesophytic leaves of biennials and winter annuals

exhibit upregulation of photosynthetic capacity and an increased capacity for phloem loading and export of sugars in response to low temperatures and winter conditions (Adams et al. 1995b, 2001a, 2002, 2013a, 2016; Verhoeven et al. 1999; Dumlao et al. 2012; Cohu et al. 2013a, b, 2014; Muller et al. 2014; Stewart et al. 2016, 2017b; Chap. 2). This upregulation allows maintenance of photosynthetic rates despite lower temperatures; photosynthetic capacity of cool-grown leaves measured under cool temperature is similar to that of warm-grown leaves measured at warm temperature (Fig. 18.7a). In addition, when both cool- and warm-grown leaves are evaluated under warm temperature, it is clear that the photosynthetic upregulation in cool-grown leaves results in much higher maximal photosynthetic capacities of these cool- versus warm-grown leaves (Fig. 18.7a). Cool-grown leaves of winter annuals are also thicker (Dumlao et al. 2012; Cohu et al. 2014; Adams et al. 2016; Stewart et al. 2016), due to a greater number of mesophyll cell layers (Fig. 18.7b), and laced with minor veins, each of which has a greater number of phloem cells (Fig. 18.7c). These acclimatory structural adjustments to cool temperature presumably facilitate the higher rates of photosynthesis coupled with a greater capacity to load and export sugars facilitated by a greater surface area of phloem cell membranes for placement of sugar-loading transporters and greater volumes of the conduits that export sucrose from the leaves, as summarized in the schematic of Fig. 18.8 (corresponding to the photosynthetic rates of Fig. 18.7a).

Figure 18.8 summarizes the coordinated response of leaf morphology, anatomy, and function to growth temperature. Leaves grown under warm conditions featured two palisade tissue cell layers exhibited modest rates of photosynthesis, and matching activity of sucrose loading into the phloem and flux capacity (volume) of sucrose-exporting phloem sieve tubes under the warm temperature (Fig. 18.8a). Active loading of sucrose

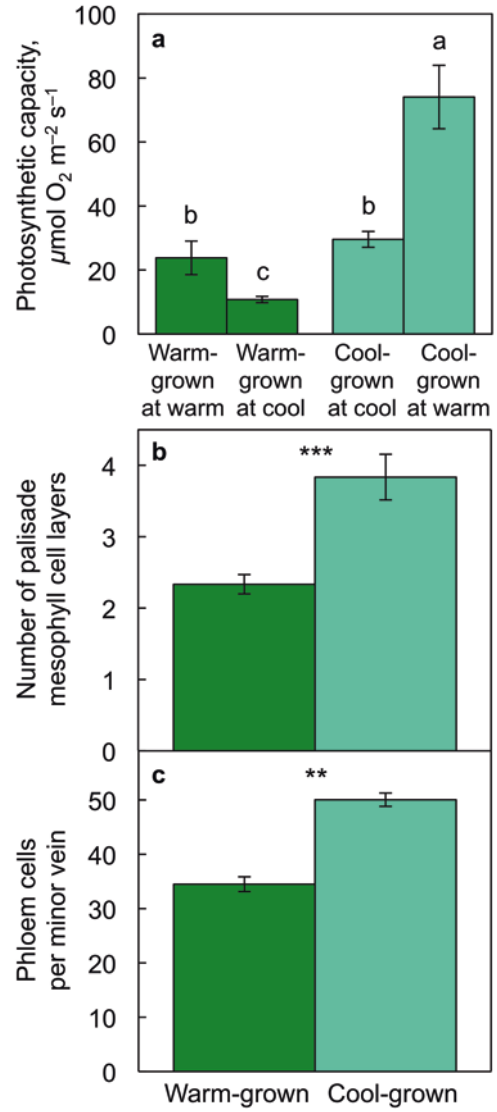

Fig. 18.7. (**a**) Light- and CO_2-saturated capacity for photosynthetic oxygen evolution determined at 25 °C (at warm) or 12.5 °C (at cool), (**b**) number of palisade mesophyll cell layers, and (**c**) number of phloem cells per minor vein for leaves of an *Arabidopsis thaliana* ecotype from Sweden (see Ågren and Schemske 2012) grown under a 9-h photoperiod of 400 μmol photons m^{-2} s^{-1} at daytime leaf temperatures of 25 °C (Warm-grown, green columns) or 14 °C (Cool-grown, blue-green columns). Mean values ± standard deviation (**a**) or ± standard error (**b**, **c**), $n = 4$ leaves from four plants. Significant differences in (**a**) indicated by different lower case letters (one-way analysis of variance coupled with a post-hoc Tukey-Kramer test for honestly significant differences) and in (**b**, **c**; Student's *t*-test) by asterisks – ** = $P < 0.01$, *** = $P < 0.001$. (Data from Cohu et al. (2013b, 2014))

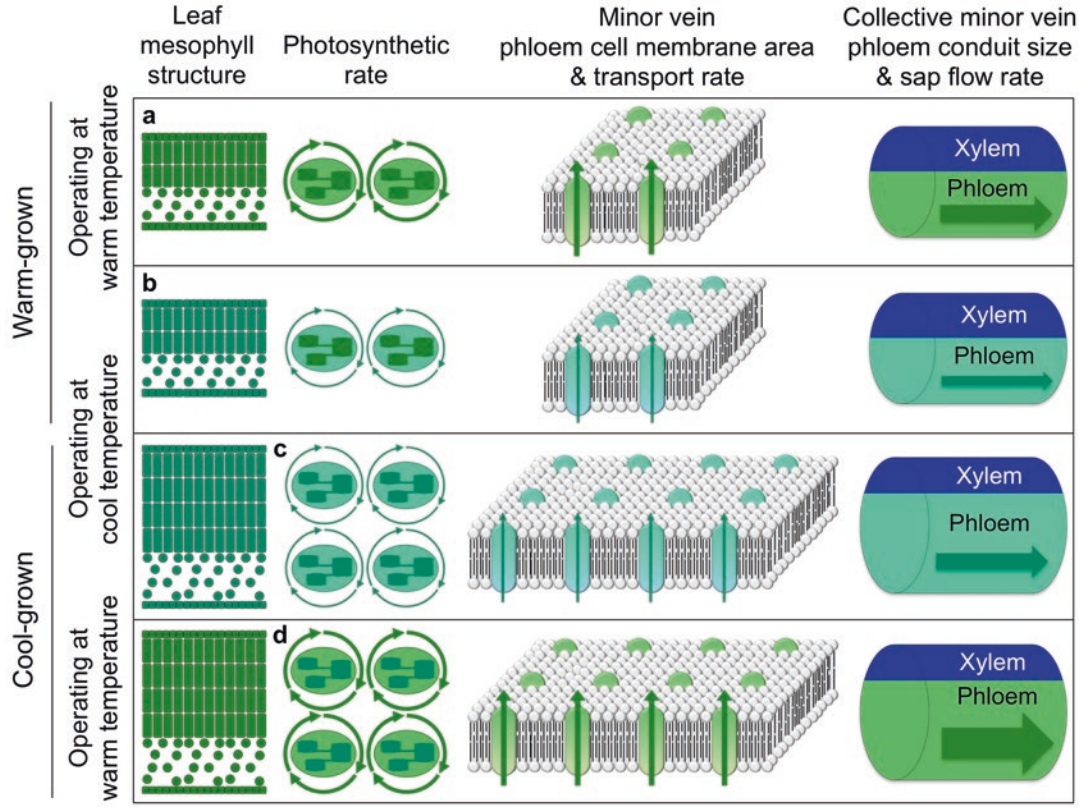

Fig. 18.8. Schematic diagram depicting features underlying the data for *Arabidopsis thaliana* shown in Fig. 18.7. Physical features of leaves that developed at (**a**, **b**) 25 °C (fewer layers of leaf mesophyll cells, fewer chloroplasts per unit leaf area, a smaller area of phloem cell membrane within which fewer transport proteins per unit leaf area can be accommodated, and a smaller cross-sectional area of phloem per unit leaf area) or (**c**, **d**) 14 °C (a greater number of leaf cell mesophyll layers, a greater number of chloroplasts per unit leaf area, a greater area of phloem cell membrane within which more transport proteins per unit leaf area can be accommodated, and a greater cross-sectional area of phloem per unit leaf area). Rates at which photosynthesis and transport occur (as denoted by the width of the arrows) at 25 °C (**a**, **d**; higher) or at 12.5 °C (**b**, **c**; lower)

by the phloem is facilitated by the coordinated action of membrane-spanning sucrose efflux carriers (permitting sucrose flow into the apoplast around the phloem), ATPases, and sucrose-H^+ symporters (for transporting sucrose into the phloem), as is the case for apoplastic loaders like *Arabidopsis thaliana*, spinach, and pea. For the same warm-grown leaves suddenly exposed to lower temperature (Fig. 18.8b), however, the rate of photosynthesis is less than half the rate observed at the warm growth temperature (Fig. 18.7a), the activity of the membrane transporters is presumably likewise diminished, and the rate at which the sugar-laden phloem sap flow is diminished as a result of a lower rate of loading and increased viscosity that occurs at lower temperature (Cohu et al. 2013a). When leaves have time to acclimate to low growth temperature, their form and function is adjusted to maintain photosynthetic activity. The thicker leaves of plants that develop at low temperature pack more chloroplasts into more numerous mesophyll cells per unit leaf area (Fig. 18.8c), which yields a photosynthetic rate at the low growth temperature that is comparable to that of leaves that developed and operate at warm temperature (Fig. 18.7a). To counteract the negative impact of low temperature on active sucrose

loading into the phloem, greater numbers of phloem cells per minor vein (Fig. 18.7c), as well as greater levels of phloem transfer cell wall invagination (Adams et al. 2014b, 2016), provide for enhanced cell membrane area for placement of phloem-loading transporters in cool-grown leaves (Fig. 18.8c). The greater number of phloem sieve elements per minor vein in the cool-grown versus warm-grown leaves also presumably permit a greater flux of sugar-laden phloem sap through the sieve tubes than would otherwise be possible at the lower temperature (cf. Fig. 18.8b, c). Transferring cool-grown plants to warm temperature relieves Calvin-Benson cycle proteins and membrane-spanning transporter proteins of the phloem from low-temperature depression of activity, permitting extraordinarily high rates of photosynthesis, high rates of sucrose loading into the phloem, and high rates of sucrose transport through the minor vein network (Fig. 18.8d) to the midrib of the leaves for distribution to the plant. This coordinated upregulation of photosynthetic capacity and sugar-export capacity in cool-grown leaves of winter annuals allows these plants to make pronounced carbon gains during narrow windows of opportunity on mild days in winter and spring (see discussion in Demmig-Adams et al. 2017).

Once a leaf has developed in a given environment, aspects of its infrastructure (mesophyll and vascular cell composition) may be fixed and prevent the mature leaf from acclimating fully to an altered set of conditions. For instance, transfer of warm-grown *Arabidopsis thaliana* plants to a lower growth temperature regime stimulated partial upregulation of photosynthesis to a level intermediate between warm- and cool-grown leaves (Fig. 18.9a). This partial acclimation to the lower growth temperature also involved an intermediate leaf mass per area (Fig. 18.9b). Similar constraints to acclimation of mature, fully expanded leaves have been reported for transfer from low to high growth light intensity (Amiard et al. 2005; Adams et al. 2007; see also Chap. 5),

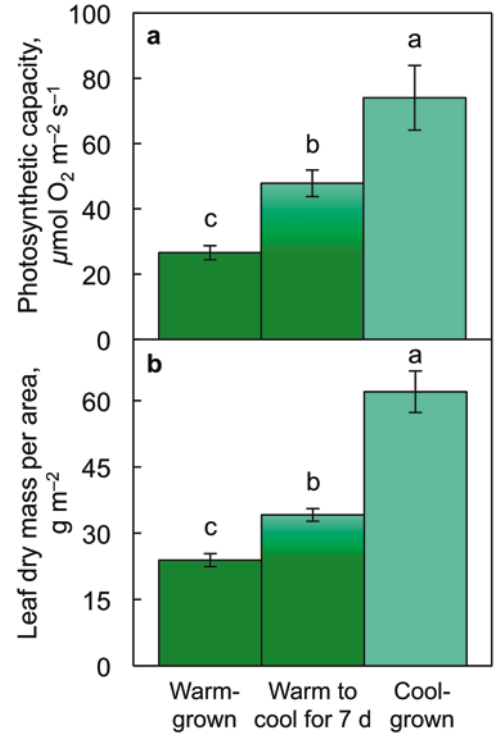

Fig. 18.9. (**a**) Light- and CO_2-saturated capacity for foliar photosynthetic oxygen evolution determined at 25 °C and (**b**) leaf dry mass per area for an *Arabidopsis thaliana* ecotype from Sweden that developed under a 9-h photoperiod of 400 μmol photons m^{-2} s^{-1} at a leaf temperature of 25 °C (Warm-grown, green), 14 °C (Cool-grown, blue-green), or at 25 °C and subsequently transferred to 14 °C for 7 days (Warm to cool for 7 days, green shading into blue-green). Mean values ± standard deviation, $n = 4$ leaves from four plants. Significant differences indicated by different lower case letters (one-way analysis of variance coupled with a post-hoc Tukey-Kramer test for honestly significant differences). (Data from Cohu et al. (2013b, 2014) and unpublished data)

illustrating the importance of foliar structural acclimation and its limitations.

As for low temperature, plant response to high temperature depends on plant evolutionary history and other factors. Plant growth at higher temperature commonly involves a shift in the temperature optimum for photosynthesis to higher temperatures (Berry and Björkman 1980; Slot and Winter 2017). Depending on species, moderately high growth temperatures can accelerate

growth and development (Suwa et al. 2010; Becker et al. 2017), which can be viewed as a strategy to escape heat/drought and complete the lifecycle before prohibitive conditions set in. Overexpression of an expansin that may facilitate accelerated growth through increased cell wall loosening yielded tobacco plants with higher rates of photosynthesis when growing under heat stress (Xu et al. 2014). Closer to the lethal point, high temperatures can inhibit plant growth and sink activity (Bussotti et al. 2014; Sunmonu and Kudo 2015; Teskey et al. 2015). Heat stress in cotton repressed genes coding for proteins involved in growth and photosynthesis (Cottee et al. 2014). Growth responses to high temperature may include development of either smaller leaves with higher rates of photosynthesis per unit leaf area (in potato; Lafta and Lorenzen 1995) or larger, thinner leaves with lower rates of photosynthesis per unit leaf area (in aspen; Rasulov et al. 2015). Higher levels of soluble sugars (and lower starch or sorbitol) are also commonly observed in leaves at elevated temperature (Lafta and Lorenzen 1995; Lie et al. 2013; Marias et al. 2017a), possibly due to impaired sugar export via the phloem (Suwa et al. 2010; Marias et al. 2017b, e.g., see heat girdling of petioles in the previous section). For species that emit isoprene (a sink for photosynthate), increased emission rates with increasing temperature provides enhanced thermotolerance to the leaves (Hanson and Sharkey 2001; Sharkey and Yeh 2001; Vickers et al. 2009). High temperature also stimulates photorespiration in C_3 plants (Zhang et al. 2013b; Sicher 2015; Bräutigam and Gowik 2016). As was introduced above for moderate temperature conditions, greater photorespiration rates can actually have positive effects on carbon fixation and growth. Overexpression of a photorespiratory enzyme yielded higher rates of photosynthesis and growth under high temperature (in rice; Cui et al. 2016) as did expression of a bacterial glycolate dehydrogenase in potato plastids (Nölke et al. 2014).

C. Water Availability

Increased leaf and air temperature are typically associated with decreased atmospheric water content, resulting in a greater difference in water vapor pressure between the leaf and surrounding air and increased rates of water loss as long as stomates remain open. This scenario contributes to water stress and reduced growth and photosynthesis (Bussotti et al. 2014; Vasseur et al. 2014; Perdomo et al. 2015; Teskey et al. 2015; Ruehr et al. 2015; Vårhammar et al. 2015). However, plant acclimation to high temperature prior to the onset of water stress mitigated the impact of reduced water availability in mesophytic, summer annual C_3 (sunflower) and C_4 (corn) species (Killi et al. 2017). Selection of wheat lines that maintained high photosystem II efficiency under elevated temperatures exhibited higher rates of growth and photosynthesis (Sharma et al. 2015).

An early response to decreasing soil water content can be enhanced root growth (Lipiec et al. 2013). As water stress develops further, the shoot experiences proportionally greater decreases in growth rate compared to the root system (leading to an enhanced ratio of roots to shoots; Sharp et al. 1988), eventually culminating in a cessation of all growth in the absence of additional precipitation (Hsiao 1973; Hsiao et al. 1976). During the development of water stress, an increasing fraction of photosynthate may be diverted to the synthesis of compatible solutes (osmotic adjustment) to aid in maintaining a water potential gradient from the soil to the plant and a positive pressure potential or turgor (Moore et al. 1997; Streeter et al. 2001; Lee et al. 2005; Lipiec et al. 2013). In the early stages of water stress, a midday depression in CO_2 uptake (Hirasawa and Hsiao 1999) and in photosystem II efficiency (involving formation and engagement of zeaxanthin in energy dissipation; Fig. 18.1) may become more pronounced (Demmig-Adams et al. 1989b). As water stress becomes progres-

sively greater, stomatal opening diminishes and photosynthesis is downregulated (Vaz et al. 2010). Nocturnal retention of zeaxanthin (and antheraxanthin) engaged for energy dissipation (i.e., associated with reduced photosystem II efficiency) can be found in some species (e.g., *Nerium oleander*) experiencing water stress (Demmig et al. 1988). In other species (*Yucca* species experiencing elevated temperatures in the Mojave desert), however, the nocturnally-retained zeaxanthin only becomes engaged as dawn breaks and light quickly becomes excessive (Barker et al. 2002). Downregulation of photosynthesis in response to water stress has been associated with whole plant sink limitation (Damour et al. 2009; Muller et al. 2011; Pinkard et al. 2011). Until recently there was an open question regarding whether plants experiencing severe water stress died from carbon starvation or hydraulic failure and dehydration. This debate was recently tipped toward the latter when it was shown that the total amount of nonstructural carbohydrates was no lower in water-stressed plants compared to plants with ample access to water and that, at death, plants still retained carbon reserves (Hartmann et al. 2013; Rowland et al. 2015; Savi et al. 2016; Saiki et al. 2017). Nonetheless, an indirect role for sugars as osmolytes that enhance recovery from xylem cavitation suggests that hydraulic functioning and carbohydrate status cannot be isolated completely from one another (Trifilò et al. 2017; see also Yoshimura et al. 2016).

Excess water supply poses similar problems as water shortage. In plants not adapted to aquatic habitats, prolonged submersion of the root system after a flooding event may result in hypoxia, arrest of root growth, and disruption of root function that can lead to wilting and severely curtail plant growth (Else et al. 2001). These events result in reduced photosynthate translocation from the shoot, foliar carbohydrate accumulation, and downregulation of rate and efficiency of photosynthesis (Wample and Thornton 1984; Fernandez et al. 1999; Pezeshki 2001; Rengifo et al. 2005; Sloan et al. 2015; Yu et al. 2015). Corn likewise undergoes photosynthetic downregulation in response to flooding (Bragina et al. 2004), followed, however, by triggering of cellular apoptosis in root cortical tissue, which creates open spaces (pseudoaerenchyma) for air flow at the interface between water and air (Drew et al. 1980). Such reestablishment of root access to oxygen allows development of new leaves with higher rates of photosynthesis (Bragina et al. 2004). However, this type of response is likely the exception than the rule.

D. Salinity

Mangroves in seawater are rather slow-growing, and, when established in sun-exposed sites, utilize only a small fraction of absorbed sunlight for photosynthesis while dissipating the remainder via high levels of photoprotective energy dissipation (Björkman et al. 1988). Plant response to low water availability and salinity stress have common features, such as allocation of photosynthate to compatible solutes among salt-tolerant species (Tattini et al. 2002; Ruiz-Lozano et al. 2012; Wu et al. 2013; Tang et al. 2015), but growth in some species may be diminished even more readily as a result of water stress (Cramer et al. 2007). A lower level of sink activity in response to salinity leads to decreased translocation of photosynthate from leaves, accumulation of foliar sugars, and downregulation of photosynthesis (Suwa et al. 2006; Hummel et al. 2010; Major et al. 2017; but see Khan et al. 2017). Various manipulative approaches demonstrated the role of root activity under salinity stress. For instance, grafting of salt-sensitive pepper plants onto rootstocks from a salt-tolerant pepper line prevented downregulation of photosynthesis in response to salinity in the sensitive line, due in part to continued function of the tolerant roots (Penella et al. 2016). Overexpression of regulators of plant growth and development (DNA-binding One Finger proteins) in tomato, a particularly salt-

sensitive species, increased growth and photosynthetic capacity of the plants under control and saline conditions (Renau-Morata et al. 2017). In addition, the phloem plays an important role in salt tolerance in *Arabidopsis thaliana*. Sodium taken up by roots and transported to the shoot in the xylem is moved into the phloem in the leaves and recirculated back to the roots, which enhances plant tolerance to salinity (Berthomieu et al. 2003).

E. Nutrients

As is the case for many other environmental factors, nutrient limitation can affect both source and sink tissues, but the evidence suggests that initial effects on sink activity drive responses of the source tissues. Limiting availability of essential nutrients limits growth, leading to foliar carbohydrate accumulation, leaves with lower chlorophyll content, and lower rates of photosynthesis (Paul and Driscoll 1997; Sexton et al. 1997; Fischer et al. 1998; Guidi et al. 1998; Hansen et al. 1998; Logan et al. 1999; Passarinho et al. 2000; Cruz et al. 2003; Wissuwa et al. 2005; Araya et al. 2010; Pan et al. 2017; however, see Pinkard et al. 2011). The impact of limiting nitrogen on foliar photosynthesis can be exacerbated when developing, non-photosynthetic sinks for carbohydrates are major sinks for nitrogen (such as pecan nuts; Heerema et al. 2014; see also Zhang et al. 2013a). Conversely, increased nutrient availability increases sink activity resulting in higher rates of photosynthesis (Kaschuk et al. 2010).

F. Pollution

Pollution of various types can impair growth and photosynthesis. High levels of heavy metals led to decreased rates of growth and photosynthesis, and foliar starch levels were either lower (Shiyab et al. 2009; de Araújo et al. 2017; Zhou et al. 2017) or higher (Rai et al. 2005; Daud et al. 2013) compared to controls. Soil alkalinization above the optimal level for aspens coupled with excessive levels of several essential nutrients (both arising from cement dust pollution) resulted in reduced growth, elevated foliar carbohydrate levels, and lower rates of photosynthesis (Mandre et al. 2012), suggestive of sink-limited downregulation of photosynthesis. Elevated levels of atmospheric ozone are thought to inhibit the activity of source leaves (Topa et al. 2001; Granz et al. 2003), including export of photosynthate to sinks (Rennenberg et al. 1996; Grantz and Farrar 2000; see also Mulchi et al. 1992 & Fialho and Bücker 1996). On the other hand, sulfur dioxide and nitrogen dioxide absorbed by leaves may enter the pools of nutrients utilized by the plant (Rennenberg et al. 1996). However, high levels of sulfur dioxide can inhibit phloem loading in C_3 plants (Minchin and Gould 1986). In a study using *Populus* leaves, photosynthesis rates decreased under foliar exposure to sulfur dioxide, but the impact of this treatment was lessened by increasing light intensity as greater proportions of the SO_2 were metabolized to other products (Adams et al. 1989).

G. Herbicides

Herbicides play a role in producing higher yields of grains, vegetables, and fruits by eliminating weedy plants that compete with crop plants for essential resources. Treatment of plants with herbicides that impair cytosol-localized anabolic pathways resulted in decreased growth, decreased translocation of photosynthate from leaves to sinks, foliar accumulation of carbohydrates, and decreased rates of photosynthesis (Zabalza et al. 2004, 2013; Yanniccari et al. 2012). In contrast, herbicides that target chloroplast-localized features act on source leaves (Pechová et al. 2003; Scarpeci and Valle 2008), an effect that can be ameliorated to some extent by higher levels of pre-existing soluble sugars (Ramel et al. 2009). Ramel et al. (2009) attributed the latter effect to the

role of sugars as signaling molecules, but their ability to act as antioxidants (e.g., Matros et al. 2015) seems just as likely to be involved. Interestingly, an *Amarathus* species resistant to one of the latter types of herbicides had significantly decreased fitness in the presence of an herbivorous beetle compared to a non-resistant line (Gassmann and Futuyma 2005). Competition among plants and the impact of herbivores are among the many biotic interactions important to photosynthetic performance, as will be elaborated upon in the next section.

IV. Adjustment of Photosynthesis in Response to the Biotic Environment

A. Competition

The impact of other, interacting organisms on plant growth, development, and on photosynthesis can be just as profound as the influence of abiotic environmental factors. Competition between plants (Craine and Dybzinski 2013; Aschehoug et al. 2016) for resources occurs both below- and aboveground. Plants compete belowground for water and essential nutrients and for alliances with mutualistic and symbiotic fungi and bacteria (Hodge and Fitter 2013; Fonseca et al. 2017; more on this below), and shoot systems compete aboveground for light. Pathogen outbreaks impact the outcome of competitive interactions (Hodge and Fitter 2013), and members of the soil microbiome compete for resources surrounding a plant's rhizophere (Ke and Miki 2015; Zhu et al. 2016). Plants actively secrete allelochemicals that can negatively influence performance and growth of their competitors, potentially interfering directly in photosynthesis (Einhellig et al. 1993; Dayan et al. 2009, 2010; Uddin et al. 2012; Hussain and Reigosa 2017). When more resources are available to the root systems of plants, vegetation and leaf density within the canopy are typically greater, which increases competition for water (with C_4 species [see Chap. 9] outcompeting C_3 species; Niu et al. 2005) and light (Anten and Bastiaans 2016).

The latter competition for light results in the range of acclimation to sun versus shade mentioned in the previous section, coupled with responses to a lower ratio of red to far-red light (Pierik et al. 2013) arising from reflection of far-red light from neighboring plants and, under increasingly closed canopies, the preferential screening of red light by chlorophyll in leaves of the upper tier(s). Photosynthesis (Aspiazú et al. 2010) and genes involved in photosynthesis (Geisler et al. 2012) may either be upregulated in plants competing with each other or can be downregulated via acclimation to more shaded conditions (Norman and Martin 1994; Gibson and Skeel 1996; Robinson et al. 2001; Reynolds et al. 2007; Concenço et al. 2008). The impact of above-ground competition typically outstrips that of below-ground competition (Jensen et al. 2011). Even at the scale of a single leaf, localized regions of long-lived foliage exhibited shade acclimation characteristics when colonized by lichens on their upper surface (Anthony et al. 2002; Zhou et al. 2014). For a recent review on acclimation to shade and its myriad interactions with abiotic and biotic factors, see Valladares et al. (2016).

Competition among plants affects abiotic (van Loon et al. 2014) and biotic (Hodge and Fitter 2013) conditions relevant to plant performance and can do so with different outcomes. Symbiotic associations with mycorrhizal fungi influence competitive outcomes depending on photosynthetic pathway, association with nitrogen-fixing symbionts, and life history (Lin et al. 2015; see also Abbot et al. 2015). Herbivory can also have varying outcomes, with either no effect on competition (Jing et al. 2015) or an impact on competition (Ibanez et al. 2013; Kim et al. 2013; Gehring et al. 2014).

B. Herbivory

The impact of herbivory on photosynthesis is likewise complex. A transcriptomic approach suggested that damage to leaves by various herbivores, as well as pathogens, leads to repression of photosynthetic genes (Bilgin et al. 2010). However, many interactions between plants and other organisms result in elevated photosynthesis and growth. Specifically, some organisms interacting with plants decrease source to sink ratio within the plant by enhancing sink activity, creating additional sinks within the plant, or diminishing foliar source activity, in each case leading to photosynthetic upregulation. On the other hand, some biotic interactions diminish sink activity and prompt photosynthetic downregulation, and yet others are simply benign or deleterious to plants. Herbivory of plants also has different effects depending on various factors, as detailed in the following paragraphs. Conversely, consumption of animals by carnivorous plants improves plant nutrient status and increases photosynthesis rates (Pavlovič et al. 2009, 2014), as does plant fertilization by animal waste (Peek and Forseth 2003; Romero et al. 2010).

The impact of herbivory on photosynthesis has received much attention, in part due to the importance of plant regeneration following grazing by livestock. Photosynthetic response to herbivory depends on which plant parts are targeted by the herbivores (e.g., old versus young, shade versus sun, shoot system versus root system; see, for example, Holland et al. 2017), the location of apical meristems, the level of herbivory, and availability of resources to the plant for investment in growth and photosynthesis (Holland and Detling 1990). As with the manipulative experiments described in Sect. 2.1, removal of source leaf tissue often results in photosynthetic upregulation in the remaining source leaf tissue. In particular, grasses, with meristems at the base of the leaf and a proclivity to produce new tillers, can respond with enhanced growth to moderate grazing or simulated grazing (Paarsons et al. 1983a, b). If sufficient resources are available, these systems exhibit photosynthetic upregulation in the ungrazed portion of the foliage and growth of new leaf tissue (Detling et al. 1979; Caldwell et al. 1981; Parsons et al. 1983a, b; Parsons and Penning 1988; Wallace 1990; Dyer et al. 1991; Senock et al. 1991; Doescher et al. 1997; Harrison et al. 2010; Anderson et al. 2013). Grazing of the foliage of various broadleaved species by animals likewise results in increased rates of photosynthesis in remaining leaves (Pearson and Brooks 1996; Thomson et al. 2003; Rhodes et al. 2017). However, the impact of grazing by mammals on photosynthesis depends on a variety of factors. In an arctic tundra dry heath community, grazing reduced net productivity, whereas in a nearby moist tussock community with greater resource availability, net productivity was enhanced by grazing (Gough et al. 2012). In a mixed plant community in China, grazing by sheep led to photosynthetic upregulation in the predominant grasses and shrubs (Peng et al. 2007), while a nearby community exhibited reduced photosynthesis following long-term overgrazing (Chen et al. 2005). Grazing of two shrub species by goats also resulted in elevated rates of photosynthesis, while a third species of shrub exhibited downregulation of photosynthesis following grazing (Redondo-Gómez et al. 2010). Insects can likewise impact photosynthesis rates. Among *Acacia* species with mutualistic ants that provide defense against herbivores, photosynthesis was elevated in trees with aggressive ant colonies (where a small amount of herbivory resulted in compensatory upregulation by remaining foliage) compared to less aggressive ant species or no colonies that suffered from overgrazing (King and Caylor 2010). As with the well-protected, lightly-grazed *Acacia*, aphid-induced defoliation of *Pinus radiata* resulted in photosynthetic upregulation in remaining needles (Eyles et al. 2011).

As stimulatory as some herbivory can be to photosynthesis and vegetative growth, there are countless instances where herbivory by arthropods has deleterious consequences for photosynthesis (both direct, through loss of leaf tissue, and indirect; see Nabity et al. 2009) and the plant. Some herbivorous insects set off signaling cascades that suppress photosynthesis (Tang et al. 2009; Kerchev et al. 2012; Nabity et al. 2012; Meza-Canales et al. 2017), although salivary secretions of other insects can enhance photosynthesis of leaf tissue surrounding the damaged region (Velikova et al. 2010; Halitschke et al. 2011). Alder trees exhibited increased photosynthesis rates in the remaining portion of leaves grazed upon by alder beetle, whereas birch leaves exhibited decreased rates when grazed by the same beetle (Oleksyn et al. 1998). In over half of herbivorous attacks by arthropods, photosynthesis of the affected leaves is estimated to be reduced (Welter 1989), as seen following feeding and damage by weevils (Venter et al. 2013; but see Peterson et al. 1992), Mexican bean beetles (Peterson et al. 1998), greenbugs (Ryan et al. 1987), leaf sap-sucking lace bugs (Cowie et al. 2016), mites (Marlin et al. 2013), thrips (Ellsworth et al. 1994), brown planthoppers (Watanabe and Kitagawa 2000), leafhoppers (Marshall et al. 1942; Ladd and Rawlins 1965; Womack 1984; Candolfi et al. 1993; Lamp et al. 2004, 2011; Lenz et al. 2012), and caterpillars (Fujiie 1982; Raimondo et al. 2003, 2013; Nardini et al. 2004; Tang et al. 2006, 2009; Delaney et al. 2008; Barron-Gafford et al. 2012; Delaney 2012; Nabity et al. 2012). Compensatory increases in the photosynthesis rates of neighboring leaves were reported in some cases (Delaney et al. 2008). Photosynthesis was unaffected by grasshopper feeding on potato leaves in the field (Bastos et al. 2011) or by *Trirhabda* beetle feeding on potted goldenrod plants growing outside (Meyer and Whitlow 1992). Different impacts can be related to which specific portions of the leaf are damaged. While aspen leaves infested by leaf miners that consumed portions of epidermis with stomata had lower rates of photosynthesis, leaves targeted by leaf miners that consumed portions of the epidermis without stomata exhibited no reduction in photosynthesis (Wagner et al. 2008). Remarkably, bacterial endosymbionts of leaf miners have been shown to maintain regions of green tissue in otherwise senescent leaves via elevated levels of cytokinins, thereby sustaining a food source for themselves and their host caterpillar (Kaiser et al. 2010).

Adelgid insects that feed on stored starch in xylem parenchyma of hemlock needles significantly reduced photosynthesis in a species with little defensive response (Nelson et al. 2014). Nematodes feeding on roots inhibited photosynthesis, presumably through decreased root sink activity and feedback inhibition of photosynthesis (Mazzafera et al. 2004; see also Arce et al. 2017). A meta-analysis of insect herbivory of the root systems of seventy-five plant species showed that photosynthesis was reduced to varying degrees depending on the nature of feeding and plant growth type (Zvereva and Kozlov 2012). For example, root herbivory by corn rootworm led to lower rates of photosynthesis (Urías-López et al. 2000), as did feeding by the European corn borer. European corn borer caterpillars tunnel through the stalk of corn plants, eating through vascular bundles, disrupting the continuity of xylem and phloem strands, inhibiting foliar photosynthate export, and triggering photosynthetic downregulation (Godfrey et al. 1991). Some studies reported different outcomes depending on growth conditions. While herbivory by a stem sawfly negatively impacted photosynthesis of wheat grown in growth chambers (14-h photoperiod at 3% of full sunlight), it had no impact on photosynthesis under greenhouse or field conditions (Macedo et al. 2005), or even resulted in an increased efficiency of photosynthesis (Macedo et al. 2006b). The latter results might be explained by the greater

availability of light in a greenhouse or in the field to fuel upregulation of photosynthesis in source leaves. In a meta-analysis of over ninety plant species, stem herbivory by insects significantly decreased plant growth and reproduction, but had no discernable impact on photosynthesis (Stephens and Westoby 2015).

C. Pathogens

A deleterious outcome of herbivory can also arise from the many pathogens herbivorous arthropods frequently carry with them (Gergerich and Scott 1988; Kluth et al. 2002; Venter et al. 2013; Perilla-Henao and Casteel 2016). Insects and other herbivores serve as vectors for pathogenic viruses, bacteria, and fungi, and wounding of plant tissue provides an entry-point for infection by additional pathogens (Imathiu et al. 2009; Alderman 2013). The impact of pathogens on photosynthesis varies (for reviews, see Berger et al. 2007; Selvaraj and Fofana 2012) and will be illustrated by a few examples. Reduced rate or efficiency of photosynthesis is a common symptom of pathogen infection (Nail and Howell 2004; Linaldeddu et al. 2009; Alves et al. 2011; Magnin-Robert et al. 2011; Rohrs-Richey et al. 2011; Gruber et al. 2012; Iqbal et al. 2012; Oliveira et al. 2012; O'Keefe et al. 2013; Pérez-Clemente et al. 2015; Ghosh et al. 2017). Such effects involve photosynthetic inactivation, decreased levels of chlorophyll, and/or decreased expression of photosynthetic genes (Funayama-Noguchi and Terashima 2006; Stullemeljer et al. 2009; Kim et al. 2010; Rodríguez-Herva et al. 2012; de Torres Zabala et al. 2015; Garcia-Seco et al. 2017; see also Copley et al. 2017). In some cases, photosynthetic downregulation may be due to feedback inhibition, since a number of pathogens limit carbohydrate export from leaves (Osmond et al. 1998; Lohaus et al. 2000; Maust et al. 2003; Kocal et al. 2008; Cimò et al. 2013). In addition, stomatal function can be impaired by fungal pathogens. Stomata become unresponsive to the environment and either remain open (leading to desiccation under drought) or closed (restricting access to CO_2 for photosynthesis; Mur et al. 2013).

Conversely, both bacterial and fungal pathogen infections can create localized sinks for photosynthate, resulting in upregulation of photosynthesis in leaf tissue surrounding the infected area (Berger et al. 2004; see also Berger et al. 2007 and Chang et al. 2013). Moreover, some powdery mildew and rust fungi synthesize cytokinins, which keeps the infected region of the leaf green and photosynthetically active (Walters et al. 2007; see also Garavaglia et al. 2010), similar to the impact of the symbiotic bacteria associated with leaf miners mentioned above. In much the same way, Synková et al. (2006) were able to inhibit the deleterious symptoms of a viral pathogen through the overexpression of cytokinins in leaves of tobacco. There are also cases where plants counteract the manipulative efforts of pathogens. For example, a grape virus increased transcripts for photosynthetic genes, but the plant downregulated photosynthesis nonetheless (Gambino et al. 2012). Furthernore, the impact of pathogens on photosynthesis has ramifications for associated processes. Increased rates of photosynthesis elicited by pathogens were associated with decreased levels of photoprotective energy dissipation (Christov et al. 2007). Conversely, reductions in photosynthesis due to pathogen infection or herbivory can be accompanied by increased levels of energy dissipation (Delaney 2008; Kyseláková et al. 2011; Barron-Gafford et al. 2012). At the larger, whole plant scale, a fungal pathogen outbreak on the first flush of aspen leaves led to reduced rates of photosynthesis, i.e., a reduced source to sink ratio, followed by a second flush of leaves that remained disease free and had significantly higher rates of photosynthesis than those of leaves from uninfected trees (Call and St. Clair 2017), presumably due to a lower source to sink

ratio than the uninfected trees with their full complement of competent first and second leaf flushes.

Photosynthesis fuels both proliferation of the pathogen and the plant's defense response against the pathogen infection. Such defense responses include the synthesis of a range of compounds (e.g., Abdel-Farid et al. 2009), some of which are common to defense against herbivores. Among such compounds are the anthocyanins (Kangatharalingam et al. 2002; Gutha et al. 2010; Menzies et al. 2016; Tian et al. 2017), although a case was reported in which anthocyanin synthesis was promoted by the fungus to decrease the plant's defense (Tanaka et al. 2014). Anthocyanins that can also accumulate in leaf epidermal cells in response to abiotic stress (see above) represent an example of the intersection between abiotic and biotic stress responses. Leaf galls induced by insects can likewise exhibit enhanced anthocyanin accumulation, but the significance of this remains unclear (Connor et al. 2012; Gerchman et al. 2013).

D. Induced Galls and Tumors

Galls or tumors consist of cells that proliferate in response to egg laying and/or feeding by arthropods and nematodes as well as infection by fungi or bacteria. Galls are strong sinks for photosynthate and nitrogen. High levels of sugars in leaf galls induced by insects or mites are associated with photosynthetic downregulation of the gall tissue itself (Burstein et al. 1994; Larson 1998; Patankar et al. 2011; Carneiro et al. 2014; Huang et al. 2014, 2015; Oliveira et al. 2017; see also Yang et al. 2007). However, chlorophyll fluorescence suggested no difference in photosynthesis between galls and non-galled leaf tissue (Fernandes et al. 2010; de Oliveira et al. 2011). On the other hand, galls formed of non-photosynthetic tissues were associated with photosynthetic upregulation. For instance, wasp-induced galls in vegetative and reproductive buds were substantially greater sinks than buds without galls, resulting in considerable photosynthetic upregulation in source leaves (Dorchin et al. 2006). Nematode-induced root galls were likewise strong sinks for photosynthate that prompted photosynthetic upregulation (Kaplan et al. 2011). In yet another example, the fungal pathogen responsible for corn smut induces large tumors that are strong sinks for photosynthate, stimulating photosynthetic upregulation (Horst et al. 2010). Leaves on plants with insect-induced shoot galls also exhibited higher rates of photosynthesis that was, however, attributed to enhanced water delivery to these leaves (Fay et al. 1993).

E. Tapping of Vascular Tissues by Insects and Parasitic Plants

A variety of insects tap directly into the sugar-rich phloem of plants as a source of nutrition, creating an additional sink for photosynthate and stimulating photosynthetic upregulation. These insects include aphids (Hawkins et al. 1987; Kaplan et al. 2011; Hussain et al. 2015; Kucharik et al. 2016) and scale insects (Retuerto et al. 2004) feeding on the broadleaved dicots tobacco, soybeans, broad beans, cowpeas, garden pea, and holly trees. On the other hand, aphids and scales negatively impacted photosynthesis when feeding on grass leaves (Burd and Elliott 1996; Haile et al. 1999; Macedo et al. 2009), cotton (Shannag et al. 1998), pecan trees (Wood and Tedders 1986), and *Euonymus* (Cockfield et al. 1987), with no impact on goldenrod (Meyer and Whitlow 1992), sugar beet (Hurej and van der Werf 1993), or poplars (Dardeau et al. 2015). It is possible that the differences in photosynthetic response to aphid feeding depends on whether or not photosynthate export via the phloem becomes impaired, as some aphid species induce the formation of callose tissue in the phloem whereas others do not (Saheed et al. 2009). Such an impairment was suggested by Veen (1985), who found reductions in photosynthesis associated with

aphid feeding to be accompanied by reduced phloem transport and foliar photosynthate accumulation. In yet other cases, lower photosynthetic rates in aphid-infested leaves may result from concomitant pathogen infection (Kaakeh et al. 1992). In addition, photosynthesis of young sink leaves may be particularly susceptible to aphid infestation (Davies et al. 2004). Furthermore, under field conditions where insufficient resources may limit investment into additional source activity, high levels of aphid infestation may be particularly deleterious to plants (Razaq et al. 2014; Kucharik et al. 2016). The impact of insects that tap into the xylem on photosynthesis has received little attention, but xylem-tapping spittlebugs also negatively impacted photosynthetic rates as well as leaf area of goldenrod (Meyer and Whitlow 1992).

Animals are not the only organisms that tap into the vascular system of plants. There are parasitic plants that tap into another plant's vascular system. Some parasitic plants tap into the roots of their host, whereas others send their roots (haustoria) into stem or branches of another plant. Some parasitic plants are annuals, such as dodder (members of the genus *Cuscuta*), that parasitize stems or roots of mesophytes. Parasites of woody plants, such as mistletoes that parasitize branches of shrubs and trees (Fig. 18.10), are longer-lived. Parasitic plants can be divided into two broad classes, hemiparasites that only draw on contents of the xylem and holoparasites that establish direct connections with both xylem and phloem of the host. A subgroup of holoparasites, endoparasites of the family Rafflesiaceae, live entirely within the host in close association with the host's vascular system. The only time these endoparasites emerge is when their flowers erupt from the host to allow access to the endoparasite's pollinators. Hemiparasites encompass an order of magnitude more species than holoparasites (Tešitel 2016), carry out their own photosynthesis, but nonetheless derive anywhere between 15 and 80% of their carbon from their host's xylem sap (Marshall and Ehleringer 1990; Schulze et al. 1991; Marshall et al. 1994; Richter et al. 1995; Wang et al. 2008; Tešitel et al. 2010). Xylem transports not only water, but also carbohydrates, albeit in lower concentrations than the phloem. Phloem-tapping parasites, such as holoparasitic dodder and Rafflesiaceae family members, have no or very reduced leaves and very low or no capacity to carry out photosynthesis.

Xylem-tapping parasitic plants generally have either no (Seel and Press 1996; Watling and Press 1998; Logan et al. 2002; Reblin et al. 2006) or negative (Johnson and Choinski 1993; Frost et al. 1997; Gurney et al. 2002; Meinzer et al. 2004; Cameron et al. 2008) impacts on their hosts' photosynthesis. In several studies, negative impacts could be offset by supplying the host with ample nitrogen (Cechin and Press 1993, 1994). Further evidence for a role of nitrogen supply comes from a study on four hosts and a single species of hemiparasite. Parasitism resulted in lower rates of photosynthesis for two of the hosts, but higher rates of photosynthesis for the parasite on another host with a symbiotic relationship with nitrogen-fixing bacteria (Lu et al. 2014b; see also Seel and Press 1994; Luo and Guo 2010). The hemiparasite *Cassytha pubescens* had no impact on the photosynthesis of a host with which it co-evolved in southern Australia under controlled growth conditions, whereas two invasive (non-native) hosts were found to have reduced rates of photosynthesis when parasitized by the same hemiparasite (Shen et al. 2010; Cirocco et al. 2017), as did the native host in the field (Prider et al. 2009) where sufficient resources may have been unavailable to support higher rates of photosynthesis in source leaves. Factors that alter the source-sink relationship, in addition to nitrogen nutrition alluded to above, also alter photosynthesis rates within the host-parasite association. Clipping some leaves of a grass host resulted in higher photosynthetic rates in the remain-

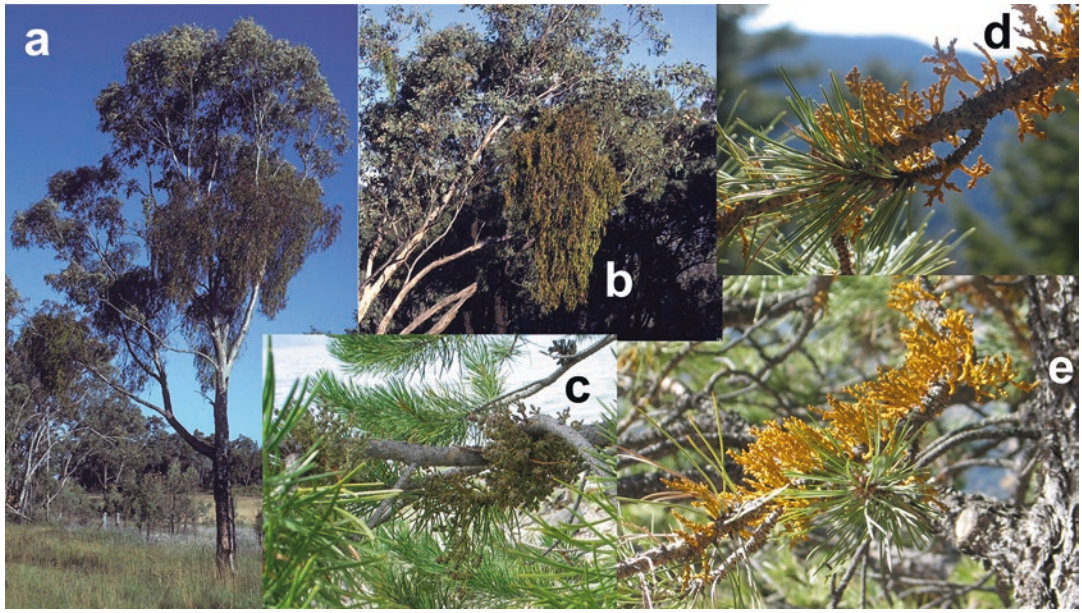

Fig. 18.10. Hemiparasitic mistletoes growing from the branches of their hosts. (**a**, **b**) Box mistletoe *Amyema miquelli* (Lehm. ex Miq.) Tiegh. growing on its broadleaved hosts (*Eucalyptus* species) in Tidbinbilla Nature Reserve, Australian Capital Territory, (**c**) dwarf mistletoe *Arceuthobium americanum* Nutt. ex Engelm. growing on its lodgepole pine (*Pinus contorta*) host in Wyoming, and (**d**, **e**) dwarf mistletoe *Arceuthobium vaginatum* (Willd.) J. Presl ssp. *cryptopodum* (Engelm.) Hawksw. & Wiens growing on its ponderosa pine (*Pinus ponderosa*) host in Colorado. (Photographs by W. Adams)

ing leaves, but reduced growth and photosynthetic rate of its root-tapping hemiparasite (Sui et al. 2015), perhaps due to greater investment of photosynthate into growth of new host leaves. Water stress reduced photosynthesis in a host to a greater extent than in its root-tapping hemiparasite (Inoue et al. 2013), and elevated CO_2 was more beneficial to a root-tapping hemiparasite than to its host (Hwangbo et al. 2003). In addition, at least two cases in which a xylem-tapping parasite increased photosynthesis rates in its host have been documented. In contrast to most studies of hemiparasite-host interactions, Hibberd et al. (1996) found that host photosynthesis was enhanced during infection with a root hemiparasite compared to uninfected control plants. In another study, on evergreen hosts and mistetoes in a climate with cold winters, photosynthesis of infected branches of the spruce host was enhanced during summer compared to non-infected branches, but was downregulated during winter to a greater extent in parasitized versus mistletoe-free host branches (Clark and Bonga 1970).

In a 2001 review, Watling and Press (2001) noted, consistent with findings reported above, that photosynthesis in host leaves of xylem-tapping parasites is generally unaffected or decreased. Photosynthate obtained by such hemiparasites from the host is withdrawn from the xylem and may be somewhat separate from the pool of carbohydrates that regulates source-sink balance of the host plant. In contrast, the phloem-tapping holoparasites were viewed by Watling and Press (2001) as additional sink tissue for the host plant, potentially contributing to a lower source to sink ratio within the host-parasite association leading to photosynthetic upregulation in host leaves. Some studies, but not others, provided evidence that phloem-tapping holoparasites are sinks for photosynthates that stimulate upregulation of host photosynthesis. A root parasite (*Orobanche*

cernua) that taps into phloem served as an added sink prompting photosynthetic upregulation in source leaves of its tobacco host (Hibberd et al. 1998, 1999). On the other hand, branched broomrape (*Orobanche ramosa*), a major pest of solanaceous crops, reduced photosynthesis of infected tomato plants (Mauromicale et al. 2008), whereas *Orobanche minor* had no impact on photosynthesis of its white clover host (Dale and Press 1998). The impact of phloem-tapping shoot parasites of the genus *Cuscuta* on their hosts was similarly varied. *Cuscuta reflexa* acted as a strong sink inducing photosynthetic upregulation in its lupine and *Coleus* (now classified as *Plectranthus*) hosts (Jeschke et al. 1994, 1997). In contrast, lower photosynthesis rates were seen in the host *Mikania* when infected with either *Cuscuta campestris* (Shen et al. 2007, 2013; Chen et al. 2011) or *Cuscuta australis* (Le et al. 2015). An endoparasite of the family Rafflesiaceae had no effect on host photosynthesis as determined through chlorophyll fluorescence measurements (Fernandes et al. 1998).

F. Witches' Brooms

Some vascular-tapping mistletoes can also influence photosynthetic activity of their hosts by inducing the host to develop a proliferation of branches and leaves at the site of infection, which is known as witches' broom or witch's broom (Godfree et al. 2002; Parker and Mathiasen 2004, Hedwall et al. 2006; Smith et al. 2013). This proliferation provides increased photosynthetic tissue for the mistletoe to draw upon for sustenance. Witches' broom-inducing pathogenic bacteria, fungi, and viruses afflict an even greater number of hosts than mistletoes (a Web of Science title search for "witches' broom" at the end of 2017 yielded over 450 references, of which only a small fraction involved mistletoes). While some of these pathogens can be quite detrimental to their host, even culminating in death in some cases despite stimulating shoot growth, there are a number of fungi and bacteria that engage in partnerships with plants that are beneficial to both parties.

G. Symbioses with Fungi and Bacteria

Mutually beneficial symbiotic relationships between plants and fungi, bacteria, or both also capitalize on the opportunity to enhance plant photosynthetic productivity by manipulating source-sink balance and increasing demand for photosynthate. Symbiotic mycorrhizal fungi form intimate partnerships with the roots of many plants and provide greatly magnified surface area in contact with soil for the acquisition of water and essential nutrients for the plant, which stimulates additional plant growth. In exchange, the plant provides photosynthate to the fungus. Both the fungus and the enhanced plant growth create additional sink demand and induce photosynthetic upregulation (Ekwebelam and Reid 1983; Azcón et al. 1992; Drüge and Schonbeck 1993; Mathur and Vyas 1995; Conjeaud et al. 1996; Wright et al. 1998a, b, 2000; Staddon et al. 1999; Loewe et al. 2000; Miller et al. 2002; Nowak 2004; Fan et al. 2008; Nehls 2008; Kaschuk et al. 2009, 2010; Nehls et al. 2010; Schweiger et al. 2014; Romero-Munar et al. 2017; but see Heinonsalo et al. 2015). Conversely, experimental removal of a substantial portion of the mycorrhizal sink led to a sudden decrease of host photosynthesis (Lamhamedi et al. 1994). Once again, abiotic factors interact with the host-symbiont relationship. Under field conditions, the enhancement of photosynthesis by mycorrhizae disappeared when temperatures decreased in autumn (Shrestha et al. 1995), perhaps as both partners ceased to grow. On the other hand, symbiotic mycorrhizal fungi can ameliorate negative impacts on host photosynthesis by limited availability of essential nutrients (Black et al. 2000; Parádi et al. 2003; Chen et al. 2014; Xiao et al. 2014; but see Huat et al. 2002 for an exception), reduced water availability (Ibrahim et al. 1990; Caravaca et al. 2003; Wu and Xia

2006; Birhane et al. 2012; Zhu et al. 2012; Yang et al. 2014; Li et al. 2015; for an early review, see Augé 2001), salinity stress (Sheng et al. 2008; Porcel et al. 2012; Ruiz-Lozano et al. 2012; Haghighi et al. 2016; Chen et al. 2017; Zhu et al. 2017), compacted soils (Polanco et al. 2008), heat stress (Zhu et al. 2011), and low temperature stress (Zhu et al. 2010). Such symbiotic relationships can furthermore stimulate production of additional leaves in the host plant (Shrestha et al. 1995; Adolfsson et al. 2015; see also Nowak 2004). Mycorrhizae may also reduce herbivory by insects feeding on roots (Frew et al. 2017) and either reduce or increase the impact of herbivory on shoots (Gilbert and Johnson 2015). The benefit of mycorrhizal symbionts to plants may become enhanced as atmospheric carbon dioxide concentrations continue to rise (Lovelock et al. 1997; Becklin et al. 2016), whereas the negative impact of fungal pathogens may be exacerbated (Aldea et al. 2006).

Nitrogen-fixing bacteria symbiotically associated with plant root systems can likewise represent a sizeable additional sink for plant photosynthate, increase the plant's own sink activity via supply of reduced nitrogen, and result in significantly elevated rates of foliar photosynthesis (Thomas et al. 1983; Thakur and Panwar 1997; Lippi et al. 1999; Peng et al. 2002; Sytnikov et al. 2006; Zhou et al. 2006; Kaschuk et al. 2009, 2010, 2012; Stefan et al. 2013). Mutualistic and symbiotic associations with bacteria can also ameliorate the negative impact of salinity and water stress on plant growth and photosynthesis (Sánchez-Díaz et al. 1990; Ben Salah et al. 2012; Dodd and Pérez-Alfocea 2012; Wang et al. 2016; Ilangumaran and Smith 2017; Radhakrishnan and Baek 2017). In addition, endophytic bacteria and fungi that live symbiotically within plants impart various benefits to their host. Some endophytes enhanced host photosynthesis in the absence of stress (Newman et al. 2003; Shi et al. 2010; Yan et al. 2013b), perhaps by serving as additional sinks for photosynthate or via production of growth-promoting substances. Many beneficial endophytes confer increased resistance against both abiotic stress and infection by pathogenic microbes, which is, once again, accompanied by elevated host photosynthesis (Bacon 1993; Bu et al. 2012; Li et al. 2012; Rozpadek et al. 2015; Xia et al. 2016; Ban et al. 2017; Su et al. 2017).

V. Conclusions

Diurnal accumulation of chloroplast-compartmentalized starch and its subsequent nocturnal degradation to sugars for export to the plant's sink tissues is closely tied to plant growth (Mengin et al. 2017; Sharkey 2017). This relationship can be altered by disruptions to the source/sink balance within a plant that can result in photosynthetic downregulation induced by foliar sugar build-up. Reduced photosynthesis may not always, however, correlate with leaf carbohydrate level (Asao and Ryan 2015). Rather than carbohydrate concentration, the rate at which sugars turnover or flux through particular pathways most likely provides the actual signal for sink regulation of photosynthesis (Nebauer et al. 2011). For instance, a transitory high level of foliar carbohydrates does not trigger downregulation of photosynthesis – as long as nocturnal export removes the photosynthate that accumulates each day, photosynthetic capacity remains elevated (Ribeiro et al. 2012). However, persistent increased levels of foliar carbohydrates resulting from an elevated ratio of source to sink activity are often accompanied by downregulation of photosynthesis. When chlorophyll content remains high during photosynthetic downregulation, and more light is absorbed than utilized in photosynthesis, photosystem II switches to a state of sustained energy dissipation and low photochemical efficiency, or photoinhibition. The extent of this photoinhibition with sustained low photosystem II efficiency is correlated with the level of foliar carbohydrate accumu-

lation (see above and Adams et al. 2013b, 2014a for reviews). These findings indicate a close link between this type of photoinhibition and source-sink imbalance.

The impact of abiotic stresses on photosynthesis at the individual plant level are also manifest at the ecosystem level when entire communities experience shifts to growth-impeding conditions. For example, net carbon dioxide uptake by subalpine forests may cease for up to 5 months as plant growth is suspended and photosynthesis is downregulated during the winter (Monson et al. 2002, 2005). A forest subjected to drought can exhibit reduced net uptake of carbon dioxide from the atmosphere (Gatti et al. 2014). At the other extreme, flooding is detectable at the ecosystem level via decreased carbon dioxide uptake from the atmosphere by vegetation (Koebsch et al. 2013; Han et al.2015). In the absence of other stresses, global warming will likely extend the total number of days during which seasonally-deciduous leaves remain active (Fu et al. 2018). Overall, recent changes in climate have led to greater sequestration of carbon from the atmosphere by plants (Nemani et al. 2003), but extreme periods of elevated temperature and drought could convert large swaths of vegetation from sinks for carbon dioxide to sources of carbon dioxide release into the atmosphere, thereby contributing to a feedforward cycle of global warming. For instance, during the summer heatwave and drought of 2003, the European continent became a source of carbon dioxide and crop productivity diminished (Ciais et al. 2005). If left unchecked, global climate change could eventually turn the entire biosphere from a sink to a source for carbon dioxide (Cox et al. 2000). Climate change is also projected to exacerbate the already enhanced frequency of intense fire, insect, and pathogen outbreaks in natural communities in the future (Olofsson et al. 2011; Rohrs-Richey et al. 2011; Appenzeller 2015; Björkman and Niemelä 2015; Hof and Svahlin 2016; Jones 2016; Ramsfield et al. 2016; Loehman et al. 2017; Wolton et al. 2017; Wyka et al. 2017). Mitigating the factors that drive climate change and extreme climate events is therefore of paramount importance. In addition, efforts to develop resilient crops that remain productive must be supported by an improved understanding of plant phenotypic and genomic controls on adaptation and acclimation to a range of abiotic and biotic factors. Strategies to enhance both source and sink activities of plants hold great potential to yield increases in plant performance (Ainsworth et al. 2012; Katoh et al. 2015), from both a carbon and nitrogen perspective (White et al. 2016), with appropriate attention to the pipelines providing for transport between the two (Ham and Lucas 2014; see Chaps. 2, 3 and 4).

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