Progress in Botany 81

Francisco M. Cánovas
Ulrich Lüttge
Christoph Leuschner
María-Carmen Risueño *Editors*

Progress in Botany

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Christoph Leuschner • María-Carmen Risueño
Editors

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Editors
Francisco M. Cánovas
Department of Molecular Biology
and Biochemistry
University of Málaga
Málaga, Spain

Ulrich Lüttge
Department of Biology
Technical University of Darmstadt
Darmstadt, Germany

Christoph Leuschner
Plant Ecology
University of Goettingen
Göttingen, Germany

María-Carmen Risueño
Pollen Biotechnology of Crop Plants Group
Centro de Investigaciones Biologicas (CIB)
CSIC Madrid
Madrid, Spain

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Contributors

Essarioui Adil National Institute for Agricultural Research, Errachidia, Morocco

Muhammad Arshad Agriculture and Agri-Food Canada, London, ON, Canada
Centre of Agriculture Biochemistry and Biotechnology, University of Agriculture, Faisalabad, Pakistan

María Jesús Cañal Plant Physiology, Department B.O.S., Faculty of Biology, University of Oviedo, Oviedo, Asturias, Spain

María Carbó Plant Physiology, Department B.O.S., Faculty of Biology, University of Oviedo, Oviedo, Asturias, Spain

María Ángeles Castillejo Agroforestry and Plant Biochemistry, Proteomics, and Systems Biology (AGR-164), Department of Biochemistry and Molecular Biology, University of Córdoba (UCO-CeiA3), Córdoba, Spain

Francisco J. Colina Department of Organisms and Systems Biology, University Institute of Biotechnology (IUBA), University of Oviedo, Oviedo, Spain
Plant Physiology, Department B.O.S., Faculty of Biology, University of Oviedo, Oviedo, Asturias, Spain

Teresa Deuchande CBQF – Centro de Biotecnologia e Química Fina Laboratório Associado, Escola Superior de Biotecnologia, Universidade Católica Portuguesa, Porto, Portugal

Mónica Escandón Plant Physiology, Department B.O.S., Faculty of Biology, University of Oviedo, Oviedo, Asturias, Spain
Agroforestry and Plant Biochemistry, Proteomics, and Systems Biology (AGR-164), Department of Biochemistry and Molecular Biology, University of Córdoba (UCO-CeiA3), Córdoba, Spain

Biruk A. Feyissa Agriculture and Agri-Food Canada, London, ON, Canada
Department of Biology, University of Western Ontario, London, ON, Canada

Margaret Y. Gruber Agriculture and Agri-Food Canada, Saskatoon, SK, Canada

Victor M. Guerrero-Sanchez Agroforestry and Plant Biochemistry, Proteomics, and Systems Biology (AGR-164), Department of Biochemistry and Molecular Biology, University of Córdoba (UCO-CeiA3), Córdoba, Spain

Noureddine Hamamouch Laboratory of Biotechnology and Sustainable Development of Natural Resources, Polydisciplinary Faculty, University Sultan Moulay Slimane, Beni Mellal, Morocco

Abdelali Hannoufa Agriculture and Agri-Food Canada, London, ON, Canada
Department of Biology, University of Western Ontario, London, ON, Canada

Jesús V. Jorrín-Novo Agroforestry and Plant Biochemistry, Proteomics, and Systems Biology (AGR-164), Department of Biochemistry and Molecular Biology, University of Córdoba (UCO-CeiA3), Córdoba, Spain

Robert Kendle Department of Botany, University of Wisconsin-Madison, Madison, WI, USA
Laboratory of Molecular and Cellular Biology, University of Wisconsin-Madison, Madison, WI, USA

J. Kreyling Experimental Plant Ecology, University of Greifswald, Greifswald, Germany

Elżbieta Kuźniak Department of Plant Physiology and Biochemistry, Faculty of Biology and Environmental Protection, University of Lodz, Łódź, Poland

Laura Lamelas Plant Physiology, Department B.O.S., Faculty of Biology, University of Oviedo, Oviedo, Asturias, Spain

Marta Libik-Konieczny Franciszek Górski Institute of Plant Physiology, Polish Academy of Sciences, Kraków, Poland

Cristina López-Hidalgo Agroforestry and Plant Biochemistry, Proteomics, and Systems Biology (AGR-164), Department of Biochemistry and Molecular Biology, University of Córdoba (UCO-CeiA3), Córdoba, Spain
Department of Organisms and Systems Biology, University Institute of Biotechnology (IUBA), University of Oviedo, Oviedo, Spain

Ulrich Lüttge Department of Biology, Technical University of Darmstadt, Darmstadt, Germany

Ana M. Maldonado-Alconada Agroforestry and Plant Biochemistry, Proteomics, and Systems Biology (AGR-164), Department of Biochemistry and Molecular Biology, University of Córdoba (UCO-CeiA3), Córdoba, Spain

Alejandra Matiz Department of Botany, Institute of Biosciences, University of São Paulo, São Paulo, SP, Brazil

Craig Matthews Agriculture and Agri-Food Canada, London, ON, Canada
Department of Biology, University of Western Ontario, London, ON, Canada

Mónica Meijón Plant Physiology, Department B.O.S., Faculty of Biology, University of Oviedo, Oviedo, Asturias, Spain

Helenice Mercier Department of Botany, Institute of Biosciences, University of São Paulo, São Paulo, SP, Brazil

Paulo Tamaso Mioto Department of Botany, Biological Sciences Center, Universidade Federal de Santa Catarina, Florianópolis, SC, Brazil

Zbigniew Miszalski Franciszek Górski Institute of Plant Physiology, Polish Academy of Sciences, Kraków, Poland
Malopolska Centre of Biotechnology, Jagiellonian University, Kraków, Poland

Tomas Morosinotto Department of Biology, University of Padova, Padova, Italy

Michał Nosek Institute of Biology, Pedagogical University, Kraków, Poland

Marisa S. Otegui Department of Botany, University of Wisconsin-Madison, Madison, WI, USA
Laboratory of Molecular and Cellular Biology, University of Wisconsin-Madison, Madison, WI, USA
Department of Genetics, University of Wisconsin-Madison, Madison, WI, USA

Giorgio Perin Department of Biology, University of Padova, Padova, Italy
Department of Life Sciences, Imperial College London, London, UK

Gloria Revilla Departamento de Biología Funcional, Facultad de Biología, Universidad de Santiago de Compostela, Campus Vida, Santiago de Compostela, Spain

María-Dolores Rey Agroforestry and Plant Biochemistry, Proteomics, and Systems Biology (AGR-164), Department of Biochemistry and Molecular Biology, University of Córdoba (UCO-CeiA3), Córdoba, Spain

Javier Sampedro Departamento de Biología Funcional, Facultad de Biología, Universidad de Santiago de Compostela, Campus Vida, Santiago de Compostela, Spain

Rosa Sánchez-Lucas Agroforestry and Plant Biochemistry, Proteomics, and Systems Biology (AGR-164), Department of Biochemistry and Molecular Biology, University of Córdoba (UCO-CeiA3), Córdoba, Spain

Carla S. Santos CBQF – Centro de Biotecnologia e Química Fina Laboratório Associado, Escola Superior de Biotecnologia, Universidade Católica Portuguesa, Porto, Portugal

Ireneusz Ślesak Franciszek Górski Institute of Plant Physiology, Polish Academy of Sciences, Kraków, Poland

Ewa Surówka Franciszek Górski Institute of Plant Physiology, Polish Academy of Sciences, Kraków, Poland

Pilar S. Testillano Pollen Biotechnology of Crop Plants Group, Biological Research Center, CIB-CSIC, Madrid, Spain

Elene R. Valdivia Departamento de Biología Funcional, Facultad de Biología, Universidad de Santiago de Compostela, Campus Vida, Santiago de Compostela, Spain

Luis Valledor Plant Physiology, Department B.O.S., Faculty of Biology, University of Oviedo, Oviedo, Asturias, Spain
Department of Organisms and Systems Biology, University Institute of Biotechnology (IUBA), University of Oviedo, Oviedo, Spain

Marta W. Vasconcelos CBQF – Centro de Biotecnologia e Química Fina Laboratório Associado, Escola Superior de Biotecnologia, Universidade Católica Portuguesa, Porto, Portugal

José M. Vega Department of Vegetal Biochemistry and Molecular Biology, Faculty of Chemistry, University of Sevilla, Sevilla, Spain

Ignacio Zarra Departamento de Biología Funcional, Facultad de Biología, Universidad de Santiago de Compostela, Campus Vida, Santiago de Compostela, Spain

Curriculum Vitae

Dr. José M. Vega was born on March 29, 1946, in Villanueva de la Serena, Province of Badajoz (Spain). He is married to Prof. Rosario Pásaro since 1972 and has two sons, José María (born in 1975) and Julio (born in 1979), and one grandson, Álvaro (born in 2016). He graduated in chemistry from the University of Seville in June 1968, and obtained his Ph.D. degree from the same university in July 1972, under the supervision of Prof. Manuel Losada. His Ph.D. thesis was entitled "NADH-nitrato reductasa de *Chlorella*."

His postdoctoral training was completed in the following three places: (a) firstly, at the Faculty of Biology, University of Virginia (1973–1974), in the research group of Prof. Reginald H. Garrett, which was working on NADPH-nitrate and nitrite reductases from the fungi *Neurospora crassa*; (b) then at Duke University Medical Center (1974–1976), under the supervision of Prof. Henry Kamin, who was working on ferredoxin-nitrite reductase from spinach leaves; and (c) finally, at the Department of Botany, University of Erlangen-Nürnberg (Germany), in the research group of Prof. Walter G. Zumft (1978), which was working on the denitrification mechanism of the bacteria *Pseudomonas perfectomarinus.*

In October 2018, he completed 50 years since his first appointment as an Assistant Professor to teach the techniques in Biochemistry to the students of the first course in Biology degree at the University of Sevilla. It was this university where he developed most of his teaching activities in the field of Biochemistry and Molecular Biology at the Faculties of Biology (1969–1980) and Chemistry (1981–2019). In 1981, he was appointed as Professor of Biochemistry and Molecular Biology at the Faculty of Chemistry, University of Sevilla, where he established his own research group that initially comprised Prof. Francisco Galván and Prof. Antonio J. Márquez. Figure 1 depicts the people belonging to the current Biochemistry group at the Faculty of Chemistry.

Fig 1 Biochemistry group at the Faculty of Chemistry. In the first line, from left to right: Prof. Marco Betti, Mrs. M. José Cubas, Mrs. Aurora Gómez, Prof. Carmen Pérez-Delgado, and Prof. Margarita García-Calderón. In the second line, from left to right: Prof. Francisco Galván, Prof. José M. Vega, and Prof. Antonio Márquez. The picture was taken on July 11, 2018

In 1986, Dr. Pedro J. Aparicio (Spanish National Research Council), Dr. Juan L. Serra (University of the Basque Country), Dr. Jacobo Cárdenas (University of Córdoba), and Dr. José M. Vega (University of Sevilla) together founded the Spanish Groups of Nitrogen Metabolism, which involved the groups working in the field of nitrogen metabolism and comprised of about 100 members. In addition, Dr. José M. Vega was a member of the Management Committee of COST 829 action budget by the European Union regarding "Fundamental, Agronomical and Environmental aspects of Sulfur Nutrition and Assimilation in Plants" (1996–2003). He was also a member of the Scientific Committee of "European Nitrate and Ammonia Assimilation Group" (ENAAG) during the period of 2001–2005.

Currently Dr. Vega has the research scopes regarding "Biotechnology of microalgae" collaborating with the research group directed by Dr. Carlos Vílchez, at the University of Huelva (Spain). This research field is gaining more and more presence in different industrial and economic sectors (Fig. 2).

Fig. 2 People of the Biochemistry area at the University of Huelva (Spain). In the first line, from left to right: Prof. Javier Vigara, Prof. Inés Garbayo, Prof. José M. Vega, Prof. Rosa León, and Prof. Carlos Vílchez. The picture was taken on September 25, 2018

To date, Dr. Vega has published 116 articles in indexed scientific journals, 43 chapters in books, and 12 books, and has also presented 240 communications to various scientific meetings. He has supervised 23 Ph.D. scholars, and has a Hirsch index (*h*-index) of 38. In addition, he has received two important awards in recognition of his excellent academic and research work. In the year 2007, the University of Seville distinguished him with the FAMA awards, and in the year 2016, the village of Villanueva de la Serena (Badajoz) declared him a distinguished person born in the village.

Nitrogen and Sulfur Metabolism in Microalgae and Plants: 50 Years of Research

José M. Vega

To my wife Rosario Pásaro

Contents

Communicated by Prof. Francisco M. Cánovas

J. M. Vega (✉)
Department of Vegetal Biochemistry and Molecular Biology, Faculty of Chemistry, University of Sevilla, Sevilla, Spain
e-mail: jmvega@us.es

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Abstract Microalgae and higher plants are primary producers which convert solar energy into chemical energy associated with biomass, which is used by the other organisms as life support. The knowledge and improvement of mineral nutrition of microalgae and plants are very important in order to obtain a good productivity, even in low fertile soils. This review describes, in chronological order, the research topics in which the author has been involved in the past 50 years, which are mostly connected with the nitrogen and sulfur metabolisms in bacteria, fungi, microalgae, and higher plants, with emphasis on the microalgal model *Chlamydomonas reinhardtii* and the plant models *Arabidopsis thaliana* and *Lotus japonicus*. It is described the outstanding contribution of the author to each topic described, and the state of art actualized. The data from our studies on the nitrate and sulfate assimilation pathways aid in clarifying plant nutrition mechanisms, indicating that there are certain key substrates which also trigger the regulatory signals, commonly participating in the photosynthetic organisms. In addition, nitrate and sulfate participate in the metabolic pathways required for plant adaptation mechanisms to abiotic stresses encountered commonly in agriculture, such as salt and metal toxicity, drought, temperature, and herbicides.

There are many species of microalgae in the world but only a few of them have been studied in terms of biomass producers as source of foods, biofuels and/or high added value products for aquaculture, functional foods, pharmaceutical, and cosmetic industries. Actually we are involved in the use of extremophilic microalga, such as *Coccomyxa onubensis*, as a natural source of C40 carotenes, such as lutein, and the halophilic bacteria *Haloferax mediterranei* as source of C50 carotenoids, such as bacterioruberin.

Keywords *Arabidopsis thaliana*, Biotechnology of microalgae, *Chlamydomonas reinhardtii*, *Coccomyxa onubensis*, *Lotus japonicus*, Nitrogen metabolism, Sulfur metabolism

1 Nitrate Assimilation Pathway in Microalgae and Plant Leaves

The term "microalgae" used in this work refers to eukaryotic microalgae and prokaryotic (cyanobacteria) organisms that convert the solar energy aerobically, with high efficiency, into chemical energy as biomass. These organisms possess high metabolic plasticity that has allowed them to adapt to the most extreme environments, even to ones in which sustenance of life is tough. In addition, microalgae are able to grow in places that are not used for crop production, and their nutritional requirements are inexpensive and simple, for example sunlight, CO_2, a source of nitrogen, phosphate, sulfur, and trace elements, which are present normally in the water that is used for the culture of microalgae (Forján et al. 2015).

In association with other research groups, we contributed to establish that the nitrate assimilation pathway in the eukaryotic microalgae and plant leaves involves four basic steps: (a) nitrate transport into the green cell, which requires ATP-dependent permease systems, of the NRT2 transporter type, to cross the cell cytoplasmic membrane; (b) nitrate reduction into nitrite in the cytosol of cells, catalyzed by NAD(P)H-nitrate reductase. With an exception of cyanobacteria, the reduction of nitrate to nitrite proceeds with reduced ferredoxin as the electron donor for a reaction catalyzed by ferredoxin-nitrate reductase; (c) nitrite transport into the chloroplast of cells, mediated by a specific permease of the NAR1 family, and its further reduction into ammonium, in a 6-electron reaction, catalyzed by ferredoxin-nitrite reductase; and (d) the incorporation of ammonium into the carbon skeleton of 2-oxoglutarate leading to the production L-glutamate, in a pathway catalyzed by the ATP-dependent glutamine synthetase (GS)-glutamate synthase (GOGAT) cycle.

The incorporation of 1N-nitrate into 1N-amino glutamate requires a minimum of four ATPs and ten electrons, which are supplied by the photosynthetic machinery. In my first contributed paper (Paneque et al. 1969), we demonstrated that the illuminated spinach grana supplemented with ferredoxin, $NADP^+$, and ferredoxin-NADP reductase (EC 1.18.1.2), are able to couple the photolysis of water with the stoichiometric reduction of nitrate into ammonium.

The reviews including most of the studies conducted by our research group in this field have been published earlier (Guerrero et al. 1981; Vega et al. 1991). An interesting phylogenetic study on the nitrogen-assimilating enzymes has been published previously (Inokuchi et al. 2002).

1.1 Nitrate Transport Systems

In *C. reinhardtii* cells growing autotrophically with nitrate or nitrite, as the N-source, ammonium per se strongly inhibits the consumption of nitrate, but not that of nitrite, indicating the possibility of different uptake systems for these N-sources (Florencio and Vega 1983a).

The nitrate/nitrite transport systems in *C. reinhardtii* have been studied in detail by the research group of Emilio Fernández and Aurora Galván, in the University of Córdoba (Spain). A minimum of four nitrate/nitrite transport systems in this microalga are responsible for the entry of nitrate into the cells, and these systems correspond to NRT2 transporter type. Three of them operate efficiently at high CO_2 concentration and are considerably sensitive to ammonium inhibition. Among these three systems, system I (NRT2;1, NAR2) is a bispecific nitrate/nitrite transporter with a high affinity for both nitrate and nitrite ($Ks = 1$ μM), system II (NRT2;2, NAR2) is nitrate specific ($Ks = 10$ μM), and system III (NRT2;3) is nitrite specific ($Ks = 3$ μM). On the other hand, system IV (NRT2;4) functions optimally at a low CO_2 concentration and is not sensitive to ammonium. It is bispecific for nitrate and nitrite ($Ks = 30$ μM). In addition, NAR 1 represents a member from another family, which is required for the specific transport of nitrite inside the chloroplasts (Galván and Fernández 2001).

In the microalga *Tisochrysis lutea*, four genes (*Nrt2*) coding for the putative high-affinity nitrate/nitrite transporters (TINrt2) have been identified, and the deduced TINRT protein sequences exhibited similarities to the NRT2 proteins from the other phyla, such as the land plants and green microalgae (Charrier et al. 2015). The high-affinity nitrate and nitrite uptake occurs in different cyanobacteria either through an ABC-type transporter or through a permease from the major facilitator superfamily (MFS) (Flores et al. 2005).

1.2 NAD(P)H-Nitrate Reductase (NAD(P)H-NR)

The NAD(P)H-NR enzyme (EC 1.7.1.3) catalyzes the following reaction:

$$\mathrm{NAD(P)H} + \mathrm{NO_3}^- + \mathrm{H}^+ \rightarrow \mathrm{NO_2}^- + \mathrm{H_2O} + \mathrm{NAD(P)}^+.$$

In the process of transfer of electrons from NAD(P)H to nitrate, two enzymatic activities occur sequentially during the catalysis; one is the NAD(P)H-diaphorase activity, which catalyzes the NAD(P)H-dependent cytochrome c reduction, and the other is the reduced flavin-dependent activity (FNH_2-nitrate reductase), which is able to stoichiometrically reduce nitrate into nitrite (Losada et al. 1970). At the beginning of my Ph.D. thesis, the attention was focused on the isolation and molecular properties of NADH-nitrate reductase (NADH-NR; EC 1.7.1.1) from the eukaryotic microalga *Chlorella fusca*, as well as on the regulation of the activity of this enzyme. The most interesting conclusions observed in our results were as follows:

(a) Molybdenum is essentially required when growing *C. fusca* cells in a culture medium containing nitrate; however, the metal is not required when using a culture medium containing ammonium as a unique nitrogen source. The role of the metal is to assist the reduction of nitrate into nitrite, particularly during FNH_2-nitrate reductase activity (Vega et al. 1971).

(b) The enzyme NADH-NR from *C. fusca* is an interconvertible enzyme, which is inactivated in vivo by the presence of ammonium and reactivated by its absence. This regulatory effect was observed during the FNH_2-nitrate reductase activity, while no such effect was observed during the NADH-diaphorase activity. The synthesis of NADH-NR enzyme is repressed completely by the presence of ammonium in the culture medium (Losada et al. 1970).
(c) Purified NADH-NR from *C. fusca* may be inactivated in vitro by treating the NADH-reduced enzyme with cyanide, which binds tightly to the nitrate-active site. The original enzyme activity may be restored by oxidizing the inactivated enzyme using ferricyanide (Vega et al. 1972). The inactivation of the purified NAD(P)H-NR obtained from the microalga *Ankistrodesmus braunii* (also known as *Monoraphidium braunii*) occurs in two steps: the first one is one-electron reduction of the enzyme, which probably involves the molybdenum cofactor, and the second step, which is also the rate-limiting step for this process, is the interaction of the reduced enzyme with a nucleophilic agent which is either a superoxide or a cyanide. The reactivation of the inactivated enzyme by cyanide is a process that requires the removal of one electron from the enzyme (de la Rosa et al. 1981a).

The absorption spectrum obtained for the purified NAD(P)H-NR from *M. braunii* revealed the presence of FAD and *b*-type cytochrome, as the prosthetic groups of the enzyme. A chemical analysis of the purified enzyme preparations indicated the presence of four FAD, four cytochrome *b557*, and two molybdenum for each enzyme molecule. The molecule was an oligomeric protein of size 467 kDa and was composed of eight similar subunits, each of which was 58.75 kDa in size. Electron micrographs depicted that the eight subunits were arranged alternately in two planes, and eightfold rotational symmetry was deduced from the highly magnified images processed by optical superimposition (de la Rosa et al. 1981b). Conversely, nitrate reductase from *C. reinhardtii* was a homodimeric protein with subunits of about 100–120 kDa in size, and including each one of the three prosthetic groups, namely, FAD, cytochrome *b*557, and molybdenum cofactor (MoCo) (Sanz-Luque et al. 2015).

The molybdenum cofactor (MoCo), which was observed to be involved in the nitrate reductase activity, was further characterized, and it was observed that it is a coordination complex formed between a pterin and molybdenum (VI) oxide; this complex is known to function in other molybdoenzymes involved in carbon, nitrogen, or sulfur metabolisms occurring in microorganisms, plants, and animals (Mendel 2013).

1.3 Ferredoxin-Nitrate Reductase (Fd-NR)

This enzyme Fd-NR (EC 1.7.7.2) has been specifically found in cyanobacteria and catalyzes the following reaction:

$$NO_3^- + 2\,Fd_{red} + 2H^+ \rightarrow NO_2^- + H_2O + 2Fd_{ox}.$$

A molecule of this enzyme has a size of 80 kDa, and contains [4Fe-4S] cluster and Mo-*bis*-molybdopterin guanine dinucleotide as prosthetic groups. The enzyme is associated with thylakoid membranes and forms a 1:1 complex with ferredoxin, the physiological electron donor. This complex is electrostatically stabilized through interactions between the lysine and arginine residues of nitrate reductase and the negatively charged residues of ferredoxin (Flores et al. 2005).

1.4 Ferredoxin-Nitrite Reductase (Fd-NiR)

The enzyme Fd-NiR (EC1.7.7.1) catalyzes the following reaction:

$$NO_2^- + 6Fd_{red} + 8H^+ \rightarrow NH_4^+ + 2H_2O + 6Fd_{ox}.$$

Evidence indicates that siroheme is a unique group present in the unusual 6-electron reductions catalyzed by sulfite reductases (Siegel et al. 1982). Siroheme is a complex of iron (II) with sirohydrochlorin, a class of hydroporphyrins consisting of eight carboxylic acid side chains (Murphy et al. 1973). We purified the NADPH-nitrite reductase (EC 1.7.1.4) obtained from *Neurospora crassa*, an enzyme that catalyzes the six-electrons reduction of nitrite into ammonium, and identified the presence of FAD and siroheme as the prosthetic groups of the molecule of this enzyme (Vega et al. 1975).

Prof. Henry Kamin (Masters 1989) at Duke University, North Carolina, was interested in the identification of prosthetic groups of Fd-NiR from spinach, in order to evaluate if siroheme was present in this enzyme as well. We purified the enzyme obtained from the spinach leaves, and identified the presence of one siroheme and one tetranuclear iron-sulfur cluster [4Fe-4S] as prosthetic group per molecule of size 61 kDa, which function in coordination during the 6-electron reduction of nitrite into ammonium (Vega and Kamin 1977; Lancaster et al. 1979; Vega et al. 1980). Later, we confirmed the presence of one siroheme in the Fd-NiR molecule from the model microalga *C. reinhardtii* (Romero et al. 1987), as well as in the enzyme from *M. braunii* (Vigara et al. 2002). Purified Fd-NiR from cyanobacteria also appears to contain siroheme and the iron-sulfur cluster as prosthetic groups, as observed in the cyanobacteria *Anabaena* sp. (Méndez and Vega 1981; Curdt et al. 2000), and *Phormidium laminosum* (Arizmendi and Serra 1990). The nitrate reducing enzymes from cyanobacteria have been substantially reviewed by Flores et al. (2005).

We established the formation of a 1:1 functional electrostatic complex (Fd:NiR) between ferredoxin and the nitrite reductase from *C. reinhardtii*, and identified two negative surface regions in Fd that were involved in its binding with NiR, namely, the Glu91/Glu92 and the Asp25/Glu28/Glu29 regions (García-Sánchez et al. 1997,

2000). An interesting in silico docking model for the1:1 Fd:NiR complex was proposed by Hirasawa et al. (2009) using the technique of site-directed mutagenesis in the putative amino acid residues involved in both the proteins. These Fd residues are also essential for the formation of a functional 1:1 complex (Fd:SiR) required for the intermolecular electron transfer between Fd and sulfite reductase (SiR) from maize leaves (Saitoh et al. 2006).

Furthermore, there has been a significant progress in the area concerning the crystal structure and reaction mechanism involved in spinach NiR. In silico docking model studies have suggested that the spinach Fd residues Phe63, Glu92, and Glu93 (which correspond to Phe65, Glu92, and Glu93 in *Anabaena* Fd) are supposed to be involved in the binding with NiR (Hirasawa et al. 2009). Using NMR spectroscopy, Sakakibara et al. (2012) studied the Fd:NiR interaction using proteins from cyanobacteria and identified three negative regions in Fd, Tyr25 to Leu37, Ser61 to Gln70, and Glu95 to Leu97, which were involved in the interaction with NiR. The spinach Fd:NiR complex is stabilized by the interaction of Ser43 and Glu93 in Fd with Lys80, Lys83, and Lys100 in NiR, by a salt bridge between Asp60 in Fd and Arg504 in NiR, and by a H-bond between Ser43 in Fd and Lys100 in NiR (Swamy et al. 2005; Hase et al. 2006).

Spectroscopic evidence has suggested that the prosthetic groups in NiR are functionally coupled, probably through the sharing of a sulfur ligand between the siroheme iron and the [4Fe-4S] cluster (Wilkerson et al. 1983). In the Fd:NiR 1:1 complex established with reduced Fd, the six-electrons required for the reduction of nitrite into ammonium flow one-to-one through the enzyme, and it is probable that NiR is first reduced by one electron and then the Fe^{2+}-siroheme binds to the nitrite rapidly. The delivery of a second electron from the reduced ferredoxin produces a well-characterized Fe^{2+}-siroheme/NO adduct as a reaction intermediate, which has already been observed in the EPR studies conducted a few years ago (Aparicio et al. 1975; Lancaster et al. 1979; Kurtnetsova et al. 2004). Another chemical species involved as a possible intermediate in the catalytic cycle of NiR is hydroxylamine (Hase et al. 2006; Hirasawa et al. 2010).

2 Ammonium Assimilation and Re-assimilation in Microalgae and Plant Leaves

Ammonium may be present in the microalgae obtained from different sources, such as those obtained directly from the environment, from the nitrate reduction, from nitrogen fixation (in certain cyanobacteria), photorespiration, protein degradation, and from nitrogen-transport compounds, particularly asparagine, which produces ammonium when it is broken down into aspartate plus ammonium under the catalytic action of asparaginase. In addition, ammonium is also formed in plants and microalgae through the first step of the biosynthetic pathway of secondary metabolites, phenolic compounds. This step is catalyzed by the enzyme

phenylalanine-ammonia-lyase (PAL, EC 4.3.1.5.), according to the following reaction:

$$\text{Phe} \rightarrow \textit{trans}\text{-Cinnamic acid} + NH_4{}^+.$$

This enzyme is very active when plant and microalgae are submitted to abiotic or biotic stress (Mrázová et al. 2017).

Photorespiration is a pathway in C3 plants and microalgae for the recovery of the glycolate formed during the oxidase activity of Ribulose-*bis*-phosphate carboxylase (RUBISCO; EC 4.1.1.39):

$$\text{Ribulose-}\textit{bis}\text{-phosphate} + O_2 \rightarrow \text{3-Phosphoglycerate} + \text{Phosphoglycolate}.$$

This pathway is an energy-dependent O_2-consuming process which involves three organelles, chloroplast, peroxisome, and mitochondria, and releases CO_2 and eventually ammonium, which is produced in the mitochondria under the catalytic action of serine-hydroxymethyltransferase (EC: 2.1.2.1) through the following reaction (Maurino and Peterhansel 2010):

$$2\text{Gly} + NAD^+ + H_2O \rightarrow \text{Ser} + CO_2 + NH_4{}^+ + \text{NADH} + H^+.$$

This ammonium is either recycled in the mitochondria, or is transported to the cytosol and chloroplast, where it is incorporated into 2-oxoglutarate under the catalytic action in the GS/GOGAT cycle (Inokuchi et al. 2002; Pérez-Delgado et al. 2014). Photorespiration is essential for the growth of microalgae and plants, and is favored by low CO_2 and light. The identification of plant-like photosynthetic and photorespiratory genes in cyanobacteria indicated that these genes were endosymbiotically conveyed to the eukaryotic oxygenic phototrophs from the ancient cyanobacteria (Kern et al. 2011).

We performed various studies on the primary assimilation of ammonium (from nitrate reduction) through by the sequential action of glutamine synthetase/glutamate synthase (GS-GOGAT) cycle in *C. reinhardtii* as well as through the putative alternative pathway the amination of 2-oxoglutarate catalyzed by NAD(P)H-glutamate dehydrogenase [NAD(P)H-GDH; (Vega et al. 1991)]. When our studies commenced, the existing knowledge regarding these enzymes in microalgae was scarce. Muro-Pastor et al. (2005) reviewed ammonium assimilation in cyanobacteria, and established that subsequent to its transport through specific permeases, ammonium is incorporated into carbon skeletons through the GS/GOGAT cycle.

The molecular and functional properties of these enzymes are as follows.

2.1 Glutamine Synthetase (GS)

GS (EC 6.3.1.2) is a ubiquitous enzyme present in all the organisms in the form of three different types of proteins: dodecameric GS-I, which is present mostly in prokaryotes, octameric or dodecameric GS-II, which is present in eukaryotes, and the hexameric GS-III, which is also present in prokaryotes (Betti et al. 2012). GS catalyzes the incorporation of ammonium into the carbon skeleton of L-glutamate through the following reaction:

$$\text{Glu} + \text{NH}_4^+ + \text{ATP} \rightarrow \text{Gln} + \text{ADP} + \text{Pi}.$$

Two isoforms of glutamine synthetase that have been isolated from *C. reinhardtii* grow autotrophically in a nutrient medium containing nitrate apparently functioning in the cytosol (GS1) and chloroplasts (GS2) (Florencio and Vega 1983b). This is logical because the source of ammonium may have diverse origins, and the microalgae are required to possess the ability to incorporate it into organic molecules in any region of the cell. On the basis of the occurrence of GS isoforms, microalgae have been classified into three groups: (a) the ones containing only cytosolic GS1; (b) the ones containing only chloroplastic GS2; and (c) a third group characterized by containing both the isoenzymes (Casselton et al. 1986). The green alga *M. braunii* contains only one isoform of GS (García-Fernández et al. 1994), while *Haematococcus pluvialis*, a microalga that is used widely in biotechnology and has high economic potential, contains two distinct isoforms of GS (Reinecke et al. 2016). GS is widely distributed in plants and occurs in two major forms, one (GS2) in the chloroplast and other (GS1) in the cytosol. However, the pattern of distribution of GS in the roots, leaves, and specialized tissues and organs has been observed to be highly sophisticated (Miflin and Habash 2002).

On the other hand, two important metabolic pathways are involved in the intracellular release of ammonium in *L. japonicus*; one is the photorespiratory metabolism, and the other is the asparagine breakdown mediated by asparaginase enzyme. Re-assimilation of the intracellular ammonium is crucial for the normal growth and development of the plant. A photorespiratory mutant of *L. japonicus* that was deficient in plastidic glutamine synthetase (GS2) exhibited ammonium accumulation in the plant, and indicated the existence of a coordinated regulation of genes involved in the photorespiratory metabolism (Betti et al. 2014).

2.2 Ferredoxin-Glutamate Synthase (Fd-GOGAT)

Fd-GOGAT (EC 1.4.7.1) catalyzes the formation of two molecules of L-glutamate, one of which would supply the acceptor for a new ammonium molecule in order to maintain the GS/GOGAT cycle, and the other glutamate molecule may assist in

continuing the nitrogen metabolism in the microalga and plant. The reaction catalyzed by Fd-GOGAT is given below:

$$\text{2-oxoglutarate} + \text{Gln} + 2\ \text{Fd}_{\text{red}} + 2\ \text{H}^{+} \rightarrow 2\ \text{Glu} + 2\text{Fd}_{\text{ox}} + \text{H}_2\text{O}.$$

All the photosynthetic organisms, higher plants, and microalgae contain this enzyme in the chloroplasts of eukaryotic organisms or associated with the photosynthetic machinery in the prokaryotic organisms. An important achievement of our research group was the isolation and purification of two enzymes with glutamate synthase activity from the *C. reinhardtii* extracts, one of which was dependent on reduced ferredoxin as its electron donor, and the other was specific for NADH. We were the first ones to report the purification to the electrophoretic homogeneity of Fd-GOGAT, and to demonstrate through chemical analysis and EPR studies the presence of flavin nucleotides, one FAD, one FMN, and one iron sulfur cluster of the [3Fe-4S] type per enzyme molecule of 146 kDa size (Galván et al. 1984; Márquez et al. 1986b). Other authors have indicated that this enzyme is composed of a single polypeptide chain of size165 kDa which contains FMN and [3Fe-4S] iron-sulfur cluster as its prosthetic groups (Suzuki and Knaff 2005; Varoni et al. 2005). Purified Fd-GOGAT may also catalyze the Fd-dependent reduction of 2-oxoglutarate, in the absence of glutamine (Márquez et al. 1986b), which is compatible with the glutaminase activity and that is the other activity associated with this enzyme complex (van den Heuvel et al. 2004). Fd-GOGAT is present in all the cyanobacteria studied and the enzyme from *Synecchocystis* PCC6803 has been well characterized (Ravasio et al. 2002).

In the cross-linking studies we established that Fd and GOGAT proteins from *C. reinhardtii* were able to form two independent covalent complexes with 1:1 and 2:1 Fd:GOGAT stoichiometry, and that the Glu92 in ferredoxin was involved directly in the stabilization of these complexes (García-Sánchez et al. 1997). Similar data were obtained in the X-ray crystallographic studies of the enzyme, in which both the complexes could be formed depending on the relative proportion of these proteins in the mixture. The E94 residue of *Anabaena* 7120 Fd (=E92 of the *Synechocystis* 6803 Fd) was observed to be necessary for the establishment of functional 1:1 enzyme:substrate complex, as well as for an efficient electron transfer from reduced Fd to GOGAT (Schmitz et al. 1996). With the use of X-ray crystallography, it was identified that the GOGAT enzyme contained an amidotransferase domain, where ammonia was formed through the glutaminase activity associated with this enzyme, thereby producing the first glutamate molecule, following which ammonia was channeled through an intramolecular tunnel to the FMN-binding domain, which was able to bind two molecules of reduced ferredoxin in order to catalyze the reductive amination of 2-oxoglutarate to form the second molecule of glutamate (van den Heuvel et al. 2004).

The ferredoxin-dependent enzymes, glutamate synthase and nitrite reductase, obtained from *C. reinhardtii*, contained a similar structural domain for binding ferredoxin and forming the functional enzyme:substrate complex (García-Sánchez

et al. 2000). The other colleagues identified two important sequence regions in NiR that were apparently similar to those regions in the ferredoxin-NADP reductase that contained amino acids involved in ferredoxin binding; this also suggested the presence of a similar specific domain for binding ferredoxin in these enzymes (Zanetti et al. 1988; Curdt et al. 2000). This appears to be a general characteristic of the ferredoxin-dependent enzymes, such as nitrate reductase from cyanobacteria, sulfite reductase, and thioredoxin-reductase (Hase et al. 2006). Ferredoxins are able to distribute electrons probably originated either from photosynthesis, fermentation, and other reductant-generating pathways, to specific redox enzymes in different organisms. In *C. reinhardtii,* six different ferredoxins have been identified, and the third ferredoxin appears to be involved in nitrogen assimilation (Peden et al. 2013).

2.3 NADH-Glutamate Synthase (NADH-GOGAT)

The NADH-GOGAT (1.4.1.14) consists of a single polypeptide chain (200 kDa) in which two different activities may be measured separately. One is the NADH-diaphorase activity, in which ferricyanide is used as an electron acceptor from NADH, and the other is the reduced methyl viologen-glutamate synthase activity, in which glutamate formation is catalyzed from glutamine and 2-oxoglutarate using reduced methyl viologen as the electron donor (Márquez et al. 1984; van den Heuvel et al. 2004).

This enzyme catalyzes the following reaction:

$$\text{Gln} + \text{2-oxoglutarate} + \text{NADH} + \text{H}^+ \rightarrow 2\ \text{Glu} + \text{NAD}^+ + \text{H}_2\text{O}.$$

In further studies, we suggested that the physiological role of this NADH-GOGAT in *C. reinhardtii* could be the assimilation of ammonium under darkness and/or the recycling of the ammonium released from protein degradation during the low availability of nitrogen (Márquez et al. 1986a). This enzyme is associated with the non-photosynthetic tissues in plants (van den Heuvel et al. 2004). NADH-GOGAT is present simultaneously with Fd-GOGAT in the plants (Miflin and Habash 2002) and eukaryotic microalgae (Márquez et al. 1984). However, this enzyme has been reported only in a few cyanobacteria (Muro-Pastor et al. 2005).

2.4 NAD(P)H-Glutamate Dehydrogenase (NAD(P)H-GDH)

In *Chlorella sorokiniana*, the NADH-GDH enzyme (EC 1.4.1.2) is located in mitochondria, and the NADPH-GDH enzyme (EC 1.4.1.4) is located in chloroplasts (Bascomb and Schmidt 1987). *C. reinhardtii* contains three GDH isoenzymes, one mitochondrial NADH-GOGAT that supports the formation of 2-oxoglutarate in

order to maintain the Krebs's cycle, and the other two NADPH-GDH isoenzymes are presumably distributed in cytosol and chloroplast, which are the main parts of the cells that are putatively involved in glutamate biosynthesis (Moyano et al. 1992). There is a possibility that L-glutamate is also synthesized inside the microalgal glyoxysome, particularly when the cells are grown heterotrophically in a medium containing acetate as a unique carbon source (Pérez-García et al. 2011). Although the deamination of glutamate to form ammonia plus 2-oxoglutarate appears to be the role of NADPH-GDH activity in plants (Forde and Lea 2007) and microalgae, this activity may also be involved in L-glutamate synthesis, particularly when the external concentration of ammonium is high. On the other hand, the alternative ammonium-assimilating GS/GOGAT cycle functions when the ammonium concentration is low. The disadvantage of the second pathway for L-glutamate biosynthesis is its additional energy requirement (Hudson and Daniel 1993).

Under abiotic stresses, such as Cd-toxicity, paraquat, high salinity, and/or high presence of ammonium, in the culture medium of *C. reinhardtii*, the amination activity of the NAD(P)H-GDH isoenzymes for producing L-glutamate manifested, indicating that these isoenzymes have a role in the adaptive mechanisms of the microalga in response to the environmental conditions (Domínguez et al. 2003). In the model legume *Lotus japonicus*, we studied the main genes involved in the assimilatory process for ammonium as well as their levels of expression, using qRT-PCR for these genes in different tissues of the plant (Pérez-Delgado et al. 2014).

Plants and microalgae contain genes for GS/GOGAT cycle and GDH enzymes, which have evolved through different evolutionary processes suggesting that the two pathways will play different physiological role in these organisms.

3 Source of 2-Oxoglutarate for Ammonium Assimilation: Isocitrate Metabolism in Microalgae

Glutamate metabolism functions through transaminase reactions that eventually produce 2-oxoglutarate as the reaction product, which may, in turn, feed the GS-GOGAT cycle, thereby sustaining the rate of glutamate synthesis in the photosynthetic organisms. However, if microalgae or plants demand a higher rate of glutamate biosynthesis, isocitrate dehydrogenase (IDH) isoenzymes may supply the new 2-oxoglutarate molecules by catalyzing the oxidative decarboxylation of isocitrate:

$$\text{Isocitrate} + \text{NAD(P)}^{+} + \text{H}_2\text{O} \rightarrow \text{2-oxoglutarate} + \text{CO}_2 + \text{NADPH} + \text{H}^{+}.$$

According to this reaction, IDH contributes to the modification of intracellular C/N ratio in green cells. There are different types of IDH isoenzymes, which are grouped into two classes on the basis of coenzyme specificity; one of these classes is

the mitochondrial NAD-IDH, which is involved in the Krebs's cycle, and the activity of this isoenzyme is modulated by an extensive allosteric regulation. This regulation, in addition to the presence of a 2-oxoglutarate transporter in the inner membrane of mitochondria, allows NAD-IDH to play an essential role in modulating the carbon flux to nitrogen metabolism in the cytosol and the chloroplasts (Lancien et al. 1998). The other class of IDH isoenzyme is NADP-IDH, which is present in the cytosol, chloroplasts, and peroxisomes, the regions where glutamate biosynthesis would eventually be demanded (Abiko et al. 2005; Leterrier et al. 2016).

We studied the isocitrate metabolism in *C. reinhardtii* to better understand the connection between carbon and nitrogen metabolisms. When the microalga was grown autotrophically in a culture medium containing nitrate, three IDH isoenzymes were identified in it. The first one was mitochondrial NAD-IDH, which was probably involved in the function of Krebs's cycle (Martínez-Rivas and Vega 1998), and two were NADP-IDH isoenzymes, one of which was NADP-IDH1, located in the cytosol and the other was the chloroplastic NADP-IDH2. This distribution of IDHs in the microalga is compatible with the two GS-GOGAT systems operating in the cytosol and chloroplast of *C. reinhardtii* (Florencio and Vega 1983b).

3.1 NAD-Isocitrate Dehydrogenase (NAD-IDH)

The NAD-IDH (EC 1.1.1.14) from *C. reinhardtii* is composed of eight similar subunits of size 45 kDa each (Martínez-Rivas and Vega 1998), while plant enzyme was heteromeric and three subunits existed in tobacco (Lancien et al. 1998). The cytosolic NADP-IDH1 isoenzyme has been purified to homogeneity, and it is a dimer of two similar subunits with an apparent molecular weight of 40 kDa each, and the physicochemical and kinetic properties are similar to the corresponding enzymes in higher plants (Martínez-Rivas et al. 1996).

3.2 NADP-Isocitrate Dehydrogenase (NADP-IDH)

The chloroplastic NADP-IDH2 (EC 1.1.1.42) isoenzyme from *C. reinhardtii* was a dimer of 45 kDa subunits. Antibodies generated against a recombinant tobacco-cytosolic NADP-IDH cross-reacted strongly with the NADP-IDH1, and weakly with the NADP-IDH2 (Martínez-Rivas and Vega 2003). Small changes in the cytosolic NADP-IDH1 activity produce significant alterations in the activities of the enzymes involved in primary nitrate assimilation as well as in the synthesis of 2-oxoglutarate-derived amino acids in tomato plant (Sulpice et al. 2010).

In *Arabidopsis*, a peroxysomal NADP-IDH has been identified; although the role of this IDH in plant metabolism appears to be minor because it is dispensable for the plant growth and redox homeostasis, this enzyme could be involved in plant responses to the environmental challenges such as a biotic stress (Mhamdi and

Noctor 2015). The peroxisome is an organelle involved in the photorespiratory pathway in chloroplast and mitochondria of the C3 plants and microalgae, in which ammonia is released within the mitochondria during the glycine decarboxylation step and is re-assimilated by the chloroplastic/cytosolic GS/GOGAT systems (Hagemann et al. 2016).

Only one type of NADP-IDH has been identified in cyanobacteria, and it is strictly dependent on the divalent cations, such as Mg^{2+} or Mn^{2+}. The enzyme from *Anabaena* PCC 7120 has been purified, and it is composed of two identical subunits (MW = 57 kDa). The corresponding gene *icd* has been cloned, and its sequence presented significant similarity with the sequence of the corresponding gene from *E. coli* (Muro-Pastor and Florencio 1994).

3.3 Isocitrate Lyase (ICL)

When *C. reinhardtii* cells were grown heterotrophically in a culture medium containing acetate, as a unique carbon source, an isocitrate lyase (ICL; 4.1.3.1) activity was induced, while the NADH-IDH activity decreased significantly; this observation was consistent with the functioning of the glyoxylate cycle and with the decreasing level of the Krebs's cycle in the mitochondria (Martínez-Rivas and Vega 2003). ICL catalyzes the following reaction:

$$\text{Isocitrate} \rightarrow \text{Succinate} + \text{Glyoxylate}.$$

This reaction allows glucose biosynthesis from acetyl-CoA and eventually from fatty acids. In general, microalgae growing heterotrophically in acetate induces isocitrate lyase and malate synthetase (EC 2.3.3.9), while light and glucose suppress the formation of isocitrate lyase in the microalgae (Pérez-García et al. 2011).

4 Regulation of Nitrate Assimilation

4.1 Eukaryotic Microalgae

We studied the regulation of nitrate assimilation in *C. reinhardtii* that was grown autotrophically with nitrate as unique N-source, and we contribute the following highlights:

(a) The first step in the regulation of this pathway is performed with the entrance of nitrate into the cells. Although ammonium is a powerful antagonist of nitrate uptake, it must be assimilated by the microalga to exhibit its negative regulatory effect (Florencio and Vega 1983b). The entrance of nitrate into the cells promotes the synthesis of nitrate reductase activity (Florencio and Vega 1982). The

activity of the high-affinity nitrate transport system I (NRT2;1, NAR2) is responsible for the efficient signaling of the nitrate assimilation genes in *C. reinhardtii* (Rexach et al. 2002). The availability of a carbon source and a healthy photosynthetic process modulates the nitrate consumption rate in the microalga.

(b) NAD(P)H-nitrate reductase is an adaptive enzyme in *C. reinhardtii*, which exhibits the highest level of its activity in the cells growing with nitrate in the culture medium, while the activity decreases dramatically in the absence of nitrate, and/or in the presence of ammonium (Florencio and Vega 1983b). Our data obtained from microalgae indicate the existence of an in vivo short-term nitrate reductase activity regulation (Vega et al. 1972; Guerrero et al. 1981).

Nitrate serves both as a nutrient and as a positive signal to activate the nitrate assimilation pathway in *C. reinhardtii*, while nitric oxide (NO) serves as a negative signal by inhibiting the expression of the high-affinity nitrate/nitrite transporters and the nitrate reductase activity (Sanz-Luque et al. 2015). NIT2 is the key transcription factor for the nitrate-dependent activation of several genes for the handling of nitrate assimilation in the microalga. It is important to mention that nitrate can be in vivo transformed into nitric oxide, in a process shared with the molybdoenzyme ARC; nitric oxide may then be converted into nitrate, in a process mediated by nitrate reductase and truncated hemoglobin THB1 (Calatrava et al. 2017).

Nitric oxide signaling regulates various physiological processes in both animals and plants. In animals, NO synthesis is catalyzed by the NO-synthase enzyme (NOS). NOS-like sequences have been identified in the genome of 15 among the 265 algal species analyzed in a study (Jeandroz et al. 2016). On the other hand, *C. reinhardtii* is apparently deficient in such sequences.

In *C. reinhardtii,* the level of cytosolic, but not chloroplastic, GS transcript is repressed by ammonium and induced by nitrate. In *Chlorella sorokiniana* seven ammonia-inducible NADP-GDH chloroplastic isoenzymes have been identified (Inokuchi et al. 2002).

4.2 Cyanobacteria

The regulation of inorganic nitrogen assimilation by the cyanobacteria has been reviewed extensively (Flores et al. 2005; Muro-Pastor et al. 2005; Ohashi et al. 2011). Our contribution in this field has been limited. We reported that ferredoxin-nitrite reductase from the cyanobacterium *Anabaena* sp. 7119 was an adaptive enzyme, the synthesis of which was nutritionally repressed by the presence of ammonium in the culture medium (Méndez et al. 1981).

Nitrate assimilation genes are commonly observed to form an operon with the structure *nir* [nitrite reductase-permease gene(s)-*narB* (nitrate reductase)] which is transcribed from a complex promoter that includes binding sites for NtcA, a global nitrogen-control regulator, and sites for NtcB, a pathway-specific regulator (Flores

et al. 2005). The major intracellular signal for the regulation of nitrate assimilation in cyanobacteria is 2-oxoglutarate (2-OG), the intracellular concentration of which changes with the cellular C/N balance. In addition, Pii, a signal transduction protein, inhibits the ABC-type nitrate transporter and nitrate reductase, under low level of 2-OG. Pii was recently shown to negatively regulate the activity of NtcA by binding to PipX, a small coactivator protein of NtcA (Ohashi et al. 2011).

In N-limited cells, the intracellular accumulation of 2-OG activates the transcription factor NtcA for inducing the transcription of the nitrate assimilation genes, and the expression of the genes encoding GS and IDH. In addition, the 2-OG binds to Pii and prevents the protein from inhibiting nitrate assimilation. In all the cyanobacteria that have been studied, the presence of ammonium in the culture medium immediately promoted the inhibition of nitrate uptake, as well as the repression of the transcription of the operon *nirAnrtABCDnarB*. However, these effects require the assimilation of ammonium, thus indicating that it is not an effect of ammonium per se (Flores et al. 2005).

5 Metabolism of L-Asparagine in *Lotus japonicus*

In legumes, L-asparagine or ureides (allantoin and allantoic acid) are crucial nitrogen-storing molecules and transporters between the organs and tissues, due to their high N/C ratio and stability. In the model legume *L. japonicus*, asparagine supports the vast majority of reduced nitrogen stored and translocated between different organs and tissues (Betti et al. 2014; García-Calderón et al. 2017), and the intracellular concentration of asparagine is dependent on a balance between its biosynthesis and degradation. The main enzymes involved in the asparagine metabolism are asparagine synthetase, asparaginase, and serine-glyoxylate aminotransferase.

5.1 Asparagine Synthetase (ASN)

This enzyme (EC 6.3.5.4) converts aspartate plus glutamine into asparagine plus glutamate, in an ATP-dependent reaction, which is given below:

$$\mathrm{Asp} + \mathrm{Gln} + \mathrm{ATP} + \mathrm{H_2O} \rightarrow \mathrm{Asn} + \mathrm{Glu} + \mathrm{AMP} + \mathrm{PP_i}.$$

ASN is widely distributed in all the living organisms, particularly in humans where it has been observed to be overexpressed in the cancer cells, such as the acute lymphoblastic leukemia. Conversely, ASN deficiency is associated with neurological disorders, such as intellectual disability, microcephaly, and progressive brain atrophy (Lomelino et al. 2017).

In *L. japonicus*, ASN is encoded by three genes, *LjASN1* being the main gene responsible for asparagine synthesis in mature leaves, while this function is reserved for *LjASN3* in the young leaves. All the three genes encoding for ASN, particularly *LjASN1* and *LjASN2*, are expressed in the roots. The differential expression of the ASN genes in *L. japonicus* highlights the relevance of each gene in the different plant regions (García-Calderón et al. 2017). In *A. thaliana*, the *ASN2* gene was expressed the most in the vegetative leaves, and it was proposed that this gene was essential for nitrogen assimilation, distribution, and remobilization (via phloem) within the plant (Gaufichon et al. 2013).

The information available regarding asparagine synthetase in microalgae is scarce.

5.2 L-Asparaginase (NSE)

NSE (EC 3.5.1.1) catalyzes an important reaction that produces ammonium inside the cells:

$$\mathrm{Asn} + \mathrm{H_2O} \rightarrow \mathrm{Asp} + \mathrm{NH_4}^{+}.$$

This ammonium must be re-assimilated by the plants or microalgae in order to have a healthy growth. Plant asparaginases belong to the superfamily of N-terminal nucleophilic hydrolases (Ntn-hydrolases) and are composed of two different subunits α (20 kDa) and β (17 kDa) disposed of as $(\alpha\beta)_2$ quaternary structure. There are two different subgroups of these enzymes based on their K^+-dependence to perform their activity. In *L. japonicus*, we identified two K^+-dependent (LjNSE1 and LjNSE3) and one K^+-independent (LjNSE2) isoenzymes. Major differences were observed in the catalytic efficiencies of these groups as K^+ apparently induces an active conformation of the enzyme, which increases the V_{max} tenfold and significantly decreases the *K*m value (from 17.9 to 7.2 mM) of the enzyme for asparagine. On the other hand, the K^+-independent LjNSE2 (*K*m for Asn = 30 mM) exhibited β-aspartyl-hydrolase activity (*K*m = 0.54 mM, for β-Asp-His), and therefore appears to be involved in the protein reparation by eliminating this anomalous bond. This activity is not exhibited by the other two isoenzymes, LjNSE1 and LjNSE3.

We purified and characterized the most active isoform of this enzyme, the K^+-dependent LjNSE1 isoform. Its optimum activity was obtained at 5–50 mM concentration of K^+, and Na^+ and Rb^+ were able to substitute K^+ in activating the isoenzyme to some extent. In addition, K^+ was able to stabilize LjNSE1 against thermal inactivation. Using protein homology modeling and the molecular dynamics studies, complemented with site-directed mutagenesis, we revealed the significance of E248, D285, and E286 amino acid residues for the catalytic activity and the K^+-dependence mechanisms of LjNSE 1 (Credali et al. 2011). In addition, Ser118, which is the last residue of the activation loop (residues Val111-Ser118), was

observed to be involved in the K^+-activation mechanism of the NSE from *Phaseolus vulgaris* (Beiger et al. 2014).

L. japonicus contains three asparaginase genes, *LjNSE1*, *LjNSE2*, and *LjNSE3.* Two of these genes, 1 and 3, encode a K^+-dependent enzyme and *LjNSE2* encodes a K^+-independent NSE. All the three genes, particularly the *LjNSE1* gene, are highly expressed in different tissues of the plant. However, *LjNSE 2* is the most highly expressed in the nodule, suggesting an important role of this isoenzyme in this region. We used reverse genetics to identify the phenotypes produced by the deficiency of LjNSE1 isoenzyme, and obtained four different mutants, two of which affected the structure and function of the *LjNSE1* gene, causing intracellular asparagine accumulation. The LjNSE1 isoenzyme appeared to be involved in the plant growth and seed production (Credali et al. 2013).

Besides its role in the nitrogen metabolism of several organisms, asparaginase has been and remains one of the most studied therapeutic enzymes. The amino acid asparagine is a nutritional requirement for normal as well as tumor cells, and asparaginase has been observed to be one of the best antileukemic agents. Therefore, microalgae have been considered a source of this enzyme, for its purification, and clinical uses (Yadav et al. 2014), and other applications in the food technology sectors. Asparaginase activity has been studied in *Chlamydomonas* species (Paul and Cooksey 1981), *Chlorella vulgaris* (Ebrahiminezhad et al. 2014), and *Spirulina maxima* (El Baky and El Baroty 2016).

In the chloroplasts and in the nongreen plastids of plants, aspartate serves as a precursor for the biosynthesis of different amino acids and derived metabolites required for plant growth, reproduction, development, or defense. Aspartate biosynthesis is mediated by an enzyme known as aspartate aminotransferase (EC 2.6.1.1), which catalyzes the reversible transamination between glutamate and oxaloacetate (OAA) in order to generate aspartate and 2-oxoglutarate. The reaction is given below:

$$\text{Glu} + \text{OAA} \rightarrow \text{2-oxoglutarate} + \text{Asp.}$$

Plastids contain two aspartate aminotransferases: the eukaryotic-type and the prokaryotic-type (de la Torre et al. 2014).

5.3 Serine-Glyoxylate Aminotransferase (SGAT)

SGAT (EC 2.6.1.45) is a peroxisomal enzyme, which is involved in the photorespiration in C3 plants and eukaryotic microalgae, and catalyzes the following reaction:

$$\text{L-Serine} + \text{Glyoxylate} \rightarrow \text{3-Hydroxy-Pyruvate} + \text{Glycine.}$$

In *L. japonicus* we observed two genes, *LjSGAT1* and *LjSGAT2*, coding for the LjSGAT enzymes; the expression of *LjSGAT2* in the leaves was observed to be about 25-fold to 30-fold higher than that of *LjSGAT1*. However, the LjSGAT1 gene was observed to be expressed in the leaves, roots, and the nodules of the plant. We observed important links between asparagine metabolism and the photorespiratory genes in *L. japonicus*, particularly between the expression of *LjNSE* and *LjSGAT* genes. Plants deficient in the *LjNSE1* gene accumulated high levels of asparagine in the leaves, and exhibited significantly diminished expressions of both *LjSGAT1* and *LjSGAT2* genes, which could be an indication of the existence of a close relationship between these pathways (Credali et al. 2011; García-Calderón et al. 2017).

This connection involving the SGAT enzyme and the asparagine metabolism was also observed in *Arabidopsis*, where the SGAT activity may operate with asparagine as the amino donor instead of L-serine. It is suggested that the leaf SGAT activity is required to be dynamically adjusted to ensure the minimal consumption of asparagine, and maintain adequate serine levels (Modde et al. 2017). The aforementioned reaction, which is also catalyzed by SGAT, can be written as:

$$\text{Glyoxylate} + \text{Asn} \rightarrow \text{Glycine} + \text{2-Oxosuccinamate}.$$

6 Sulfate Assimilation in Microalgae and Plant Leaves

Sulfur is considered an essential macronutrient for the microalgae and plants because it is present in about 0.3–0.5% of the dry matter, which is a considerable low level as compared to that of nitrogen (about 5–10%) and carbon (about 50%). Elementary analysis of *C. reinhardtii* dry matter exhibited 50.33 $\pm$ 0.15% of carbon, 10.35 $\pm$ 0.10% of nitrogen, 7.81 $\pm$ 0.08% of hydrogen, and 0.68 $\pm$ 0.04% of sulfur (Mosulén et al. 2003). However, sulfur is structurally transcendent due to its presence in two proteinogenic amino acids, L-cysteine and L-methionine, and several primary metabolites, such as glutathione or lipoic acid, and secondary metabolites, such as glucosinolates, among others. Sulfur compounds play a critical role in the metabolism as well as in the protection of the photosynthetic organisms against several kinds of environmental or metabolic stresses (Saito 2000). Sulfate assimilation pathway proceeds in microalgae just as in higher plants (Barroso et al. 1997; Giordano et al. 2005) and includes the following steps:

(a) Sulfate transport into the green cells, mediated by a cytoplasmic ATP-dependent permease. In order to be assimilated sulfate should proceed inside the chloroplast through an ABC-type ATP-dependent permease; however, sulfate may be stored into the vacuoles of vegetal cells.
(b) Once inside the chloroplast, sulfate is activated by ATP, in a reaction catalyzed by the ATP-sulfurylase, to form adenosine-5′-phosphosulfate (APS).

(c) The reduction of sulfate to sulfide proceeds through two consecutive reactions. The two-electron reduction of APS to sulfite, which is catalyzed by APS-reductase, and the further six-electron reduction of sulfite to sulfide, which is catalyzed by the ferredoxin-sulfite reductase.
(d) The incorporation of sulfide into the carbon structure of O-acetyl-serine (OAS) to produce L-cysteine catalyzes by the enzymatic complex cysteine synthase.

6.1 Sulfate Transport

Sulfate transport and uptake are similar terms used all along the scientific literature. Sulfate uptake in *C. reinhardtii* is a light-dependent saturable process, mediated by permeases, which exhibits biphasic kinetics and apparently involves two different transporter systems, one with high affinity (Km = 10 μM) and the other with low affinity (Km = 40 μM) for sulfate, and a Vmax value of 180 nmol/h mg Chl. When *C. reinhardtii* cells were pre-incubated for 24 h under S-limited growing conditions, their capacity to absorb sulfate was dramatically increased, registering a Vmax value that was tenfold higher than that in the S-repleted cells (Pérez-Castiñeira et al. 1992). Yildiz et al. (1994) demonstrated that the *C. reinhardtii* cells growing under S-sufficient or S-limited conditions absorb sulfate following monophasic kinetics, indicating that a single transporter system predominates under each of these conditions. However, the S-sufficient cells have a transporter with low affinity for sulfate, and the S-starved cells synthesize a different transporter system, with a high affinity for sulfate, resulting in a tenfold increase in the Vmax compared to that exhibited by the S-sufficient cells. Furthermore, an ABC-type sulfate permease holocomplex has been identified in the chloroplasts envelope of *C. reinhardtii*, which consists of one sulfate-binding protein (Sbp), localized in the exterior region of the chloroplast envelope, and two ATP-binding transmembrane proteins, SulP and SulP2, localized in the chloroplast stroma. All these proteins are encoded by four nuclear genes (Melis and Chen 2005). This is consistent with the idea that sulfate assimilation proceeds inside the chloroplasts (Chen and Melis 2004), although, exceptionally in the photosynthetic organisms, only the microalga *Euglena gracilis* translocates the sulfate-reducing enzymes into mitochondria (Patron et al. 2008).

In the unicellular green alga *C. fusca* sulfate uptake was described as a pH, temperature, and energy dependent process, which follows triphasic kinetics (Biedlingmaier and Schmidt 1989). In *Chlorella elipsoidea* sulfate transporter systems are driven by a proton gradient across the plasma membrane (Matsuda and Colman 1995), and the sulfate uptake by *M. braunii* follows monophasic kinetics according to the typical Michaelis process, with Km = 5.0 μM and Vmax = 30 nmol/h mg Chl (Pérez-Castiñeira et al. 1998). Cyanobacteria, such as *Anacystis nidulans*, contain only a single transporter system for sulfate (Green and Grossman 1988).

Sulfate transporters have been localized in the plasma membrane of the cells of plant roots, functioning as a 3 H^+/1 sulfate symport, and exhibiting either a high or low affinity for the sulfate ions in the plants exhibiting distinct and regulatory

patterns of expression. The high-affinity forms (*K*m for sulfate = 9 μM) are expressed exclusively in the roots, while the lower-affinity form (*K*m = 100 μM) is expressed mainly in the leaves, and also in the roots (Leustek and Saito 1999). Three functional sulfate transporters involved in the uptake and translocation of sulfate have been isolated from *A. thaliana*. The in vivo translocation of sulfate into the chloroplasts must occur because reduction of sulfate occurs inside this organelle (Takahashi et al. 2000).

Sulfate is usually taken from the environment, but when its availability is too short, the *C. reinhardtii* cells, but not the higher plants, induces the sulfur-scavenging system arylsulfatase (EC 3.1.6.1), a periplasmic localized enzyme, which is able to generate sulfate from the extracellular aromatic sulfate esters. An example is given below:

$$\text{Phenol-sulfate} + H_2O \rightarrow \text{Phenol} + \text{sulfate}.$$

Two different arylsulfatase genes designated as *ARS1* and *ARS2* were identified in the *C. reinhardtii* genome (Ravina et al. 2002).

6.2 Activation of Sulfate

Once inside the chloroplast, sulfate should be reduced to sulfide, in an eight-electron transfer pathway. Among the photosynthetic electron donors, the most negative normal potential corresponds to reduced ferredoxin (Fd_{ox}/Fd_{red}, $E'_0 = -420$ mV); however, this value is not large enough to promote the direct 2-electron reduction of sulfate to sulfite (SO_4^{2-}/SO_3^{2-}, $E'_0 = -454$ mV, pH 7, and 25°C). Therefore, the sulfate anion must be activated with ATP prior to its reduction, according to the following reaction:

$$SO_4^{2-} + ATP^{4-} + H^+ \rightarrow APS^{2-} + PPi^{3-}.$$

ATP-sulfurylase (EC 2.7.7.4) from green microalgae is a plastidial enzyme which is encoded by a single gene, with the only exception of the freshwater green microalga *C. reinhardtii*, which contains two isoenzymes that are encoded by two distinct nuclear genes, termed *ATS1* and *ATS2*. According to Prioretti et al. (2014), algal ATP-sulfurylase proteins exhibit a great diversity of isoforms, and a high content of cysteine residues, the positions of which are often conserved.

However, APS must not be accumulated inside the chloroplasts, because it is a potent inhibitor of the enzyme ATP-sulfurylase, and therefore, a second enzyme, APS-kinase (EC 2.7.1.25), should become functional, catalyzing the transformation of APS into 3′-phosphoadenosine-5′-phosphosulfate (PAPS), according to the following reaction:

$$APS^{2-} + ATP^{4-} \rightarrow PAPS^{4-} + ADP^{3-} + H^{+}$$

The physiological function of PAPS is either to serve as a reserve for the active sulfate ready for assimilation or as a donor of the activated sulfate for several sulfation reactions. When required, PAPS produces APS again, by the action of the enzyme 3′(2′),5′-bisphosphate nucleotidase (EC 3.1.3.7), according to the following reaction:

$$PAPS^{4-} + ADP^{3-} + H^{+} \rightarrow APS^{2-} + ATP^{4-}.$$

Both the enzymes ATP-sulfurylase (Yildiz et al. 1996) and APS-kinase (Jender and Schwenn 1984) have been identified in and purified from *C. reinhardtii*.

6.3 The APS Reduction to Sulfite and Sulfide

The first redox reaction of the sulfate assimilation pathway is catalyzed by APS-reductase (EC 1.8.99.2) and proceeds according to the following reaction:

$$APS^{2-} + 2GSH \rightarrow {SO_3}^{2-} + AMP^{2-} + GSSG.$$

In this reaction glutathione (GSH) serves as the two electrons donor (Gao et al. 2000). APS-reductase has been well characterized in marine algae, such as *Tetraselmis* sp. (Gao et al. 2000) and the cyanobacteria (Prioretti et al. 2016). We have identified in *C. reinhardtii* genes encoding for ATP-sulfurylase and APS-reductase (Ravina et al. 2002).

The second reduction which is the six-electron transfer reduction of sulfite to sulfide, catalyzed by ferredoxin-sulfite reductase (Fd-SiR, EC 1.8.7.1), proceeds according to the following reaction:

$${SO_3}^{2-} + 6Fd_{red} + 6H^{+} \rightarrow S^{2-} + 6Fd_{ox} + 3H_2O.$$

Evidence of the presence of Fd-SiR in *C. reinhardtii* has been reported (Godman and Balk 2008).

6.4 Sulfide Incorporation into Carbon Skeletons to Produce L-Cysteine

Sulfide is further incorporated into the carbon skeleton of the activated L-serine, O-acetyl-L-serine (OAS), in order to form L-cysteine. OAS is synthesized through

the catalytic action of enzyme serine-acetyl transferase (SAT, EC 2.1.3.30), according to the following reaction:

$$\text{Serine} + \text{Acetyl-CoA} \rightarrow \text{OAS} + \text{CoA-SH}.$$

O-Acetylserine(thiol)lyase (OAS-TL, EC 2.5.1.47) catalyzes the indicated synthesis of L-cysteine, according to the following reaction:

$$SH_2 + \text{OAS} \rightarrow \text{L-Cysteine} + \text{Acetate} + H^+.$$

L-Cysteine serves as a precursor or donor of reduced sulfur for a diverse range of S-compounds, such as L-methionine, glutathione, and phytochelatins (Takahashi et al. 2011). Both SAT and OAS-TL enzymes are present in association with each other in a complex named cysteine synthase (CSC), which is formed by a 2:1 ratio of OAS-TL homodimer and SAT homohexamer. This complex controls the cellular sulfur homeostasis in plants (Wirtz et al. 2010; Feldman-Salit et al. 2012) and microalgae (Salbitani et al. 2014) through the reversible association of its subunits. While the sulfide production occurs almost exclusively inside the chloroplasts, the L-cysteine biosynthesis occurs in the three main compartments of the photosynthetic cells: chloroplasts, mitochondria, and cytosol. We separated and purified from the *C. reinhardtii* extract three OAS-TL isoenzymes (León et al. 1987; Prieto et al. 1998), which are structurally more similar to the OAS-TL from higher plants than to those from the prokaryotes (León and Vega 1991; Ravina et al. 1999).

Cysteine synthase genes *Sat1Acr* and *Cys1Acr*, which are responsible for the SAT and OAS-TL activity, respectively, were identified in *C. reinhardtii* (Ravina et al. 1999). The gene *Atcys-3A* encodes the cytosolic OAS-TL in *Arabidopsis*, where it is present in the different organs of the mature plants, with its expression being more pronounced in the roots, and declining by about 40–50% in the leaves (Gotor et al. 1997). The *Atcys-3A*, *sat5* (SAT), *gsh1* (ϒ-glutamylcysteine synthase), and *gsh2* (glutathione synthetase) genes were highly expressed in the leaf trichomes of *A. thaliana* and exhibited a total GSH content that was about 300-fold higher than that in the basement cells or epidermal cells, suggesting an important L-cysteine and glutathione biosynthesis in the trichomes (Barroso et al. 1995; Gutiérrez-Alcalá et al. 2000).

6.5 Regulation of Sulfate Assimilation

In microalgae and plants, the biosynthetic pathway for the conversion of sulfate into L-cysteine is regulated at both transcriptional and post-transcriptional level on the basis of availability of sulfate. Plants and freshwater microalgae respond to sulfate deficiency primarily by increasing the sulfate uptake and assimilation capacity, while *C. reinhardtii* is, in addition, capable of inducing arylsulfatases that are required for

the utilization of the alternative sources of sulfur (de Hostos et al. 1988). The marine microalgae which are adapted to grow in water containing high sulfate concentration (25 mM) retained the ability to reprogram their gene expression in response to a decrease in the sulfate concentration (Bochenek et al. 2013).

Sulfate permease and ATP-sulfurylase, the two earliest components of the sulfate assimilation pathway in microalgae and plants, appear to be the important points of regulation for the sulfate assimilation pathway. The transcription of the two genes *ATS1* and *ATS2*, which encode ATP-sulfurylase in *C. reinhardtii*, was strongly stimulated by S-deprivation. The expression of these genes was under the control of *SAC1* gene, which is responsible for the acclimation of the microalga to the S-limited conditions. In addition, a redox regulation has been proposed for the ATP-sulfurylase activity in microalgae (Prioretti et al. 2016). In *Brassica napus* L., the regulation of sulfate uptake and ATP-sulfurylase activity in the roots is related to the level of reduced glutathione (GSH) rather than to the GSH/GSSG ratio, and is independent of the oxidative-stress response generated by the S-limited conditions (Lappartient and Touraine 1997). An interesting regulatory mechanism has been proposed for maize, in which the expressions of the sulfate permease (*ZmST1*) and ATP-sulfurylase (*ZmAS1*) genes increased in both roots and shoots of the seedlings that were grown under S-deprived conditions, and the expressions decreased rapidly when the external sulfate supply was restored. This coordinated response correlated with the depletion of the intracellular L-cysteine level rather than with the sulfate stores (Bolchi et al. 1999). Vuclare et al. (2002) studied the flux control of sulfate assimilation in *A. thaliana*, and concluded that APS-reductase exhibits high susceptibility to the negative control by thiols compared to ATP-sulfurylase.

The cysteine synthase complex controls cellular sulfur homeostasis in photosynthetic organisms. The SAT and OAS-TL activities are dependent on the associations of these enzymes in the cysteine synthase complex. In plants, the catalytic activity of OASTL is decreased when associated in the complex, while the free OAS-TL is the main enzyme that is responsible for the cysteine biosynthesis. The addition of OAS causes the dissociation of the cysteine synthase components, while the addition of sulfide induces complex stability in vitro (Droux et al. 1998).

The steady state of the transcripts and the activity of ATP-sulfurylase, APS-reductase, SAT, and OAS-TL increase when the cells are deprived of sulfate (Ravina et al. 1999, 2002; Takahashi et al. 2011). In *C. reinhardtii* and *A. thaliana*, the OAS-TL activity and the expression of the gene *Atcys-3A* were induced by S-limiting conditions in the presence of sufficient carbon and nitrogen sources in the culture medium for maximal activation (Barroso et al. 1995). Two genes encoding SAT and OAS-TL have been identified in the cyanobacterium *Synechococcus* sp. PCC 7942, the expression of which is stimulated by S-limitation (Nicholson et al. 1995). In general, the expression rate of the aforementioned genes and the activity of the enzymes involved in the sulfate assimilation pathway reached lower levels in S-repleted cells.

In microalgae, a molecular mechanism that integrates all these observations has been proposed. Under the S-limiting conditions, the intracellular levels of OAS

increase and trigger all the activities involved in the sulfate assimilation pathway. On the other hand, under S-sufficient conditions, the intracellular OAS levels decrease, and, in turn, the corresponding levels of cysteine and glutathione increase, which then serve as moderators for the sulfate assimilation pathway. In *Chlorella sorokiniana*, S-deprivation caused a decrease in the intracellular levels of cysteine and glutathione, and an increase in the levels of serine, OAS, and OAS-TL activity (Carfagna et al. 2011). Similar mechanism has been observed in higher plants as well (Barroso et al. 1997).

6.6 *Effect of Abiotic Stress on Cysteine and Glutathione Biosynthesis*

S-containing compounds, such as glutathione (GSH, ®-glutamyl-cysteinyl-glycine) and its analogue homo-glutathione (h-GSH, ®-glutamyl-cysteinyl-β-alanine), phytochelatins [PCs, (®-glutamyl-cysteinyl)$_n$-Gly, where $n = 2–11$], metallothioneins (MTs, Cys rich peptides), and secondary metabolites glucosinolates, are known to be involved in the microalgal adaptation mechanisms for survival during the nutritional or abiotic stresses, such as UV-light, high-PAR light intensity, salinity, drought, temperature, pH, or heavy metal contamination, and this is the reason for the induction of gene transcription and enzyme activity of the sulfate assimilation pathway in the plants in response to abiotic stresses (Anjum et al. 2015). In *C. reinhardtii*, the presence of NaCl (200 mM), Cd^{2+}(0.3 mM), or paraquat (0.2 μM) in the culture medium induces a strong oxidative stress, which in turn inhibits the photosynthetic activity drastically, and as a consequence, the biomass productivity, while the respiratory activity in the microalga remains significantly high in all the cases. Salt induces, by 20-fold, the intracellular catalase activity and the presence of Cd^{2+} in the culture medium stimulates the sulfate uptake rate in the microalga by 40% and it inhibits the nitrate uptake rate in the cells by 20% (Vega et al. 2006).

The intracellular levels of cysteine and glutathione were increased threefold in the *Arabidopsis* leaves after salt-treatment, and the expression of the *Atcys-3A* gene was significantly induced. This salt-specific regulation was mediated by the abscisic acid as the *Arabidopsis* mutants, *aba-1* and *aba-2*, which did not possess the capacity to produce abscisic acid were unable to respond to NaCl in the way that the wild-type plant responded (Barroso et al. 1999). The metal detoxification mechanism in photosynthetic organisms proceeds through the formation of a complex between phytochelatins and Cd^{2+}, which remains confined in the cell vacuoles (Cobbett 2000). Phytochelatins are synthesized in the cells from glutathione, in a reaction catalyzed by phytochelatin synthetase, which implies the requirement of an additional glutamate and cysteine synthesis in the plant or microalgae for the purpose of protection. Consistent with this, we observed that the NADH-glutamate dehydrogenase activity (aminating activity) and the OAS-TL activity increase in the

C. reinhardtii cells submitted to cadmium stress, and that Cd^{2+} is accumulated at $0.90 \pm 0.02\%$ in the dry microalgal biomass (Mosulén et al. 2003; Vega et al. 2005). The synthesis rate for L-cysteine increased significantly in the *A. thaliana* leaves post 18 h of treatment of the plant with Cd_2Cl. In addition, transformed plants overexpressing the *Atcys-3* gene by ninefold exhibit increased Cd-tolerance, accumulating the metal in the leaves (Domínguez-Solís et al. 2001).

7 Biotechnology of Microalgae

Our first work in the field of biotechnology of microalgae was published much earlier in Vílchez et al. (1991), and in that work, we studied the glycolate photoproduction by the free and the alginate-entrapped cells of *C. reinhardtii.* Since the year 2001, I have been collaborating actively with the research group of Prof. Carlos Vílchez at the University of Huelva (Spain), which is involved in this research field, and is gaining increasing presence in different industrial sectors.

Microalgae have simple requirements for growth. They are photosynthetic organisms that are able to utilize atmospheric CO_2 and require a source of nitrogen (certain cyanobacteria are also able to fix N_2), phosphorous, and sulfur. It is possible to culture microalgae in freshwater, marine water, or wastewater, which contain the required micronutrients. Industrial, agricultural, or domestic wastewater may be used with the goal to produce biomass and remove contaminants. In addition, microalgae possess metabolic plasticity, which allows them to adapt to different environmental changes by using metabolic mechanisms that involve the synthesis of novel chemicals absent in the other organisms, rendering their biomass particularly valuable. Certain microalgae are able to grow, in addition to phototrophic conditions, under both heterotrophic and mixotrophic conditions (Forján et al. 2015).

Particularly interesting are the outdoor high-scale cultures, in which several types of photobioreactors (PBRs) may be used. Among others, the following PBRs are available with us: (a) *Bach-type*, which are similar to swimming pools, where the microalgae grow with a continuous supply of nutrients and are mixed by mechanical agitation. This system is adequate for robust microalgal species and the main problem with such types of cultures is the possibility of contamination by other microalgae; (b) *Tubular type*, which are closed cultures in transparent tubes, equipped with propeller pumps, and having an attached column for gaseous exchange with the exteriors. This system is adequate for non-robust and slow-growing microalgae, because the conditions can be controlled at any time; and (c) *Airlift reactor*, which are constructed as a transparent column, with aeration of the cultures from the bottom of the reactor. This system is adequate for cultures which need to be well aerated (Xu et al. 2009).

Nutritional stress, such as N-starvation, induces in most microalgae accumulation of lipids in the form of neutral fats and carotenes. The abundance of saturated fatty acids renders the biomass suitable for biofuel production; however, the high intracellular content of polyunsaturated fatty acids (PUFAs) in the microalgae renders the

biomass suitable for animal nutrition (Navarro et al. 2017b). Abiotic stress is used to induce synthesis of high added-value compounds in microalgae and these compounds have biotechnological applications in the industries of different sectors, such as foods, pharmaceuticals, and cosmetic (Forján et al. 2015; Liang et al. 2018).

In general, any kind of nutritional and abiotic stress induces oxidative stress in microalgae, causing the generation of reactive oxygen species (ROS), which promotes the synthesis of antioxidants, either enzymes or metabolites, as a part of the adaptation mechanism (Vega et al. 2005; Paliwal et al. 2017). Among these antioxidants are carotenes, with broad applications in foods, pharmaceutical and cosmetic industries. In a recent work, Zhang et al. (2018) showed that melatonin, a strong antioxidant, confers salt-stress tolerance to *C. reinhardtii* by improving redox homeostasis.

Transcription factors control gene expression through interaction with RNA polymerase and by binding to the *cis*-acting elements in the target gene promoters, especially in response to abiotic stress. The MYB-related transcription factor ROC40 plays a role in the nitrogen-starvation induced lipid accumulation in green algae (Goncalves et al. 2016). Transcriptional engineering, therefore, could be a viable way to manipulate the biosynthesis of a specific high-value metabolite in microalgae (Bajhaiya et al. 2017).

Certain microalgae are able to live under extreme environmental conditions, where life is considerably difficult due to extreme temperatures or pH, high salinity, intensive UV-illumination, or due to the presence of contaminants, such as high levels of CO_2, heavy metals, and the other pollutants. These extremophile microalgae are particularly interesting because their biomass could be rich in certain exceptional and unusual chemicals and/or they could serve as sources of enzymes with exceptional functional properties. Such varieties of microalgae are an important source of opportunities for the phycologists to discover certain exceptional microalgae for industrial use, which are unknown to date (Forján et al. 2015).

On the other hand, the techniques for the genetic modification of microalgae are progressing rapidly, and it is possible to adapt the metabolism of a microalga in a manner that it would be useful for us, which is certainly revolutionary and could assist us in solving technical problems that appear unsolvable to date. These are the reasons that cause the biotechnology of microalgae to be a fascinating field of research. CRISPR (clustered regularly interspaced short palindromic repeats)-Cas9 (CRISPR-associated nuclease 9) technology, which has been identified as an important defense system against viruses in the archaebacteria, has become a rapid, powerful, and precise tool for genome editing in the microalgae (Ng et al. 2017; Banerjee et al. 2018; Liang et al. 2018).

Biotechnology of microalgae encompasses the orientation of microalgal cultures toward commercial benefits and is usually based on the production of raw materials and the utilization of the residual biomass. The most prominent one among the various biotechnological uses of microalgae is their use as a raw material for the following industries:

(a) Aquaculture and Food technology
(b) Biofuels production: Biodiesel, bioethanol, biogas

(c) Production of high-added value metabolites
(d) Biofertilizers for agriculture
(e) Bioelimination of contaminants

Microalgae are at the base of the trophic food pyramid for fish and animals, and they serve as the source of proteins, polyunsaturated fatty acids, and vitamins, which may be used in aquaculture and in the functional food industry. Among the main products that may be produced from the microalgae are carotenoids and PUFAs, and our research group at the university of Huelva is working in this area. We have published reviews regarding the different biotechnological uses of microalgae (Vílchez et al. 1997, 2011; Forján et al. 2015).

The river Tinto (Huelva, Spain), also known as the "Red River" due to the high concentration of iron in its water, exhibits a constant pH value that ranges between 2 and 3 along its length of 80 km. The low pH value is a result of sulfuric acid formation as a consequence of both chemical and biological iron redox reactions. The water of this river exhibits a high contamination of heavy metals, especially iron, copper, magnesium, and aluminum. The extreme oxidant conditions of the environment (intensive illumination comprising high levels of UV radiations) require the microorganisms to adopt protective antioxidant mechanisms. No higher life is present in the river water, which is otherwise rich in the microorganism species, including the green eukaryotic microalga *Coccomyxa* sp. (strain *onubensis*), which was isolated by our research group and was adapted to grow autotrophically in the laboratory using a mineral culture medium (Garbayo et al. 2012).

7.1 Aquaculture and Food Technology

Aquaculture is expected to grow in the near future, as the population of the world is increasing, and therefore, there is high demand for the fish products, such as fish oil, particularly PUFAs, carotenoids, vitamins, and proteins. The fish-capture activity worldwide is limited until approximately 95–100 million tonnes/year, and it is not possible to increase these numbers without causing a severe harm to the equilibrium of the species in the oceans. Therefore, aquaculture is required to satisfy the increasing demand for fish products. It is estimated that by 2025 the amount of the fish food generated from aquaculture will equal that which is generated from captures in the wild. There are interesting reviews concerning the valuable biomolecules from microalgae for growing fish (Yaakob et al. 2014), with a special discussion of the nutritional value in fish dietetics (Roy and Pal 2015).

The biomass of *C. onubensis* is rich in proteins (44.60%) and dietary fiber (15.73%); it has moderate carbohydrate content (24.80%) and low lipid content (5.4%), in which the polyunsaturated fatty acids represent about 65% of the total fatty acids content. This biomass may be used as antimicrobials source (Navarro et al. 2017a), food supplement for the laboratory animals, and it may also serve as a source of nutraceuticals for the functional foods. In addition, the microalgae-supplemented diets have been

demonstrated to exert significant hypocholesterolemic and hypotriglyceridemic effects on animals (Navarro et al. 2017b).

7.2 Biofuel Production: Biodiesel, Bioethanol, and Biogas

Microalgae are a promising feedstock for the sustainable production of biofuels, due to their unique capacity to reach high lipid productivities; however, in order to be profitable, the production of biomass should be highly economical, the cells harvesting technology should be simple and economically acceptable, the process of extraction of fuels must be optimized, and the residual biomass should be suitable for other applications (Barbosa and Wijffels 2013). Producing biodiesel for transport is the main objective for the production of biofuel from microalgae. The procedure is simple; the microalgal biomass enriched with lipids, particularly in saturated fatty acids, is extracted using hexane, and the lipid fraction is treated with methanol, in order to obtain the fatty acid methanol esters (FAMEs), which may then be used directly as biodiesel. The residual biomass should be viable for anaerobic fermentation, to produce biogas, which is a mixture of mainly CH_4 and CO_2, and the proportion of these gases depends on the organic matter from which the biogas is generated. Alternatively, the residual biomass may be used for alcoholic fermentation to produce bioethanol (Brennan and Owende 2010). In order to make the microalgae biomass economically attractive, the concept of "biorefinery" has been introduced to produce biofuels from microalgae (Cherubini 2010). This means the successive treatment of the microalga biomass to produce biodiesel, bioethanol, and biogas. The capacity of microalgae to photoproduce hydrogen is other interesting aspect of the biorefinery possibility (Kruse et al. 2005).

Nutrient-starved cultures of *C. onubensis* accumulated fatty acids in their biomass, linolenic fatty acid (C18:3) being the most abundant one; this accumulation rendered the microalga more suitable for food, rather than for the biofuel industry (Ruiz-Domínguez et al. 2015). However, there are several other microalgae which contain saturated fatty acids that cause them to be suitable for biodiesel production (Chisty 2007).

7.3 Production of High-Added Value Metabolites

Microalgal biomass may contain and accumulate high-added value metabolites, such as antioxidants, antimicrobials, antitumor, and anti-inflammatory compounds (Forján et al. 2015). In humans, the most relevant biological functions of the carotenoids are linked to their antioxidant property, the particularly relevant ones being the immunoresponse modulation, signal transduction between the cells, and the anti-inflammatory response mechanisms. Humans are not able to synthesize the carotenoids de novo, although they are able to convert them into vitamin A, which is

essential for healthy functional eyes. In this context, lutein and zeaxanthin are present in the macula of the eye, where they protect the tissue against it against the macular degenerative disease. In general, a carotenoid-enriched diet is considerably effective for the human health, as it diminishes the risk of degenerative diseases, such as Alzheimer's disease and Parkinson's disease, and it also provides protection to the skin against UV radiation (Vílchez et al. 2011).

Carotenoid pigments are common in nature and are synthesized by the photosynthetic organisms and fungi. These pigments are considered the key molecules for life, as they perform important functions vital for life, such as capturing light during photosynthesis, protecting the thylakoid membranes in the chloroplast by allowing dissipation of excess light, and quenching the singlet oxygen and free radicals generated against high UV-irradiation. The carotenoid pigments are hydrophobic compounds essentially consisting of a C40 (eight isoprene residues) hydrocarbon backbone, which may be modified with oxygen-derivative functional groups to produce cyclic or acyclic xanthophylls. Microalgae are recognized as a natural source of carotenoids and various other beneficial products (Gong and Bassi 2016).

In recent years, the production of carotenoids has become one of the most successful activities in the field of microalgal biotechnology (Torregrosa-Crespo et al. 2018). The main microalgae candidates to serve as natural source of carotenes are *Dunaliella saline*, as source of β-carotene and *Haematococcus pluvialis* to produce astaxanthine (Gong and Bassi 2016). Submitting the acidophilic microalga *C. onubensis* to nutritional (N-starvation) and abiotic stresses (UVA-radiation or high salinity) produces biomass that is enriched with carotenes, particularly lutein (7.07 ± 0.15 mg/g dw) and polyunsaturated fatty acids, which are in high demand in the foods and pharmacological industries (Bermejo et al. 2018).

Halophilic archaea are the extremely halophilic microorganisms, which are mainly grouped into the *Haloferacaceae* family, phylum *Euryarchaeota*, and domain *Archaea*. They are mostly aerobic, non-photosynthetic, and red-pigmented. Among these archaea, there is *Haloferax mediterranei*, which produces C50 carotenoids, mainly bacterioruberin, a strong antioxidant, which is even stronger than the C40 carotenoids, such as β-carotene. This outstanding property makes bacterioruberin particularly interesting for foods and pharmaceutical industries (Rodrigo-Baños et al. 2015; Montero-Lobato et al. 2018).

7.4 Biofertilizers in Agriculture

Microalgae also represent a potentially sustainable alternative for the enhancement and protection of agricultural crops (Uysal et al. 2015). As a representative example, the *Acutodesmus dimorphus* culture, its cellular extract, and the dry biomass generated from it, which were applied as biostimulants, foliar spray, and biofertilizer, respectively, were able to trigger faster germination, and also enhanced the plant growth and flower production in tomato plants (García-González and Sommerfeld 2016).

7.5 *Bioelimination of Contaminants*

Microalgae consume CO_2 and may contribute significantly to greenhouse mitigation effect. In parallel, microalgae may also be employed in the contaminant bioelimination processes, especially for the nitrogen, phosphorous, and sulfur compounds. It is particularly relevant that they are used for the removal of the heavy metals from the wastewaters (Vílchez et al. 1997).

Depuration of wastewater (agricultural, domestic, or industrial) may be efficiently performed through the association of bacteria with microalgae. Bacteria are able to grow in the presence of organic contaminants, with the consumption of O_2 and the simultaneous production of CO_2. On the other hand, microalgae would be able to grow by consuming minerals and the CO_2 produced by the bacteria. Oxygenic photosynthesis allows the microalgae to generate O_2 for bacterial respiration. In addition, the microalgal biomass could be used for the production of biofuels (Rawat et al. 2011).

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Progress Toward Deep Sequencing-Based Discovery of Stress-Related MicroRNA in Plants and Available Bioinformatics Tools

Abdelali Hannoufa, Craig Matthews, Biruk A. Feyissa, Margaret Y. Gruber, and Muhammad Arshad

Contents

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A. Hannoufa, C. Matthews, and B. A. Feyissa
Agriculture and Agri-Food Canada, London, ON, Canada

Department of Biology, University of Western Ontario, London, ON, Canada

M. Y. Gruber
Agriculture and Agri-Food Canada, Saskatoon, SK, Canada

M. Arshad (✉)
Agriculture and Agri-Food Canada, London, ON, Canada

Centre of Agriculture Biochemistry and Biotechnology, University of Agriculture, Faisalabad, Pakistan
e-mail: muhammad.arshad3@ucalgary.ca

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Abstract MicroRNAs (miRNAs) are small endogenous RNA molecules that regulate gene expression at the posttranscriptional level. Recently, studies have discovered that miRNAs are induced by various stresses, suggesting that miRNAs may be an efficient target to improve crop resilience. Studies that are more recent have demonstrated that the response of miRNAs to stresses stimuli depends on type and level of stress, tissue, and genotype. Stress conditions induce several miRNAs, which alter downstream signaling cascades by regulating target genes and lead to adaptive responses to stress. During the past decade, research was focused on identification of plant miRNAs in response to environmental stressors, dissecting their expression patterns and studying their role in plant stress responses and tolerance. This has been accomplished principally by using next-generation sequencing (NGS) technologies, which have proven to be a powerful tool for this purpose. Altering miRNAs expression resulted in changes in plant growth and development under abiotic stress including cold, drought, salinity, nutrient, high temperature, and heavy metal stress as well as biotic stress such as fungus, bacteria, and virus infection. These findings implicate potential targets for genetic manipulation to improve stress tolerance in plants. This review is aimed to provide updates on recent miRNA research in improving plant resistance to biotic and abiotic stress. Moreover, we discuss availability of computational tools and genomics platforms for identification of stress-related miRNAs in plants.

1 Introduction

MicroRNAs (miRNAs) are small noncoding RNAs, which target genes that encode mostly transcription factors in eukaryotes (Ambros 2001; Hobert 2008). In plants, these miRNAs are approximately 21–24 nucleotides long and cause posttranscriptional gene dysfunction mainly by transcript cleavage or translation inhibition (Huntzinger and Izaurralde 2011; Rogers and Chen 2013). RNA polymerase II transcribes the miRNA genes and produces the primary transcript or pri-miRNA (Lee et al. 2002), which is converted to mature miRNA in two steps. The first step involves formation of 60–70 bp long pre-miRNAs in the nucleus from primary transcripts (Denli et al. 2004; Kumar 2014), while the second step is carried out in the cytoplasm. The latter step includes formation of 21–24 bp mature miRNA from pre-miRNA (Kumar 2014). Primary miRNA transcripts (pri-miRNA) are embedded into hairpin structures that are recognized by members of Dicer-like (DCL) family proteins. Within this family, DCL1 plays a vital role in the cleavage of pri-miRNA into precursor miRNA (Axtell et al. 2011). Plant microRNA then matures in steps (before engaging with sequence complementary transcripts) by first being methylated at the 3′ overhang and then interacting with a silencing effector protein ARGONAUTE1 (AGO1). Translocation of mature miRNAs and miRNA-mediated silencing requires AGO1 to shuttle between nucleic acid and cytosol (Bologna et al. 2018).

Along with miRNA, plants also produce many short interfering RNAs (siRNAs) that are distinguished from miRNAs based on their biogenesis and functions. Regarding biogenesis, siRNAs are processed from long double-stranded RNA

instead of single-stranded hairpins, and they typically function in transcriptional instead of posttranscriptional regulation (Axtel and Meyers et al. 2018).

miRNAs are found throughout the plant kingdom and are evolutionary conserved in species ranging from mosses to higher flowering plants, including monocots and dicots (Axtell and Bartel 2005; Zhang et al. 2006). Eight plant miRNA families, including miR156, miR159/319, miR160, miR166, miR171, miR408, miR390/391, and miR395, are found to be conserved in the ancestral Embryophyta (Cuperus et al. 2011). The functional and evolutionary conserved nature of some plant miRNAs has expedited their studies in several plant species such as *Arabidopsis*, alfalfa, and wheat (Aung et al. 2015; Budak et al. 2015; Lei et al. 2016). Recent findings revealed that conserved plant miRNAs regulate many common traits in plant growth and development such as plant morphology and growth phase change (Aung et al. 2015; Cui et al. 2014). Species-specific miRNAs regulate vital traits in an individual plant species, for instance, fiber growth and development in cotton (Xie et al. 2015). Among plant species, nine Leguminosae family species have been found to have different numbers of mature and precursor miRNAs (Kozomara and Griffiths-Jones 2014). Different types of miRNAs have also been identified in monocot crops, for example, rice (Lan et al. 2012). Moreover, deep sequencing of small RNA from soybean seeds and vegetative parts has revealed tissue-specific expression of some miRNAs (Zabala et al. 2012).

Plant miRNAs affect diverse aspects of plant growth, development, and stress response by regulating transcription factors and downstream effector genes. For example, miR156 targets SPL family genes in *Arabidopsis* and alfalfa (Poethig 2009; Huijser and Schmid 2011; Gao et al. 2016). Similarly, miR395 functions in sulfate uptake and its metabolism in plants (Zhang 2015), whereas miR398 regulates heat stress tolerance by downregulating its target genes *Cu/Zn SUPEROXIDE DISMUTASE1 (CSD1)*, *CSD2*, and *CCS* (a gene encoding a copper chaperone for both *CSD1* and *CSD2*) (Guan et al. 2013; Naya et al. 2014). Several studies show a vital role for plant miRNAs in mediating the stress response in various plant species (Liu et al. 2008b; Zhou et al. 2008; Arshad et al. 2017a, b; Arshad et al. 2018). Similarly, miR160 regulates hormone signaling under stress conditions by targeting an auxin response factor (ARF) family gene (Wang et al. 2016). Moreover, miR156 is induced by stress (Arshad et al. 2017a; Stief et al. 2014) and targets non-SPL *WD40* genes in a stress-specific manner in alfalfa (Arshad et al. 2018).

A number of approaches have been used to identify plant miRNAs and their targets in different plants, such as microarray (Voinnet 2009), next-generation sequencing (Bonnet et al. 2010), homology search of public databases, and RNA ligase-mediated amplification of cDNA ends (5′ RLM-RACE) (Aung et al. 2015; Gao et al. 2016; Sun et al. 2012). The large numbers of identified miRNAs in one species, however, make it almost impossible to analyze expression of each miRNA using conventional methods, such as RNA blotting and qRT-PCR. Instead, next-generation RNA sequencing (NGS) has proven to be an efficient technique, particularly in species without a sequenced genome (Unamba et al. 2015), as NGS not only provides information regarding expression profiles but also gives information on sequences of the small RNAs. Deep sequencing technologies have been helpful in

efficiently revealing the hidden genetic information from a plant genome. Hence, identification and functional characterization of plant miRNAs using in silico approaches have emerged as a fast, efficient, and reliable way compared to laboratory-based cloning (Gupta et al. 2017; Tyagi et al. 2018). In the current report, we review the latest literature on miRNA involvement in stress response in plants and provide an overview of miRNA discovery using deep sequencing approaches.

2 MicroRNAs and Abiotic Stress Response

Abiotic stresses, such as drought, salt, cold, heat, and nutrient deficiency, are factors that negatively affect global agriculture production. Along with physiological modifications, plants need to regulate gene expression in order to survive under abiotic stress. The efficiency with which plants can make use of miRNAs to regulate expression of vital genes decides the survival of plants (Jeong and Green 2013). The role of miRNAs in abiotic stress responses is evident from the fact that miR159 and its target *GIBBERELLIN AND ABSCISIC ACID MYB* (*GAMYB*) genes are differentially regulated under heat stress in wheat, and *miR159* mutants are heat stress-sensitive (Wang et al. 2012b). Similarly, *Arabidopsis* mutants *hyponastic leaves-1* (*hyl1*), *hua enhancer-1* (*hen1*), and *dicer-like1* (*dcl1*) are defective in miRNA metabolism and exhibit hypersensitivity to abiotic stress and to the stress hormone ABA (Lu and Fedoroff 2000). This suggests a role of some miRNAs in modulating abiotic stress response in plants. Using different approaches, several abiotic stress-responsive miRNAs have been identified (Table 1) (Cui et al. 2014; Wang et al. 2013; Aung et al. 2015; Xie et al. 2015). Genomics approaches have proven to be efficient in the identification of stress-responsive miRNA in a range of plant species (Sahu et al. 2016; Khan et al. 2014), including rice (Zhou et al. 2010), peach (Eldem et al. 2012), cotton (Xie et al. 2015), and wheat (Ragupathy et al. 2016). Moreover, advancement in NGS technologies has expedited microRNA discovery and the dissection of their role in crop improvement in model and non-model plants (Fig. 1).

2.1 Cold Stress

Low temperature exerts negative effects on plant growth and development by inducing tissue injury that can reduce crop productivity (Mahajan and Tuteja 2005). Cold acclimation involves several physiological and biochemical modifications such as synthesis of soluble sugars and free amino acids, as well as altering expression of cold-regulated genes such as cold responsive (*COR*), cold induced (*KIN*), and low temperature induced (*LTI*) and C-repeat (*CRT*) genes. Studying the *CRT* family demonstrated that these genes play vital roles in cold tolerance by regulating downstream effector genes (Thomashow 2010). Besides genes encoding

Table 1 Role of recently discovered conserved plant miRNAs with function in stress tolerance

Type of stress	Role of miRNA	miRNA family	Target gene/ families	Reference(s)
Heat	Heat stress tolerance and memory	miR156	*SPLs*	Stief et al. (2014), Wang and Zhang (2017)
	Hormone signaling under heat stress	miR160	*ARF*	Wang et al. (2016)
	Regulatory changes under heat stress	miR169	*NF-YA*	Leyva-Gonzalez et al. (2012), Wang and Zhang (2017)
	Heat stress tolerance	miR396	*GRF, WRKY*	Giacomelli et al. (2012), Hivrale et al. (2016)
	Repress antioxidant enzyme activity	miR398	*Cu/Zn SOD*	Guan et al. (2013), Kumar et al. (2015)
Oxidative	Hormone signaling under oxidative stress	miR160	*ARF*	Lv et al. (2016)
	Regulatory changes under oxidative stress	miR169	*NF-YA*	Lv et al. (2016)
	Hormone signaling under oxidative stress	miR390	*TAS3* tasiRNAs	Iyer et al. (2012)
	Nutrient assimilation/ allocation under stress	miR395	*ATPS, RRF*	Li et al. (2017a, b)
	Repress antioxidant enzyme activity	miR398	*Cu/Zn SOD*	Sunkar et al. (2006), Lv et al. (2016)
Heavy metal	Downregulate PC biosynthesis-associated genes	miR156	*SPLs, GGT, GST*	Zhou et al. (2012)
	Hormone signaling under heavy metal stress	miR167	*ARF*	Wang et al. (2016)
	Downregulate PC biosynthesis-associated genes	miR393	*PCS*	Xu et al. (2013)
	Nutrient assimilation, increase GSH	miR395	*ATPS, RRF*	Zhang et al. (2013), Jagadeeswaran et al. (2014)
	Repress antioxidants enzyme activity, metal ion usage	miR398	*Cu/Zn SOD*	Xu et al. (2013)
Fungus	Exported to downregulate fungal virulence gene	miR159	*HiC-51*	Zhang et al. (2016)
	Hormone signaling after fungal infection	miR160	*ARF*	Li et al. (2014a, b)
	Exported to downregulate fungal virulence gene	miR166	*Clp-1*	Zhang et al. (2016)
	Fungal infection susceptibility	miR396	*GRF*	Soto-Suarez et al. (2017)

(continued)

Table 1 (continued)

Type of stress	Role of miRNA	miRNA family	Target gene/ families	Reference(s)
	Negatively regulate antioxidant enzyme activity	miR398	*Cu/Zn SOD*	Li et al. (2014a, b)
	Negative regulation of fungal infection via phenylpropanoid pathway PSM	miR858	*MYB*	Camargo-Ramirez et al. (2018)
Cold	Cold tolerance in rice	miR319	*PCF5, PCF8*	Yang et al. (2013)
	Cold tolerance in rice	miR156	*SPL*	Cui et al. (2015)
	Enhanced cold tolerance	miR394a	*LCR*	Song et al. (2017)
	Improves growth under cold	miR399	*IPS-1*	Gao et al. (2015)
Drought	Altered drought stress response in various plant species	miR156 miR156 miR164, 397,399,528 miR1432	*SPL13* *SPL9/DFR, PAP1* *NAC transcription F.* *B-ZIP*	Arshad et al. (2017a), Cui et al. (2014), Ferreira et al. (2012)
Salinity	Altered salinity stress response in various plant species	miR156 miR161, 173, 172 miR394 miR394	*SPL* *SSAC1* *DIF1* *LCR*	Arshad et al. (2017b), Dolata et al. (2016), Pan et al. (2016), Gao et al. (2017), Song et al. (2013)
Nutrient	Altered nutrient stress response in various plant species	miR395 miR827 miR1193	*SULTR1, SULTR2, AST68, APS1, APS3 APS4* *PHT5, VPT1* *NtPT*	Jagadeeswarn et al. (2013), Liu et al. (2015), (2016), (2018)
Bacteria	Involved in regulating response under microorganism (bacteria) stress bacterial	miR393 miR844	*+Flg22, −TIR, −AFB2, −AFB3* *CDS3*	Robert-Seilaniantz et al. (2011), Yu et al. (2015), Lee et al. (2015)
Viruses	Involved in regulating response under microorganism (virus) stress bacterial	miR169 miR171	*NF-YA, GA3-oxidase, SCL6-IIa, SCL6-IIb, SCL6-IIc*	Križnik et al. (2017), Kis et al. (2015), Tong et al. (2017)

transcription factors, cold also alters expression of miRNAs, including miR393, miR319c, miR398a (Sunkar and Zhu 2004), miR408, miR397, miR396, miR319, miR172, miR171, miR169, miR168, miR165, and miR399 (Liu et al. 2008a; Leyva-Gonzalez et al. 2012; Yang et al. 2013; Gao et al. 2015). All these mRNAs play important regulatory roles in plant growth, development, and stress responses (Khraiwesh et al. 2012).

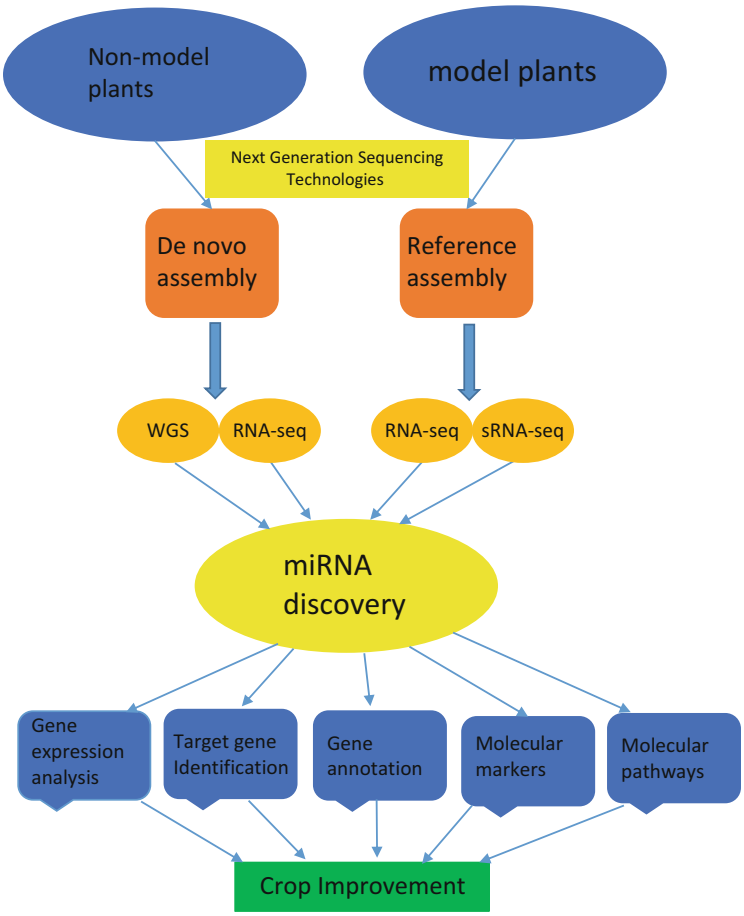

Fig. 1 A flowchart showing the role of high-throughput technologies in microRNA discovery and improving crop plants

Many miRNAs have been identified by high-throughput sequencing to be involved in cold response in plants. These include miR156/157, miR169, miR393, miR396, miR394, and miR398 in *Brachypodium distachyon* (Zhang et al. 2009), *Arabidopsis* (Zhou et al. 2008), *poplar* (Chen et al. 2012b), *rice* (Lv et al. 2010), and *wheat* (Rajwanshi et al. 2014). A transcriptomic study revealed that about 17% of *Arabidopsis* miRNAs are upregulated in response to cold (Lee et al. 2005). In other studies, Solexa sequencing exhibited altered expression of 3 conserved miRNAs (miR169e, miR172b, and miR397) and 25 predicted miRNAs in response to cold stress in *B. distachyon* (Zhang et al. 2009) and 64 cold-inducible miRNAs (21 novel) in *Musa balbisiana* (Wang et al. 2016). The deep sequencing approach was also employed to identify 30 cold-responsive miRNAs in *Populus tomentosa* (Chen et al. 2012a) and tea plants

(*Camellia sinensis*) (Prabu and Mandal 2010). Six unique miRNAs in the latter species were subsequently cloned and functionally characterized to verify their role in cold response (Mohanpuria and Yadav 2012). Microarray analysis identified 18 cold-responsive miRNAs in rice where the miR171 family exhibited variable expression patterns (Lv et al. 2010). Another tea study combined Solexa sequencing technology with microarray-based hybridization and identified 215 candidate miRNAs, including genotype-specific ones in cold stress conditions, out of which 98 miRNAs were found to have novel sequences (Zhang et al. 2014b). Several studies have also been conducted in forage crops like alfalfa to identify 35 miRNAs involved in cold tolerance, and their subsequent analyses showed that these miRNAs directly target 105 gene transcripts associated with biological regulation, cellular and metabolic processes, and stress response (Shu et al. 2016).

Genomics approaches have also been employed to identify and study miRNAs in vegetable and fruit plants. Whole genome sequencing revealed 60 novel miRNAs in potato, and further degradome analysis identified 70 target genes for these miRNAs (Ou et al. 2015). A similar study was also conducted in tomato in which 236 novel miRNAs were found to be differentially expressed in response to cold (Cao et al. 2014). Degradome analysis revealed 62 target genes of conserved miRNAs (including miR390, miR408, miR6024, miR8007) and 9 novel miRNAs (including miR01, miR02, and miR03). In another study conducted in trifoliate orange (*Poncirus trifoliata*), 107 conserved miRNAs and five novel miRNAs were identified in response to cold (Zhang et al. 2014a). The mRNA-seq analysis found that downregulation of *miR159-5p, miR858, miR8029-3p,* and a *novel miR0048-3p upregulated several transcription factors (CDPK, BHLH, WRKY, MYB, LEA,* and *DREB) involved in cold responses in Citrullus lanatus* (Li et al. 2016)*, and miR396-regulated WRKY were involved in temperature damage in sunflower* (Giacomelli et al. 2012). Similarly, a total of 318 long noncoding RNAs were found to be responsive to cold and/or drought in cassava (*Manihot esculenta*) (Li et al. 2017a). *In grass species, 203 novel, 246 known, and 102 differentially expressed miRNAs were found in response to salt and cold stress in bermudagrass* (*Cynodon dactylon)* (Hu et al. 2018). Similarly, high-throughput sequencing identified that 34 conserved and 5 novel miRNAs were differentially expressed under cold stress in wheat (Song et al. 2017). Moreover, a study conducted in maize revealed 28 known and 24 novel miRNAs to be differentially expressed under cold treatment (Li et al. 2016).

2.2 Heat Stress

High temperatures adversely affect cellular function through changes to protein folding and stability, membrane fluidity, biochemical reactions, and metabolism (Bernstam 1978; Chen et al. 1982; Wu and Wallner 1985; Smertenko et al. 1997). Heat also exacerbates water stress by increasing potential evapotranspiration (Rind et al. 1990). Altogether, excessive heat disrupts plant growth and development and ultimately negatively impacts yield.

Silencing of miR398, which targets a Cu/ZnSOD, improved heat tolerance in *Arabidopsis*, whereas the opposite effect was observed in plants with *miR398* overexpression (Guan et al. 2013). *miR398* showed decreased expression in cotton (*Gossypium hirsutum*) under extreme temperature (42°C) compared to moderately high temperature (35°C) (Wang et al. 2016) but was upregulated in wheat at 42°C (Kumar et al. 2015). Additionally, it was found that miR160, which targets auxin response factor (ARF) transcription factors and is associated with plant development (Meng et al. 2010), also targets heat shock protein 70 (HSP70) in wheat (Kumar et al. 2015) and is downregulated in response to heat stress. A similar silencing of miR160 was also reported in heat-treated *Populus tomentosa*, with a 0.13-fold change (Chen et al. 2012a). However, these results contrast with the expression pattern of *miR160* reported in barley (*Hordeum vulgare*) (Kruszka et al. 2014) and sunflower (*Helianthus annuus*) (Khaksefidi et al. 2015), where miR160 expression was enhanced under heat stress. These results may indicate a divergence in targets of conserved miRNAs across different plant species. Similar species variability was also observed when comparing the expression patterns of other miRNAs in *Arabidopsis* and rice. For example, under heat stress, miR169 is upregulated in rice and downregulated in *Arabidopsis*, whereas miR156, miR319, and miR396 are downregulated in rice and upregulated in *Arabidopsis* under heat (Wang et al. 2016). RNA sequencing in wheat under heat stress also revealed that miR169, a regulator of the nuclear factor Y subunit A (*NF-YA*) (Li et al. 2008), was upregulated (Xin et al. 2010).

P. tomentosa small RNA sequencing also showed differential expression of seven conserved miRNAs under 37°C heat stress (Chen et al. 2012a). These included the downregulation of miR395, which may indirectly affect glutathione metabolism (Hell and Wirtz 2011) via its target ATP sulfurylase (Jones-Rhoades and Bartel 2004); miR408, which may play a role in apoptosis via plastocyanin-like protein gene targets (Lu et al. 2005); and miR168, which targets AGO1 (Lu et al. 2005). Unlike *Populus tomentosa*, heat stress upregulated miR395 and miR168 in wheat (Kumar et al. 2015), celery (Li et al. 2014a), and switchgrass (Hivrale et al. 2016); however, miR168 was downregulated in rice under heat stress (Sailaja et al. 2014).

Two different heat-tolerant cultivars of wheat seedlings also showed contrasting regulation of heat stress-responsive miR159 and miR160 genes (Yu et al. 2012). In both of these cultivars, miR156, a regulator of SPL transcription factors and of heat stress tolerance and memory (Wang and Zhang 2017; Stief et al. 2014), was upregulated under heat. In addition, RNA sequencing and microarray analyses showed miR156 was upregulated under dehydration stress in Chinese cabbage (Yu et al. 2012) and under heat stress in *Arabidopsis* (Stief et al. 2014), respectively. However, RNA sequencing revealed that miR156 was downregulated in rice shoots but upregulated in the roots of some rice cultivars (Sailaja et al. 2014; Mangrauthia et al. 2017). RNA sequencing by Xin et al. (2010) and Hirvale et al. (2016) showed miR827 was upregulated in response to heat in wheat and switchgrass. miR827 targets SPX-MFS, a regulator of phosphate homeostasis (Wang et al. 2012a) that has been implicated in nutrient management under heat stress in barley (Barciszewska-Pacak et al. 2015).

In addition to the association of known miRNAs to heat stress, genomic technologies have allowed for the identification of a large number of novel stress-responsive miRNAs in a wide variety of crop plants. RNA-base sequencing analyses have identified hundreds of novel heat-responsive miRNAs in *Arabidopsis* (Barciszewska-Pacak et al. 2015), rice (*Oryza sativa*) (Mangrauthia et al. 2017), cotton (*Gossypium hirsutum*) (Wang et al. 2016), *Populus tomentosa* (Chen et al. 2012a), wheat (*Triticum aestivum*) (Xin et al. 2010; Kumar et al. 2015), Chinese cabbage (*Brassica rapa*) (Yu et al. 2012), celery (*Apium graveolens*) (Li et al. 2014a), and switchgrass (*Panicum virgatum*) (Hivrale et al. 2016). Identification of novel miRNAs and their targets may provide insights into unique stress tolerance strategies in individual plants, strategies that can be harnessed for broader use.

2.3 Oxidative Stress

Reactive oxygen species (ROS) are produced naturally in chloroplasts and mitochondria during photosynthetic and respiratory processes, respectively. In addition to the ROS present under normal cellular conditions, ROS production is enhanced under various biotic and abiotic stresses from disrupted function of the electron transport chain and photosystems I and II and from the increased activity of oxidases and peroxidases. ROS production is not limited to plastids, but extends to peroxisomes at the cell membrane and cell wall and outside of the cell in the apoplast. While ROS accumulation is damaging to cells, it also plays roles in cell signaling and apoptosis.

A miRNA filter array was used by (Iyer et al. 2012) to identify miRNAs that are differentially regulated under ozone-induced oxidative stress. This study identified 22 different miRNAs from 21 miRNA families that showed increased expression under oxidative stress, including an initial increase in miR398 followed by a decrease at 4 h and afterward. The *Arabidopsis* Cu-Zn superoxide dismutases (SODs) encoded by *CSD1* and *CSD2* are targeted by miR398 (Sunkar et al. 2006). SODs play a crucial role in ROS scavenging by converting superoxide (O^{2-}) to hydrogen peroxide (H_2O_2) for detoxification (Wang et al. 2018). Previous studies revealed decreased expression of miR398 under oxidative stress treatments and induced total CSD1 and CSD2 activity, while nuclear CSD1 and CSD2 mRNA levels were unaffected, suggesting that these SODs are regulated posttranscriptionally by miR398 in response to oxidative stress (Sunkar et al. 2006). *CSD2* overexpression lines and miR398 insensitive *CSD2* expression lines showed increased germination, fresh weight, chlorophyll content, and PSII activity while having reduced lipid peroxide and antioxidant molecule accumulation (inferred from malondialdehyde and anthocyanin content, respectively) (Sunkar et al. 2006). *Arabidopsis* plants grown under ozone treatment also showed an initial increase in miR390 before a long-term reduction (Iyer et al. 2012). miR390 indirectly regulated various *ARFs* via *tasi*RNA intermediates (Cho et al. 2012), which may affect growth and development regulation under oxidative stress. This study identified 20 other miRNAs that were upregulated

in response to ozone treatment, including miR156, miR159, miR165, miR167, miR168, and miR171, all of which are differentially expressed in response to other abiotic stresses as well (Iyer et al. 2012). miR159 targets *GIBBERELLIN MYB*-like genes (*GAMYB-like*), *MYB33*, and *MYB65*, associated with development (Reyes and Chua 2007).

Seven other miRNAs were differentially regulated in response to H_2O_2 in rice, including the upregulation of miR169, miR397, miR1425, miR408, and miR827 and the downregulation of miR528 and miR319 (Li et al. 2011). miR169 targets the NF-YA also known as HAP2 and CBF-B. miR169 is also drought responsive in an ABA-dependent manner in *Arabidopsis* and regulates antioxidant enzymes including peroxidases and glutathione S-transferases (Li et al. 2008). The rice-specific *miR1425* targets some *PENTATRICOPEPTIDE REPEAT* (*PPR*) genes that are targeted to plastids and are involved in posttranscriptional processing of plastid genes (Sunkar et al. 2008). The differential expression of *PPR* genes would affect the expression of genes transcribed in the chloroplasts and mitochondria, major sites of ROS production. The upregulation of miR397, miR408-5p, and miR827 were hypothesized to limit nonessential processes (Li et al. 2011). The downregulation of miR319 would then result in the increase of apoptosis-associated metacaspase, a C14 cysteine protease inducing cell death (Li et al. 2011). Induced cell death is also involved in the hypersensitive response to pathogens, which uses ROS as a signaling molecule. Contrary to the results in rice, miR319 was upregulated in response to ozone treatment in *Arabidopsis* (Iyer et al. 2012).

RNA sequencing of *B. distachyon* identified many of the oxidative stress-responsive miRNAs in rice (miR159, miR160, miR169, miR397, and miR528) (Li et al. 2011), as well as 26 others (Lv et al. 2016). A total of 10 *B. distachyon* miRNAs, including members of the miR160 and miR169 families, were downregulated, while miR395 and miR159 were upregulated. Moreover, 30 of the novel *B. distachyon* miRNAs were identified as oxidative stress responsive (Lv et al. 2016).

The aforementioned findings highlight how RNA sequencing in the case of rice (Li et al. 2011) and *B. distachyon* (Lv et al. 2016) showed the importance of conserved miRNAs to oxidative stress but also identified novel responsive miRNAs for further investigation.

2.4 Heavy Metal Toxicity

Heavy metals are widely used in various industrial and technological applications, leading to widespread heavy metal pollution (Wei and Yang 2010). The presence of heavy metal pollutants leads to various adverse physiological and biochemical effects on plants and may result in plant death.

miRNAs are involved directly with biochemical response to the presence of heavy metals by altering the biosynthesis of sulfur-containing metabolites involved in the sequestration of heavy metals (Na and Salt 2011). For example, *miR395* is

induced under various heavy metal stresses, and its overexpression in *Brassica napus* conferred cadmium tolerance (Zhang et al. 2011). The increased sulfur transportation would allow for increased glutathione (GSH) biosynthesis, the precursor for phytochelatins (PCs) that contribute to heavy metal sequestration and detoxification (Yang and Chen 2013). Additionally, miR156 targets glutathione-γ-glutamylcysteinyl transferase (GGT) in *B. napus*, which interacts with PHYTOCHELATIN SYNTHASE (PCS) in heavy metal chelation by PCs (Zhou et al. 2012). Additionally, a review by Yang and Chen (2013) cited that aluminum and manganese toxicity sometimes share miRNA responses and that cadmium, mercury, and other heavy metals often have the opposite miRNA expression changes compared to the response common to aluminum and manganese.

miRNAs' role in response to heavy metal stress appears to be related more to their function in regulating phytohormone biosynthesis than to a direct involvement in the response. Many miRNAs, including miR160, miR167, and miR393, indirectly affect auxin biosynthesis via their target ARFs (Mallory et al. 2005), indole-3-acetic acid (IAA), alanine resistant 3 (IAR3) (Kinoshita et al. 2012), and transport inhibitor response1 (TIR1) (Teale et al. 2006), respectively. By inducing the miRNA-negative regulators of auxin biosynthesis at the root, root growth is reduced, which can result in stress avoidance (Yang and Chen 2013).

miR390 indirectly regulates auxin signaling (Marin et al. 2010) through the cleavage of transcripts that lead to the production of *trans*-acting small interfering RNAs (*tasi*RNAs) that, in turn, downregulate ARFs posttranscriptionally. miR390 is differentially expressed in response to cadmium stress in rice (Ding et al. 2016), radish (Xu et al. 2013), and barley (Cao et al. 2014) and manganese stress in common bean (Valdes-Lopez et al. 2010). *miR319* has also been implicated in growth control in response to heavy metal stress by repressing the expression of its target teosinte branched/cycloidea/PCF (TCP) transcription factors. The decreased expression of TCPs halts plant growth and induces senescence (Schommer et al. 2008).

Additionally, heavy metal stress induces ROS production, which was shown to alter expression of *miR398* in many plant species and resulting in increased expression of CSD1 and CSD2 under metal stress (Sunkar et al. 2006; Valdes-Lopez et al. 2010). In some cases, miR398 was upregulated in response to cadmium stress (Zhou et al. 2012). In contrast, miR164 was downregulated in *B. napus*, leading to the upregulation of miR164-targeted monothiol-glutaredoxin-S12, a member of the glutaredoxin family involved in ROS regulation in plants (Cheng et al. 2011).

High-throughput genomic techniques not only allow for the recapitulation of roles that conserved miRNAs play in heavy metal tolerance but also point to possible new and unique heavy metal stress tolerance pathways and adaptations by identifying novel miRNAs and their potential gene targets in different species. The investigation of different cultivars using genomic techniques may also allow for the determination of different stress tolerance strategies within one species. For example in one study, a comparison of tobacco cultivars under cadmium stress identified differences in miRNA expression between tolerant and susceptible cultivars and identified 33 tolerance-associated miRNAs, 5 of which were novel miRNAs

(He et al. 2016). Additionally, sequencing of *B. napus* under cadmium stress identified eight conserved cadmium-responsive miRNAs and five novel ones (Huang et al. 2010b).

2.5 The Role of MicroRNA in Regulating Drought Stress

To identify the molecular mechanism for drought stress tolerance, transcriptomics-based analyses of plants exposed to stress have been used in different plant species (Ferreira et al. 2012; Wu et al. 2017). In line with this, Liu et al. (2008a) used microarray-based transcriptomics to identify salt, drought, and cold temperature-responsive microRNAs in *Arabidopsis*. The putative differentially expressed microRNAs such as miR168 and miR396 were expressed highly in *Arabidopsis* and tobacco (Liu et al. 2008a; Frazier et al. 2011) while reduced in rice (Zhou et al. 2010) when exposed to similar drought stress. This emphasizes the species-specific role of miRNAs in regulating stress and the need for a case-by-case investigation of the plant's response.

The overexpression of miR156 improved drought tolerance in alfalfa (Arshad et al. 2017a). Using genotypes with reduced expression of *SPL13* further confirmed that drought tolerance mechanism in alfalfa is regulated in a miR156-SPL13-dependent manner (Arshad et al. 2017a). Similarly, *Arabidopsis* plants with enhanced expression of miR156 showed drought tolerance by reducing the transcript level of *SPL9* and subsequently increasing *DFR* and *PRODUCTION OF ANTHOCYANIN PIGMENT-1*, *PAP-1*, to accumulate secondary metabolites (Cui et al. 2014). A recent transcriptome study conducted on miR156 overexpressing (miR156OE) alfalfa identified a WD40 family gene (*WD40-2*) with expression reduced under drought stress. Subsequent functional characterization revealed that alfalfa plants overexpressing *WD40-2* exhibited reduced tolerance to drought, whereas transgenic alfalfa with reduced expression of this gene showed enhanced tolerance to drought (Arshad et al. 2018).

Other microRNAs have been identified in response to drought stress in other plant species (Ferreira et al. 2012; Wu et al. 2017). Wu et al. (2017) used drought-susceptible and drought-tolerant cultivars of common bean to identify 16 microRNAs associated with drought response using RNA sequencing followed by qRT-PCR validation. MicroRNA target genes included transcription factors, protein kinases, and a nuclear transcription factor. Ferreira et al. (2012) used small RNA deep sequencing on drought-tolerant and drought-susceptible sugarcane cultivars subjected to different periods of drought (2, 4, 6, and 8 days) to identify seven microRNAs potentially contributing to drought stress. Six microRNAs were differentially expressed after 2 days, while five of them were expressed after 4 days. Among these miRNAs, *ssp*-miR164, *ssp*-miR394, *ssp*-miR397, *ssp*-miR399-seq1, and *ssp*-miR528 were most affected by drought (Ferreira et al. 2012). Accordingly, increased *ssp*miR164 resulted in reduced expression of the target *NAC* transcription factor by mRNA cleavage, while *ssp*miR397 reduced laccase 23-like gene expression

by inhibiting the translation process (Ferreira et al. 2012). On the other hand, *ssp*miR1432, which regulates a B-ZIP transcription factor, was significantly reduced upon drought stress in sugarcane, while B-ZIP expression was increased significantly (Gentile et al. 2013).

2.6 MicroRNAs Regulate Salinity Stress in Plants in a Species-Dependent Manner

Soil salinity results from overuse of inorganic fertilizers, poor irrigation water quality, or reduced soil moisture (Ju et al. 2007; Han et al. 2015; Miller et al. 2017). In the search for molecular factors responsible for salinity stress tolerance, miRNAs were found to regulate other genes contributing to stress tolerance (Dolata et al. 2016; Arshad et al. 2017b). A total of 116 miRNAs were differentially expressed out of 289 in an *Arabidopsis* suspension cell line (Columbia 0-T87) during drought and salinity stress (Dolata et al. 2016). Among the differentially expressed miRNAs, miR161 and miR173 were increased significantly during salinity, contributing to stress tolerance despite a reduction in pri-miR161 and pri-miR173 regulation by AGO1 (Ma and Zhang 2018). Similarly, miR172a was significantly increased in soybean plants upon salinity stress resulting in a reduction of salt suppressed AP2 domain containing 1 (SSAC1) (Pan et al. 2016). A mutated recognition site resulted in an increased level of SSAC1 and sensitivity to salt stress, indicating specific cleavage by miR172a.

miR156 was found to regulate salinity stress tolerance in alfalfa (Arshad et al. 2017b). Investigation of alfalfa plants during mild (7.0 electric conductivity, EC) and severe salinity (14.0 EC) stress at the transcriptional, physiological, and phenotypic levels showed that miR156 orchestrated various strategies leading to stress tolerance. The authors demonstrated that miR156 overexpressing genotypes reduced toxic ion uptake and accumulation while increasing beneficial ions for normal cellular activity by regulating SPL and non-SPL genes that coordinate expression of downstream genes (Arshad et al. 2017b). In addition, (Gao et al. 2017) demonstrated that *AtDIF1*, a member of the F-box E3 ubiquitin ligase-coding genes which are negatively regulated by miR394, regulates salinity stress in an ABA-dependent manner. Young *AtDIF1* overexpressing *Arabidopsis* seedlings reduced Na^+ content, while *dif1* mutants showed a strong salinity stress phenotype. Moreover, miR394 negatively regulated *LCR* (*LEAF CURLING RESPONSIVENESS*) in *LCR* overexpressing *Arabidopsis* genotypes with enhanced salt tolerance, while miR394 overexpressing and *lcr* loss-of-function plants exhibited salt sensitivity (Song et al. 2013).

Fu et al. (2017) used small RNA deep sequencing together with degradome libraries in maize to investigate microRNAs potentially involved in salinity tolerance. Accordingly, the authors were able to identify more than 1,000 previously known microRNAs and 37 novel ones associated with salinity response (Fu et al. 2017). Of these, the most

significantly increased novel microRNAs, miR29 and miR36, play a major role in salinity stress together with the well-known miR164 and miR167 (Deng et al. 2015; Fu et al. 2017).

2.7 miRNAs and Nutrient Acquisition in Plants

Both micro- and macronutrients are equally essential for plant cellular growth and development. These nutrients function as metabolic and biochemical markers and mediate various molecular, phenotypic, and physiological aspects of the plant. Plants need to maintain nutrients at homeostatic levels, and microRNAs are found to play a major role in these processes (Kawashima et al. 2009; Liu et al. 2015, 2016).

In *Arabidopsis*, miR395 contributes to sulfur homeostasis by regulating the expression of sulfur transporters SULTR1 and SULTR2, the low-affinity sulfate transporter AST68, and ATP sulfurylases APS1, 3, and 4 (Takahashi et al. 2000; Maruyama-Nakashita et al. 2003; Kawashima et al. 2009; Jagadeeswaran et al. 2017). In contrast, miR395 expression is reduced in *Arabidopsis* during phosphorus and nitrogen starvation (Hsieh et al. 2009). Regarding phosphorus homeostasis, miR399 regulates the expression level of the ubiquitin-conjugating E2 enzyme to reduce the degradation of protein during phosphorus scarcity or to enhance the acquisition of phosphorus from the soil (Bachmair et al. 2001; Kraft et al. 2005).

Phosphorus levels in plant cells are regulated by phosphorus membrane transporters *PHOSPHATE TRANSPORTER 5*, *PHT5* (Liu et al. 2016), or *VASCULAR PHT-1* family genes residing on vacuolar membranes (Liu et al. 2015). miR827 expression is induced by phosphorus, and, in *Arabidopsis*, there are three members of the *PHT5* family which are regulated by miR827 (Liu et al. 2016). Overexpression of *PHT5* increased phosphorus accumulation in vacuoles, while loss-of-function *pht5* plants showed a reduction (Liu et al. 2016). Reduced expression levels of this gene resulted in stunted plant growth associated with a lack of phosphorus homeostasis, making the plant vulnerable to both low and high phosphorus concentrations (Liu et al. 2015, 2016). Moreover, to investigate the direct contribution of PHT5 proteins to phosphorus influx into the vacuole, ^{31}P-MRS (31-phosphorus magnetic resonance spectroscopy)-based analysis was performed in *Arabidopsis* (Liu et al. 2016). The analysis differentiated between cytoplasmic and vacuolar phosphorus in plant tissues due to pH differences and revealed that *pht5* plants accumulated less phosphorus in their vacuoles, while *PHT5* overexpressing genotypes increased phosphorus significantly (Liu et al. 2016). Given that three *PHT5* genes are regulated by miR827 (Liu et al. 2016), above observation suggests that miR827 may play a role in phosphorus homeostasis by regulating the *PHT5* gene.

Other microRNAs have been found in response to nitrogen and phosphorus deficiencies (Nguyen et al. 2015). In response to phosphorus limitation, plants increased the expression level of miR444a in rice (Yongsheng et al. 2014), PvmiR399 in common bean (Valdes-Lopez et al. 2010), miR398 and miR399 in

tomato (Gu et al. 2010), and miR399 in *Medicago truncatula* (Branscheid et al. 2010). This indicated that microRNAs may have a role in developing tolerance to phosphorus deficiency in various plant species. In line with this, miR399 was reported to mediate phosphorus deficiency by regulating the expression of PHO_2, a negative phosphorus level regulator that encodes an ubiquitin-conjugating enzyme E2 enzyme (Aung et al. 2006; Chiou et al. 2006). Accordingly, *pho2* silenced plants had increased phosphorus content under low phosphorus conditions, which is similar to the phenotype of miR399 overexpression (Chiou et al. 2006). Similarly, a wheat miRNA TaMIR1139 has been shown to play a critical role in modulating Pi (inorganic phosphorus) deficiency tolerance in plant by directly or indirectly regulating a phosphate transporter gene (*NtPT*). Tobacco plants overexpressing TaMIR1139 showed enhanced expression of *NtPT* that improved Pi acquisition upon Pi starvation (Liu et al. 2018).

3 Biotic Stress

miRNAs repress gene expression through RNA silencing, and they are known for their role in mediating plant responses to biotic stress. Studies have shown that plant endogenous (not pathogen-derived) miRNAs regulate broad physiological functions particularly in response to various microbes. As a defense strategy, microbes have also evolved mechanisms to undermine and inhibit RNA silencing by miRNAs, triggering counter defensive measures by plants. This points to a never-ending molecular arms race between hosts and parasites (Ruiz-Ferrer and Voinnet 2009).

3.1 Fungal Defense

Phytopathogenic fungi are one of many microbial pathogens that attack plants to obtain nutrients, adversely affecting plant growth and development and resulting in reduced yield and ultimately plant death. miRNAs have been associated with the initial and more general basal defense mechanisms consisting of pathogen-associated molecular patterns (PAMP) and pattern-triggered immunity (PTI) (Jones and Dangl 2006). PTI is initiated by pattern-recognition receptors (PRRs) that recognize PAMPs and induce physiological and biochemical changes and reprogramming in the plant in order to defend against and isolate the pathogen (Segonzac and Zipfel 2011). During this process, plants recognize fungal cell wall components such as chitin (Baureithel et al. 1994) and β-glucan (Klarzynski et al. 2000), cell membrane components such as ergosterol (Granado et al. 1995), infection-associated enzymes such as xylanase (Hanania and Avni 1997) and elicitin (Osman et al. 2001), and other proteins (Nurnberger et al. 1994). In *Arabidopsis*, miR396 regulates PTI via its target growth-regulating factor (GRF) transcription factors (Soto-Suarez et al. 2017). After challenging *Arabidopsis* with

various fungi, miR396 expression is reduced, and this confers fungal resistance (Soto-Suarez et al. 2017).

miRNAs also target various resistance (R) genes associated with the acquired defense effector-triggered immunity (ETI) plant response which is more specific to individual pathogens (Jones and Dangl 2006). miR1448 targets the nucleotide-binding site-leucine-rich repeat (NBS-LRR) of the R gene, as well as the *POLYPHENOL OXIDASE* (*PPO*) gene, and deep sequencing determined that miR1448 was downregulated in both cotton (Yin et al. 2012) and poplar (Zhao et al. 2012) after fungal infection. Additionally, another R gene, *TOLL INTERLEUKIN-1 RECEPTOR* (*TIR*), is targeted at its *NBS-LRR* site by miR2118 (Jagadeeswaran et al. 2017) and is downregulated under *Verticillium* infection of cotton roots (Yin et al. 2012).

Like in many other stress conditions, miRNAs play a role in fungal resistance by altering hormone regulation to induce plant defense response. The expression of five hormone signaling-associated miRNAs was differentially regulated in response to fungal infection in wheat (Gupta et al. 2012), while nine were differentially regulated in pine (Lu et al. 2007). Similarly, increased IAA is associated with fungal susceptibility in barley, while fungal resistant barley accumulated less hormone (Vizarova 1968). Finally, ethylene signaling was altered in cotton infected by *Verticillium dahlia* by silencing *miR1917* via its four target genes which regulate ethylene signaling (Yin et al. 2012). In addition to the regulation of plant genes in fungal response, cotton actually exports miRNAs, including miR159 and miR166, to downregulate fungal virulence genes *HiC-51* and *CLP-1*, respectively (Zhang et al. 2016).

High-throughput sequencing technologies have identified a range of fungal-responsive miRNAs across various plant species in response to various fungi. The expression of 65 miRNAs was differentially expressed in response to *Verticillium* infection (Yin et al. 2012). Sequencing identified 27 novel miRNAs that were differentially expressed in poplar in response to *Dothiorella gregaria* in addition to members of 37 conserved miRNA families (Chen et al. 2012a). Ten out of 93 identified novel miRNAs were differentially regulated in response to *Plasmodiophora brassicae* infection in Chinese cabbage as well as 14 conserved miRNAs (Wei et al. 2015).

Microarray-based analysis of miRNAs in soybean infected by *Phytophthora sojae* revealed potential fungal resistance-associated miRNA expression patterns by comparing susceptible and resistant soybean cultivars (Guo et al. 2011). This study identified nine soybean miRNAs from six families associated with fungal resistance. One miRNA, miR858, is a negative regulator of *Plectosphaerella cucumerina* infection by reducing the abundance of specialized metabolites in the phenylpropanoid pathway (which plays an antifungal role) (Camargo-Ramirez et al. 2018).

Microarray assays also identified 41 *Botryosphaeria dothidea*-responsive miRNAs in poplar (Zhao et al. 2012), 87 fungal-responsive miRNAs in wheat (Inal et al. 2014), and 33 *Magnaporthe oryzae*-responsive miRNAs in rice (Li et al. 2014b). In addition to the latter responsive genes, rice plants overexpressing

the auxin regulation-associated miR160 and CSD-associated miR398 were resistant to *M. oryzae* (Li et al. 2014b). Genomics approaches to miRNA identification can reveal novel miRNAs associated with pathogen response as well as reveal the roles of miRNAs whose targets may not be directly associated with fungal resistance.

3.2 The Role of MicroRNAs in Mediating Bacterial and Viral Infections in Plants

Plants perceive disease predominantly using PAMPs such as flagellin from bacteria (Zipfel 2014). Tolerant varieties perceive PAMPS and then induce highly specific defense mechanisms (Bigeard et al. 2015; Cui et al. 2016). In *Arabidopsis*, a flagellin-dependent 22-amino acid peptide (flg22) was found to increase the abundance of miR393 that reduces the expression of auxin receptors from the F-box such as TIR1, AFB2, and AFB3 (Navarro et al. 2006; Yu et al. 2015). Accordingly, flg22-based enhancement of miR393 reduces the expression of *TIR1* and also causes the accumulation of glucosinolate defense metabolites (Robert-Seilaniantz et al. 2011). *Arabidopsis* plants with increased expression of TIR1 showed susceptibility to the virulent bacteria, *Pseudomonas syringae*, while enhancing miR393 increased resistance (Navarro et al. 2006).

Apart from the model plant *Arabidopsis*, immunity to the blast fungus *Magnaporthe oryzae* was negatively regulated by miR169 in rice (Li et al. 2017b). The increased expression of miR169 reduced the transcript level of nuclear factor YA (NF-YA), one of the three heterotrimeric transcription factors that binds at the CCAAT promoter-binding motif, while simultaneously promoting growth, development, and stress tolerance (Mantovani 1999; Zhao et al. 2016). This is also demonstrated with target mimicry genotypes (*MIM169*) that increased the expression of downstream miR169-regulated genes and increased tolerance to *M. oryzae*. Apparently, the increased abundance of miR169 was found in concert with a potato-virus-Y-infected potato defense mechanism that lowered the level of gibberellin signaling (Kriznik et al. 2017). Upon viral infection, the plants showed reduced transcript levels of genes coding for gibberellin biosynthesis (such as GA3-oxidase) under the direct regulation of *phasi*RNA393 (Križnik et al. 2017).

A network of miRNA with *si*RNAs regulating viral infection tolerance was suggested earlier by (Pérez-Quintero et al. 2010). These authors used bioinformatics tools to understand microRNA and *si*RNA involvement using miRNA sequences of six plant species (*Arabidopsis*, soybean, rice, sorghum, grape vine, and maize) (Pérez-Quintero et al. 2010). *si*RNAs and miRNAs are involved in posttranscriptional gene regulation by forming double-stranded RNA, which forms a defense mechanism against the intruding viral nucleic acid (Baulcombe 2005; Bazzini et al. 2007). Others reported the use of artificial miRNA, *ami*RNA, in developing barley cultivars resistant to wheat dwarf virus (Kis et al. 2015). The authors constructed an artificial polycistronic *ami*RNA precursor (composed of three *ami*RNAs) from

miR171 and then developed stable transgenic barley plants resistant to wheat dwarf virus at lower temperatures (Kis et al. 2015). Contrasting studies showed that a reduced level of miR171 resulted in severe strip virus in rice with reduced expression level of the target *SCARECROW-LIKE6*, (*OsSCL6-IIa*, *OsSCL6-IIb*, and *OsSCL6-IIc*) (Tong et al. 2017).

Lee et al. (2015) reported miR844 as a negative regulator of cytidinephosphate diacylglycerol synthase3, CDS3, which is involved in tolerance to *Pseudomonas syringae* in tomato DC3000 and the fungus *Botrytis cinerea* in *Arabidopsis*. The authors showed that *Arabidopsis* plants with enhanced expression of miR844 had reduced CDS3 expression resulting in disease susceptibility, while plants with reduced expression of miR844 had enhanced CDS3 expression and improved bacterial and fungal tolerance (Lee et al. 2015).

4 Identifying Plant miRNA from Deep Sequencing Using Bioinformatics Approaches

MicroRNAs (miRNAs) are vital regulatory sequences, and identifying their targets is a critical step toward developing crops resilient to environmental stress. There are few reliable tools available for identification of miRNA targets in plants, and next-generation RNA sequencing is one of the most promising ones. An emergence of NGS technologies, such as the Roche 454 GS System®, the Illumina Genome Analyzer®, the Applied Biosystems SOLiD® System, and the Helicos Heliscope®, have revolutionized the identification of miRNAs, as well as their profiling and expression analysis in eukaryotes (including in plants). These tasks can be accomplished for already known and novel miRNAs with low to high levels of expression (Huang et al. 2010a). Being relatively less expensive and having high efficiency, NGS has been established as one of the most promising tools for miRNA research. To utilize NGS platform to its maximum capacity, however, extensive bioinformatics tools still need to be developed to handle large and ever growing genomics datasets. Below, we described tools available to identify miRNAs and their targets in plants under stress treatment or non-stressed conditions. Figure 2 shows the schematic workflow to discover stress-related miRNA in plants. Five major components of this workflow include raw reads processing, read mapping, miRNA annotation, de novo assembly, and downstream analysis.

4.1 MicroRNA Databases

The rapid advancement in the next-generation DNA sequencing methods has created an enormous amount of plant small RNAs data, resulting in more than one miRNA annotation. This makes many miRNA annotations dubious and annotation criteria in

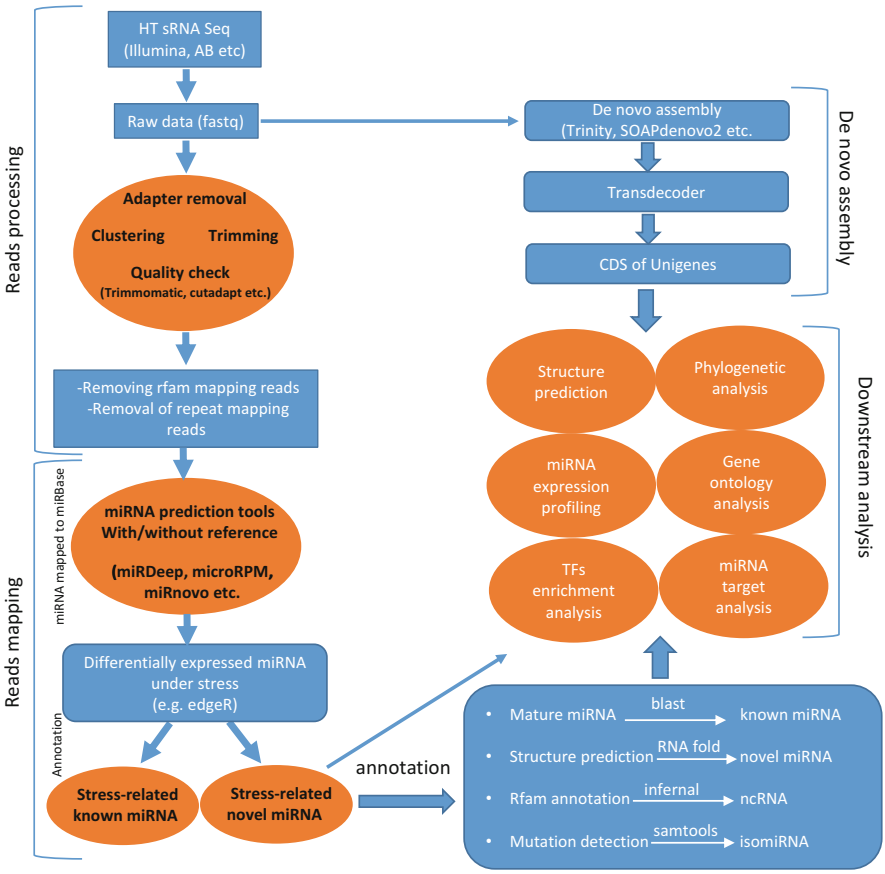

Fig. 2 The pipeline workflow for stress-related miRNA discovery based on small RNA sequencing analysis in plants

need of evaluation. The updated criteria are focused on NGS of small RNAs and replication to minimize false positives (Axtell and Meyers 2018). The large number of small RNAs, the abundance of RNA sequence diversity contributed by siRNAs, and the rapid increase in plant small RNA discovery require an increased focus on miRNA annotations (Meyers et al. 2008).

The recent miRBase (www.miRBase.org) is a database of published miRNA sequences and annotation across all the studied species (Kozomara and Griffiths-Jones 2014). This database serves the purpose of assigning miRNA loci nomenclature and provides an easy access to all known miRNAs and their sequences. As of this writing, miRBase is at version 22 (released in March 2018), containing 38,589 hairpin precursor miRNA entries expressing 48,885 mature miRNA products in 271 species. Despite all this information, miRBase is not primarily used to enforce the quality of miRNA annotations. Instead, the responsibility for miRNA annotation mainly lies on researchers, peer reviewers, and journal editors. Taylor et al. (2014)

have provided an important analysis regarding plant miRNA annotations present in miRBase version 20 where they marked questionable authenticity of approximately 75% of miRNA annotation (1,351 out of 1,802).

At par with Taylor et al. (2014), Axtell and Meyers (2018) set out criteria for miRNA annotation that could be helpful in avoiding false annotation. These include (1) excluding secondary stem or large loop in the miRNA/miRNA* duplex and limiting precursor length to 300 bp; (2) accepting confirmation by sRNA-seq and rejecting confirmation by blots; (3) allowing up to five mismatches out of which only three should be nucleotides in asymmetric bulges; (4) while calculating precision, one-nucleotide positional variants of miRNA and miRNA* should be included; (5) for annotation, at least two biological replications should be required for sRNA-seq; (6) homology-based annotation should be considered as provisional, which should include confirmation by sRNA-seq only; and (7) RNAs with length more than 20 nt and less than 24 nt should not be considered as miRNAs unless strong evidence is provided (Axtell and Meyers 2018). The authors have suggested that the current system of annotation should be replaced with a more efficient and automated one, which would allow uploading of candidate sequences in a standardized format, and the abovementioned criteria should be integrated into this system.

Several databases have been made to identify miRNAs that regulate genes responsible for agronomic traits such as yield and stress responses. For example, (Remita et al. 2016) developed a resource that provides information on miRNAs responsive to abiotic stress in wheat. This database also provides access to miRNA prediction software in wheat and other plant species (Remita et al. 2016). A study in cardamom identified 20 novel and 150 conserved miRNAs in control and drought stress conditions including 17 differentially expressed (Anjali et al. 2017). Similarly, Munusamy et al. (2017) used a bioinformatics approach to predict miRNA target sites in the *Arabidopsis* transcriptome to help understand gene regulation during stress in plants. A similar study was conducted in cowpea where 46 new miRNAs were identified for abiotic and abiotic stress responses (Gul et al. 2017). Such studies may provide insights into the miRNA-mediated regulatory mechanisms in response to various stresses and allow for the development of tools to improve stress tolerance.

4.2 Bioinformatics Tools Available for miRNAs Prediction

There are several plant microRNA prediction tools available, but these tools come with some problems such as (1) a high false-positive rate, (2) a long run time, (3) working only for genomes with existing databases, and (4) packages that are hard to install or use (Lei and Sun 2014). Of these, the miRDeep uses a probabilistic model of miRNA biogenesis to predict the position and frequency of sequenced RNA having secondary structures typical of miRNA precursors (Friedlander et al. 2008). (Xie et al. 2011) developed the target-align algorithm, which is similar to Smith-Waterman alignment tool (Smith and Waterman 1981) and can accurately

predict miRNA targets based on complementary nucleotide sequences by considering maximum mismatches between miRNAs and their targets. An improved version of miRDeep, miRDeep-P, that is highly accurate in predicting miRNAs in plants has also been developed (Yang and Li 2011). Similarly, miRDeep2 can identify hundreds of novel miRNAs mainly in animals with 98.6–99.9% accuracy (Friedlander et al. 2012). The miRDeepFinder is a software package available for identification and functional analysis of plant miRNAs including their targets using NGS data. miRDeepFinder design is based on miRNA biogenesis, miRNA-regulated gene expression, and identification of targets (Xie et al. 2012).

The miRPlant is another software tool available for miRNA prediction. This software seems to be user-friendly and shows about 10% improved accuracy when compared with miRDeep-P – the more popular plant miRNA prediction tool (An et al. 2014). The *miR*NA *PRE*diction *f*rom small *R*NA-seq data (miR-PREFeR) uses miRNA expression patterns for prediction of plant miRNAs from RNA-seq data. After being tested on several plant species, miR-PREFeR was found to be highly sensitive and accurate (Lei and Sun 2014).

The *P*lant *S*mall RN*A* *M*aker *S*ite (P-SAMS) is a web-based tool to design artificial miRNAs (*ami*RNAs) as well as synthetic *trans*-acting small interfering RNAs (*syn-tasi*RNAs) in plants. The P-SAMS has two different applications, called P-SAMS *ami*RNA Designer and P-SAMS RNA Designer, which can be used for customized purposes (Fahlgren et al. 2016). The miRCat2 is another miRNAs prediction tool for animals and plants, and comparative analysis shows miRCat2 has an improved efficiency and accuracy compared to miRCat, miRDeep2, miRPlant, and miReap. Furthermore, miRCat2 can predict novel miRNAs differentially expressed in control wild type when compared to mutants in the miRNA biogenesis pathway (Paicu et al. 2017).

Yu et al. (2017) introduced a bioinformatics-based workflow to analyze miRNA-modulated transcripts. Plant microRNA-mediated nascent transcript slicing analyzer (PmiRNTSA) is a package that can be used for comprehensive search for miRNA cleavage sites, which is supported also by degradome sequencing data. This package along with workflow helps in investigating the regulatory models of miRNAs in plant species of choice. Jha and Shankar (2013) introduced for the first time, miReader, for discovery of novel miRNAs without using reference sequences. Authors tested this tool for NGS data and a range of species including plant, human, and animals and reported above 90% accuracy.

Recently, the Mirnovo tool was developed utilizing a machine learning-based algorithm to predict sequences of both novel and known miRNA from the small RNA-seq data, with or without using a reference genome in plants (Vitsios et al. 2017). Mirnovo is simple, user-friendly, web-based tool with a shorter run time that provides robust miRNA prediction particularly in non-model plants with a poorly assembled genome. Another important feature of Mirnovo is to make its use in miRNA prediction from single cell sequencing by using genomic features. Single-cell RNA sequencing has already become an active area of research (Gawad et al. 2016). The fact that some miRNAs have been shown to be cell type specific suggests that tools like this may be extremely useful for novel miRNA discovery in plants

(Vitsios et al. 2017). Without using a reference genome, Mirnovo performs comparably or better than miRDeep2 for known miRNAs prediction. Regarding novel miRNA, a higher prediction rate was observed in Mirnovo compared to miRDeep2. Moreover, Mirnovo also runs significantly faster than miRDeep2. When a reference genome is used in both tools, a slightly improved sensitivity is observed in Mirnovo compared to miRDeep2 (Vitsios et al. 2017).

More recently, Tseng et al. (2018) developed a tool called MicroRPM that accurately predicts miRNA from small RNA sequencing data of plants using a support vector machine algorithm. MicroRPM performs more efficiently than miRDeep2 and miReader with prediction accuracy up to 98% without using a reference genome (Tseng et al. 2018). The MicroRPM contains three effective models (no-reference-required, dicot-reference-required, and monocot-reference-required) and can freely be downloaded at http://microRPM.itps.ncku.edu.tw. Both the miRDeep2 (Friedlander et al. 2008; Mackowiak 2011) and miReader (Jha and Shankar 2013) are considered efficient tools for miRNA prediction using NGS data. miReader is the only tool that does not require an already sequenced genome for prediction of miRNAs. Given that miReader consumes more time and had limited accuracy (56.95%) for miRNA detection in *Arabidopsis*, it is important to note that both the "reference-required" and "reference-not-required" model of MicroRPM showed better performance compared to other tools. Sensitivity higher than 95%, specificity, precision, and accuracy were recorded in MicroRPM when an *Arabidopsis* dataset was used to predict miRNAs based on the reference-not-required model (Tseng et al. 2018). Previously, NGS reads have been equally mapped to both strands of pre-miRNA in some studies including miR822 and miR839 (Rajagopalan et al. 2006). Therefore, the miReader prediction may be biased (Jha and Shankar 2013) because its model was built based on read count differences, and this problem can be overcome by using MicroRPM. Moreover, the MicroRPM "reference-required" model is not only suitable for dicots but also monocots such as rice (Tseng et al. 2018).

5 Future Prospects

Current climate change models predict an increase in average surface temperatures of 3 to 5°C in the next 5 to 10 decades. This stress may have deleterious effects on crop plant growth and productivity (Teixeira et al. 2013). Identification of miRNAs using genomics-based approaches has emerged as an efficient and versatile approach and hence has become one of the most vital tools currently being used in molecular biology research, particularly to enhance tolerance to environmental stress. In the era of NGS, large datasets can rapidly be produced, and candidate miRNAs, genes for the trait of interest, and signaling pathways can be explored in a variety of plant species. To take full advantage of this technology, a rapid, precise, and effective method is highly desired to dissect the function of miRNAs to fill gaps in our understanding about their role in crop improvement under stress conditions.

Using computational power and its affiliated algorithms, precision in miRNAs identification can be improved manifold. The lack of complete knowledge on (1) the steps involved in miRNA processing and biogenesis, (2) enzymes needed to properly carry out miRNA function, and (3) miRNAs recruiting machinery is a big hindrance in rapidly dissecting miRNA families and their function in crop improvement. For example, functional characterization of AGO1 protein family members that contain vital regulatory functions has helped to fulfill previous goals of functional exploration of these proteins (Meng et al. 2011).

Taken together, decreasing costs and fast sequencing reactions provided by NGS technologies have positively influenced miRNA discovery, application, and crop improvement through various genetic, genomics, and molecular approaches both in model and non-model plants (Fig. 1). NGS technology has enabled researchers and scientists to understand molecular mechanisms underlying miRNA and its target genes and explore processes of gene expression, gene identification, and annotation particularly in non-model plants with unavailable sequenced genomes (Fig. 1).

Predicted climate changes may intensify stress on crop plants, and thus the type of data that are needed to improve crops for stress tolerance need to be accessed by combining scientific knowledge with new technologies. Thus, molecular tools such as miRNAs will certainly continue to make substantial contributions at improving crop plants for stress management.

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Recent Advances in MS-Based Plant Proteomics: Proteomics Data Validation Through Integration with Other Classic and -Omics Approaches

María-Dolores Rey, Luis Valledor, María Ángeles Castillejo, Rosa Sánchez-Lucas, Cristina López-Hidalgo, Victor M. Guerrero-Sanchez, Francisco J. Colina, Mónica Escandón, Ana M. Maldonado-Alconada, and Jesús V. Jorrín-Novo

Contents

Abstract While reviewing the plant proteomics topic, both from a conceptual and methodological point of view and with a historical perspective, recent advances, current states, challenges, and future directions of the field are discussed. Proteomics

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M.-D. Rey, M. Á. Castillejo, R. Sánchez-Lucas, V. M. Guerrero-Sanchez, M. Escandón, A. M. Maldonado-Alconada, and J. V. Jorrín-Novo (✉)
Agroforestry and Plant Biochemistry, Proteomics, and Systems Biology (AGR-164), Department of Biochemistry and Molecular Biology, University of Córdoba (UCO-CeiA3), Córdoba, Spain
e-mail: bf1jonoj@uco.es

L. Valledor and F. J. Colina
Department of Organisms and Systems Biology, University Institute of Biotechnology (IUBA), University of Oviedo, Oviedo, Spain

C. López-Hidalgo
Agroforestry and Plant Biochemistry, Proteomics, and Systems Biology (AGR-164), Department of Biochemistry and Molecular Biology, University of Córdoba (UCO-CeiA3), Córdoba, Spain

Department of Organisms and Systems Biology, University Institute of Biotechnology (IUBA), University of Oviedo, Oviedo, Spain

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is moving, even though very slowly, from the descriptive era to a new era in which data start to be validated and integrated with other classic and -omics approaches, in the Systems Biology direction. This review is organized in different sections, starting with a general and historical introduction, moving to platforms and techniques employed currently, data validation to confidently conclude from a biological point of view, workflow in a multi-omics experiment, highlighting some illustrative investigations, and finishing with some conclusions and remarks. As quite reviews have been already published in the field of mass spectrometry-based proteomics and to avoid being repetitive, this review only reports the most relevant contributions published recently by our research groups and those found in the literature.

1 Introduction

In the simplest sense of the term, proteomics, considered as a scientific discipline or methodological approach, deals with the study of proteins, whose cellular complete set constitutes the proteome. What was originally termed protein chemistry or protein biochemistry becomes nowadays proteomics. This transition, during the 1990s, was the result of genome sequencing projects, development of mass spectrometry (MS) for proteins, and bioinformatics tools to analyze the huge amount of data generated. Proteomics includes all the techniques employed in protein/peptide analysis, regardless of the living organism from which proteins come or the procedure employed (in situ or in vitro experiments, in vitro mRNA translation, or chemical synthesis). Although it is mostly associated with MS (MS-based proteomics), proteomics also includes other techniques such as those based on immunochemistry (e.g., ELISA, western) and structural biology (X-ray diffraction and cryo-electronic microscopy, among others). It addresses to characterize proteins, in either pure or crude preparations, from a physicochemical and biological point of view. The present chapter is focused on in vitro MS-based techniques, directed at interpreting plant biology using the protein language. In this direction, the main goal is to understand "...how, where, when, and what for are the several hundred thousand individual protein species/forms produced in a living organism; how they interact with each other and with other molecules to construct the cellular building, and how they can be modified and work in order to be assimilated into a programmed growth and development and interact with their biotic and abiotic environments" (Jorrín 2015).

It was by 1994, just after J. B. Fenn and K. Tanaka developed the matrix-assisted laser desorption/ionization, MALDI, and electrospray ionization, ESI, soft ionization methods for MS analysis of proteins (Tanaka et al. 1988; Fenn et al. 1989), that the two first papers reporting the MS-based analysis of plant protein were published (Klabunde et al. 1994; Egorov et al. 1994). In both works, the technique was

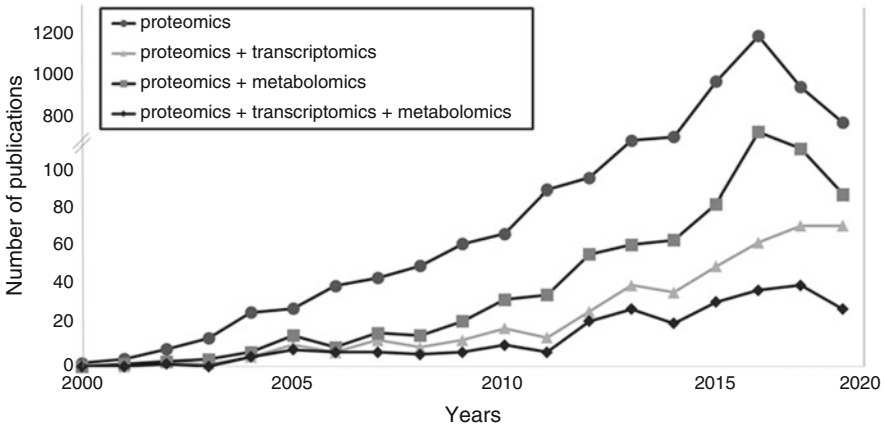

Fig. 1 Number of references reported at PubMed (2000–2018). The search was performed with the key words: plant + proteomics (circle), plant + proteomics + transcriptomics (triangle), plant + proteomics + metabolomics (square), and plant + proteomics + transcriptomics + metabolomics (diamond)

employed in a classic targeted approach, directed at sequencing a phosphatase and an avenin, respectively. It was not until 1990–2000, once the proteomics term was coined and admitted by the scientific community (Wilkins et al. 1996), that the first holistic analysis of plant protein extracts was reported (Kehr et al. 1999; Natera et al. 2000; Chang et al. 2000; Peltier et al. 2000). The potential of MS in plant protein analysis (Li and Assmann 2000), biological research (Thiellement et al. 1999), and translational application (Alomirah et al. 2000) was immediately recognized. The first review and monography (compilating current protocols for plant proteomics analysis) was by the French pioneering group (Thiellement et al. 1999). Since 2000, the number of plant proteomics papers has exponentially increased (Fig. 1), with a total of 8,141 items reported at PubMed up to 2018 ("plant proteomics" as search terms). Original papers belong to the following areas: descriptive (e.g., whole plants, organs, tissues, cells, subcellular fractions), comparative (e.g., species, genotypes, developmental stages, responses to environmental conditions, biotic and abiotic stresses), posttranslational modifications (e.g., phosphorylation, glycosylation, methylation, acetylation), interactomics (e.g., protein-protein, protein-nucleic acids, protein-metabolite), proteinomics (e.g., MS analysis of individual proteins), and, finally, translational proteomics. The latter, dealing with the practical application of the technique, will not be discussed in this chapter because it has been widely discussed in previous reviews (Jorrín-Novo and Valledor 2013; Komatsu and Jorrín-Novo 2016). Most of the plant proteomics papers published so far belong to the descriptive, subcellular, and comparative proteomics subgroups, especially in the 2000–2010 decade (Jorrín-Novo et al. 2009, 2015). The proteome of plants in different biological contexts has been reported for at least a hundred species, including model systems (e.g., *Arabidopsis*; Baginsky and Gruissem 2006), crops (mostly rice; Kim et al. 2014), and wild species

(e.g., forest trees; Abril et al. 2011). As the main objective of proteomics is to identify and quantify as many proteoforms as possible in a single experiment, the success will depend on the experimental system, the MS strategy, equipment, and algorithms employed. As for the experimental system, the challenges are related to the recalcitrant nature of the species of interest that is a consequence of the chemical composition of the sample and quality of the protein extract, as well as whether the genome has been sequenced and availability of protein sequences in databases.

The topic of plant proteomics has been widely and continuously reviewed, with a considerable number from our research group (Park 2004; Corpillo et al. 2004; Chen and Harmon 2006; Cánovas et al. 2004; Rossignol et al. 2006; Jorrín-Novo et al. 2007, 2018; Carpentier et al. 2008; Abril et al. 2011; Whitelegge et al. 2011; Kosová et al. 2011; Jorrín-Novo and Valledor 2013; Jorrín-Novo 2014; Sanchez-Lucas et al. 2016; Xing et al. 2016; Rey et al. 2019). In order to prevent repeated reviews, the present chapter has been written based on our original publications and those that appeared published by other groups in the last 5 years. Following the recommendations from *Progress in Botany*, we have intended "to give a critical point of view, draw conclusions, suggest further research directions, and hint at applications of new techniques."

2 Technical Platforms and Methods

Since the early 2000s, when the only employed platform for plant proteomics analysis was gel electrophoresis coupled to MS, mostly matrix-assisted laser desorption ionization time of flight (MALDI-TOF), continuous improvements in methods, protocols, and equipment have taken place (Thelen and Peck 2007; Jorrín-Novo 2014). In a very short period of time, less than 20 years, we have moved quickly from first generation proteomics (two-dimensional gel electrophoresis, 2-DE-based) (Jorrín-Novo et al. 2018), to second (difference gel electrophoresis, DIGE, and multidimensional protein identification technology, MudPIT) (Koller et al. 2002; Arruda et al. 2011), to third (labeling techniques, with isobaric tag for relative and absolute quantitation, being isobaric tags for relative and absolute quantitation, iTRAQ, the most representative technology) (Sankaranarayanan et al. 2013; Velez-Bermudez et al. 2016), to fourth (gel-free/label-free or shotgun) (Neilson et al. 2011), to fifth (single reaction monitoring, SRM, multiple reaction monitoring, MRM, targeted or mass western) (Picotti et al. 2013; Lyon et al. 2014), and to sixth (data-independent acquisition, DIA, and its sequential windowed data independent acquisition of the total high-resolution mass spectra, SWATH, variant) (Gillet et al. 2012; Lau et al. 2018; Aguilar-Hernandez and Loyola-Vargas 2018; Arsova et al. 2018). Beyond getting to the limit, wet classical techniques of protein chemistry (protein extraction and separation by electrophoresis or chromatography), improvements in MS analyzers, and bioinformatics tools have taken proteomics to an unimaginable achievement in terms of number of protein species confidently identified, quantified, and characterized (Matthiesen 2007; Jorrín-Novo 2014; Calvete 2014; Chen 2017; Mirzaei and Carrasco 2016; Calderon-Gonzalez et al. 2016; Misra 2018). Using technical developments, we have moved from hundreds to

thousands protein species confidently identified and quantified in a single experiment. As a result, a high number of plant protein databases and repositories have been created (Sakata and Komatsu 2014; Misra 2018).

The workflow of a typical proteomics experiment (Fig. 2) includes well-defined steps, in which the mentioned techniques above are employed, either alone or in

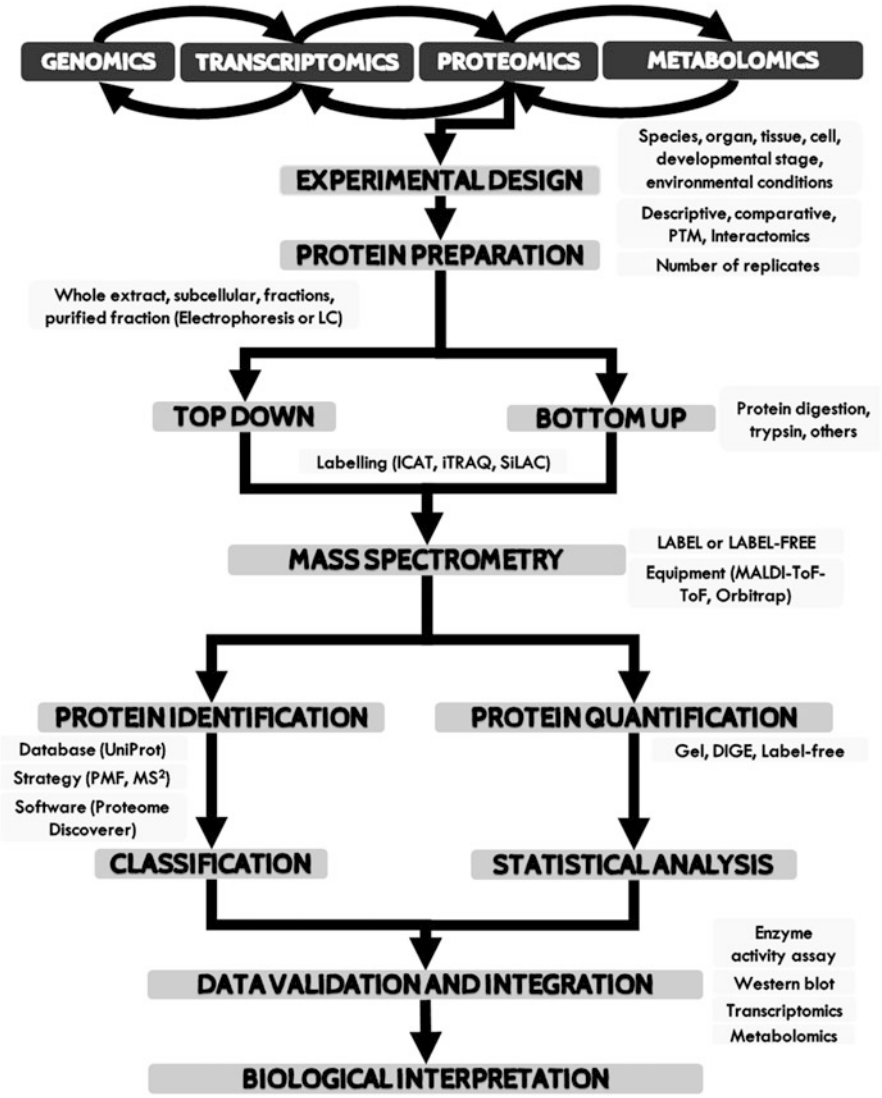

Fig. 2 Workflow of a proteomics experiment, from experimental design to biological interpretation through sample preparation, protein purification, mass spectrometry analysis, identification, and quantification. It includes alternative, complementary approaches, or strategies, based on MS analysis of proteins (top-down) or tryptic peptides (bottom-up), using gel-based or gel-free platforms

combination. The platforms and protocols of choice will depend on the experimental system, objectives, and starting hypothesis. This workflow includes stages that are dependent (experimental unit and protein identification) and independent (MS analysis) of the experimental system. The first phase of the experiment corresponds to the definition of the experimental unit [plant, organ, tissue, cell, subcellular fraction, electrophoresis, or liquid chromatography purified fractions (Jorrín-Novo et al. 2015)] and the experimental design (e.g., biological and analytical), conditioning the statistical analysis of the data (Valledor and Jorríin 2011). Once plant samples are collected and frozen in liquid nitrogen, protein extraction must be immediately conducted or else stored at −80°C or lyophilized. Mass spectrometry is, except for the imaging technique (Qin et al. 2018), an in vitro technique in which proteins must be extracted from tissue, cells, or subcellular fractions and then solubilized, and, although not necessary, fractionated, or purified using electrophoretic or chromatographic techniques. The possibilities and limitations of the protocols employed for extraction and fractionation have been deeply discussed in previous reviews (Jorrín-Novo 2014; Jorrín-Novo et al. 2015; Rey et al. 2019). So far, the results obtained, in terms of captured proteins in solution, depend on the plant system as well as organs and their chemical composition.

The second phase of the workflow encompasses the MS analysis, which is independent of the experimental system. This analysis can be performed with whole crude extracts, partial purified fractions, or pure proteins. Protein fractionation and depletion of major proteins, as, for example, RuBisCO in leaf samples, are good strategies for detecting minor proteins and, hence, obtaining a deeper proteome coverage (Widjaja et al. 2009). MS can be performed with intact proteins (top-down) (Thangaraj et al. 2010; Zhang et al. 2011) or peptides obtained by trypsin proteolysis (bottom-up; Blein-Nicolas and Zivy 2016), or by combining them with bottom-up, which is the most common option (Thangaraj et al. 2010). For comparative purposes or relative quantitation of proteins or their derived peptides, those can be in vitro or in vivo labeled with isotopic or isobaric tags, resulting in different techniques, being iTRAQ the most employed technique (Schulze and Usadel 2010). So far, the MS analysis is the end of the wet part of the workflow (Fig. 2).

The third phase corresponds to protein identification from MS spectra. As the first phase of the workflow, it is also dependent of the experimental system as the proteoform identification relies on the comparison between both the experimental m/z and theoretical data, deduced from the sequences found in databases or by searching in spectral libraries. For that, the classical data-dependent or the more recent data-independent strategies can be employed (Rose et al. 2004; Friso et al. 2004; Law and Lim 2013; Bauer et al. 2014; Chapman et al. 2014). Data-dependent acquisition is the most employed approach, in which a MS scan survey is performed, creating a list of parent ions with different m/z values and intensities. These ions are selected for a second MS/MS scan, in order of decreasing intensity. In this strategy, the second scan depends on both the first survey and selected parental ions. In data-independent acquisition, a previous survey is not necessary. Instead, a m/z window is selected, and all the ions fragmented, obtaining at the same time the m/z

of the parental ions and derived fragments. For such approaches, different algorithms have been developed (Bakalarski et al. 2007; Zhu et al. 2018).

The workflow and techniques employed should be optimized for each experimental system, objectives, and hypothesis, considering that not only proteins but also the remaining molecular components of the cell may affect the experiment results. As any analytical technique, MS should be validated, and its properties in terms of resolution, sensitivity, detection limit, and dynamic range should be taken into consideration; otherwise the conclusions in terms of relative quantitation may be erroneous (Gonzalez-Fernandez et al. 2013).

Even though the huge potential of proteomics, it is still not enough to cover and decipher the simplest whole proteome. If we consider the most successful experiment, it is even impossible to reach 10,000 proteoforms identified, which would represent a small fraction of the total proteome (Rey et al. 2019). This problem is partially solved by purifying the subcellular organelles or fractionating proteins by electrophoretic or chromatographic techniques. The limitations are most related to the proteins themselves than to the techniques. We now realize the complexity of the proteomics analysis, related to the number of genes, protein species per gene, dynamic range, and the physicochemical complexity. DNA and RNA molecules are simpler from a chemical point of view and can be amplified by PCR which simplifies the experimental design, avoiding sample limitations. On the other side, despite metabolites are much simpler molecules than proteins, due to its size, they are more complex from a physicochemical point of view (Rey et al. 2019) and, in general, more difficult to be identified (especially those secondary metabolites where purified or synthetic reference compounds are not available).

In a standard workflow such as 2-DE-MS, the covered proteome is generally low due to the presence of minor proteins and the decrease of the number of proteins obtained in each step of the workflow. Hence, when planning a proteomics experiment, some questions arise:

1. How many proteins are extracted and solubilized? From those, how many unmodified?
2. How many proteins are lost during separation (gel electrophoresis or liquid chromatography)?
3. How many proteins are visualized or provide signals during separation?
4. How many peptides are generated during the digestion? How many are lost during the digestion step?
5. Do all the peptides generate a signal in the mass spectrometer? How many of them are fragmented?
6. Do the MS data lead to protein identification depending on algorithms, databases?

Although proteomics is not the "panacea" or "Pandora box," its potential and invaluable contribution to biological knowledge should be recognized. In the case of plants, with the exception of *Arabidopsis* and rice, and despite all the technical possibilities, the full potential of proteomics is far from being fully exploited, mainly if it is compared to other organisms, especially humans (as an example, have a look

to the web pages of the Human Proteome Organization, HUPO, https://www.hupo.org/, and the Multinational *Arabidopsis* Steering Committee, MASC, http://www.masc-proteomics.org/databases.html). Even in this stage, most of the plant proteomics works published employ well-established techniques, moving from gel-based to label (iTRAQ) or to shotgun approaches.

3 From Proteomics to Biology Through Data Validation and Integration with Other Classic and -Omics Approaches

In 2011, in the Tsukuba 3rd International Symposium "Frontiers in Agriculture Proteome Research. Contribution of proteomics technology in agricultural sciences," while researchers were discussing about the contribution of proteomics to plant biology knowledge, it was concluded that *proteomics by itself is mostly descriptive and speculative* (by Jan Myernik, University of Missouri). Most of the published papers up to that date only reported a list of putative identified proteins (hits, orthologs, and gene products) intending to make conclusions from a biological point of view. It was like writing the life movie or building the biology puzzle with just a photogram or small number of the total pieces. As stated above, just a small fraction of the proteome is visualized, since proteomics has constraints and biases (Fernie and Stitt 2012), and the presence of a protein species does not necessarily indicate that is an active form. In order to overcome these limitations and confidently conclude from a biological point of view, it was clear that proteomics data had to be validated by means of other approaches in the Systems Biology direction. The logical transition from a reductionist to a holistic strategy and integration of multidimensional biological information is currently accepted by the scientific community as the only way to decipher the complexity of living organisms and predict through multiscale networks and models (Jansson and Douglas 2007; Yuan et al. 2008; Cramer et al. 2011).

The Systems Biology approach has been made possible due to the advancements in methods and techniques in any biological disciplines (Kitano 2002; Aderem 2005; Fukushima et al. 2009; Potters 2010), especially related to cellular and molecular biology, ranging from macroscopic to molecular characterization through microscopic and physiological observations (Flexas and Gago 2018). Thus, all -omics approaches developed independently have started to be used in an integrated manner since 2010 (Fig. 3). Nowadays, it is feasible to survey the journey from genotype to phenotype through genome sequencing (Bolger et al. 2014), epigenome characterization (Kohler and Springer 2017; Kapazoglou et al. 2018), gene expression, and molecular phenotype analysis together with transcriptomics (Agarwal et al. 2014), proteomics (Takac et al. 2017), and metabolomics (Hall 2006) approaches. The use of only one approach is not possible to conclude on gene or gene interactions, so that the integration of all the -omics approaches are required as it has been discussed

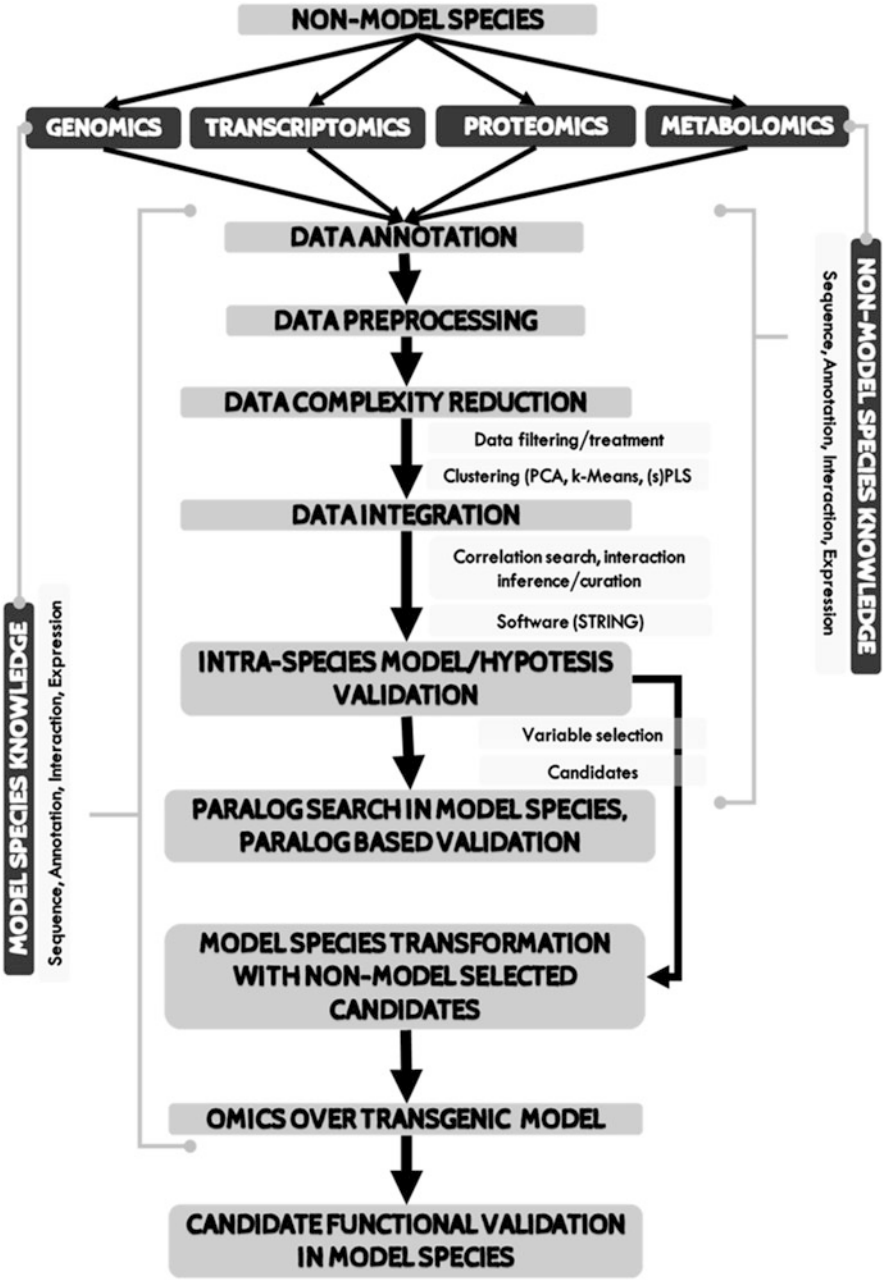

Fig. 3 Workflow of omics data integration. It includes since different omics raw data preprocessing until the hypothesis generation, prediction of plant physiology processes, and the candidate functional validation in model species. This figure was adapted from Valledor et al. (2018)

in early reviews on structural and functional genomics (Holtorf et al. 2002). The integrated use of the -omics techniques will reveal mechanisms of gene expression regulation, including posttranscriptional (RNA splicing, microRNAs, small interfering RNAs, long noncoding RNAs) and posttranslational (phosphorylation, glycosylation) events. The mentioned techniques above, combined with novel phenomics strategies based on sensors and imaging systems, will help in predicting plant performance and contributing to the identification of molecular markers for selecting more productive and resilient crops (Kole et al. 2015). Other biochemical classical approaches can also be employed in the validation of proteomics data, with in vivo or in vitro immunochemical techniques and enzyme activity assays, being these the most used, especially in metabolic and signal transduction pathways (Mann 2008; Stitt and Gibon 2014; Wisniewski and Mann 2016; Demir et al. 2018).

Bioinformatics and computer capabilities could be considered a real bottleneck in Systems Biology research. Algorithms and bioinformatics tools are requested to handle the huge amount of data generated, to perform statistical analysis, identify genes, correlate approaches, and establish networks and, in short, functional interpretation (Edwards and Batley 2004; Ambrosino et al. 2017; Mochida and Shinozaki 2011; Gomez-Casati et al. 2018).

Research results integrating -omics approaches in plant species started to be published in the middle of the 2000 (Fig. 1). The records found at PubMed were of 380, 617, and 228, when proteomics together with transcriptomics, metabolomics, or all of them were used as searching words, respectively. This represents a minimum percentage of the total when only proteomics was used in the search, 8,141. So, this implicates that plant investigation is taking the Systems Biology direction at much lower speed than other organisms such as humans, animals, and other eukaryote model systems (Joyce and Palsson 2006; Hasin et al. 2017).

The number of publications in which two -omics approaches have been used is higher for proteomics and metabolomics due to the use of the same MS-based platform (Fig. 1). Curiously, an important percentage of the publications, around 40%, corresponded to reviews, showing that the expectations given to this field are still far from experimental reality. In short, all the publications present either a general overview (Weckwerth 2011; Varshney et al. 2018) or focused on species or biological processes, including development (Hennig 2007; Proust et al. 2018), autophagy (Liu et al. 2018), senescence (Kim et al. 2016a), priming (Balmer et al. 2015), and stress responses (Zivy et al. 2015; Singh et al. 2016). At the same time, a few databases presenting -omics plant data have been created (Suwabe and Yano 2008) such as plaBiPD (http://plabipd.de/), OPTIMAS-DW (http://www.optimas-bioenergy.org/optimas_dw), Soybean (http://proteome.dc.affrc.go.jp/Soybean/), and HMOD (http://herbalplant.ynau.edu.cn/).

Original papers about integration of -omics approaches in plant biology have been mainly focused on model species like *Arabidopsis* and *Medicago*, major crops (rice, maize, wheat, soybean, grape, potato), and forest trees (poplar, eucalyptus). Beyond its importance as model species or from an economic point of view, they are species whose genome has been already sequenced as well as the availability of protocols to perform in vitro culture, genome edition, and other reverse genetics

tools for functional studies and gene validation (Muller and Grossniklaus 2010). On the other hand, the number of works carried out in orphan species remains anecdotal (Kim et al. 2016b; Batista et al. 2017; Li et al. 2017). Despite the difficulty and complexity of these species, Systems Biology approaches are being employed by several research groups (Abril et al. 2011). For example, our research group is progressing in the knowledge of forest tree species, mainly in *Quercus ilex*, from a classic and proteomics approaches. We soon realized the difficulty of performing a multi-omics approach when genomics or transcriptomics databases were not available. However, we took advantage of three recently published genomes of *Q. robur* (Plomion et al. 2016), *Q. lobata* (Sork et al. 2016), and *Q. suber* (Ramos et al. 2018) to investigate in *Q. ilex*. By using RNA sequencing Illumina and Ion Torrent platforms, we constructed the first transcriptome for *Q. ilex*, which is now employed in our current multi-omics investigations (Guerrero-Sanchez et al. 2017, 2019). From transcriptomics data, we have partially reconstructed metabolic pathways that occur in *Q. ilex* (López-Hidalgo et al. 2018). Moreover, we are about to initiate the genome sequencing project of this forest tree species, as a necessary step for progressing in its Systems Biology research.

4 Experimental Workflow in a Multi-omics Experiment

The integration of proteomics with other omic levels (phenomics, epigenomics, transcriptomics and metabolomics) in a single panoramic study is one of the major aims of Systems Biology nowadays. This approach will give us a real and comprehensive picture of what is happening in our biological system, discovering and unraveling the complex mechanisms behind plant biology, also allowing the modeling of the interactions between different organization levels, the individual molecular variability, and the discovery and unravel of complex mechanisms, in consequence predicting plant behavior linking molecular level with phenotype (Fukushima et al. 2014; Großkinsky et al. 2018).

However, this approach is currently a challenge for many reasons: from a biochemical perspective, the heterogeneity of molecular populations to be studied (proteins, metabolites, or nucleic acids) and analytical levels (almost any methodology can be employed for phenomic analyses, MS in proteomics and metabolomics, next-generation sequencing (NGS)-derived methods for nucleic acids) make this approach complex considering the need of purification and analysis of many different molecules; but from a bioinformatics approach, difficulties are even greater, considering the high amount of data and its heterogeneity, the inherent artifacts owing of the different omics platforms employed such as variation between manufacturers and omics technologies (Singh et al. 2016), the lack of curated databases for most species (genomes, transcriptomes, and interactomes), and the need of adequate algorithms and computing capacities not available for all required modeling objectives and laboratories.

At the wet lab, each advance of the techniques is usually geared toward increasing the quantity (number of analyzed molecules) and quality (sensibility and stability) of the data. However, the best analytical method will fail if the samples are not reflecting the reality of the tissue to be analyzed. One of the first problems to be faced is the fact that usually different biomolecules require different extraction methods and in consequence that different biomolecules will be extracted from different samples. Samples can come from a pool and be really similar, but not identical. These small differences between datasets can later introduce biases or even false discoveries when integrating omic levels, since one of the major aims of integrating omics datasets is the prediction of nonlinear behaviors (Weckwerth 2011).

To face this problem, a number of protocols have been developed to simultaneously isolate two or more classes of biomolecules (Weckwerth 2004; Sambrook and Russel 2006; Xiong et al. 2011; Roume et al. 2013; Gunnigle et al. 2014), but few are capable of extracting metabolites, proteins, DNA, and large and small RNAs and have been successfully tested on recalcitrant species (such as forest species) or with minimal amounts of starting material. To address this problem, in our group we have recently developed a protocol (Valledor et al. 2014) working in a broad range of organisms (*Cyanothece*, *Chlamydomonas*, *Lemna*, *Arabidopsis*, *Oryza*, *Populus*, *Pinus*, and *Eucalyptus*) or in even recalcitrant species, capable of isolating previously referred biomolecules which require minimum amount of sample and lab equipment, being also considerably cheaper than other available alternatives. Analyzing each of the different levels will require different sample preparation and instrumentation. In this sense current workflows of proteomics, metabolomics, when employing state of the art mass spectrometers (i.e., Orbitrap Fusion or qTOFs) or transcriptome analysis employing advanced platforms (Illumina, PacBio, or Ion Torrent), will provide an unprecedented amount of data in terms of quantity and quality. The analysis of these data is often considered the bottleneck of omic studies (Bino et al. 2004; Ritchie et al. 2015), despite novel bioinformatics tools are constantly being developed or updated to overcome this issue (Manzoni et al. 2016; Rajasundaram and Selbig 2016; Leonavicius et al. 2019; Singh et al. 2019). However, obtaining this global picture may be still challenging especially in non-model species, which require initial steps for building and annotating databases.

Proteomics, transcriptomics, and (epi)genomics approaches rely on the existence of a reference genome, ideally profusely annotated, that can be used for defining protein species and quantifying gene expression and DNA modifications (chromatin immunoprecipitation sequencing, ChIP-Seq, methylated DNA immunoprecipitation, MeDIP). In the case of not having this reference genome, building a reference transcriptome, if possible, is mandatory since protein identification and RNA quantification will be much more reliable (Guerrero-Sanchez et al. 2019). If datasets are available, a proteogenomics approach will undoubtedly and significantly increase the knowledge of our species and stablish highly efficient databases before starting omics processing pipeline.

The raw data of the different analytical methods often come from two main platforms, NGS and/or MS, so it must be preprocessed according to their origin

(Kim and Tagkopoulos 2018). Before starting with data integration, it is essential to manage initial data heterogeneity (Fukushima et al. 2009), being available in different pipelines for preprocessing the inputs of the different platforms and performing initial expression/abundance calculations (Trapnell et al. 2012; Zhang et al. 2013; Kuehnbaum and Britz-McKibbin 2013). Initial filtering steps are aimed to remove those reads (NGS) or masses (MS based data) not fulfilling minimal quality thresholds (signal to noise ratio, total intensity ratio, consistency between biological replicates). After this step, data pretreatment commonly continues with the imputation of missing values, filtration of noisy variables, normalization, and transformation of the data (van den Berg et al. 2006; Fukushima et al. 2009; Valledor and Jorrín 2011; Gardinassi et al. 2017). To ease these data imputation steps, several R and Python methods are available.

After these initial processing steps, the researcher will face thousands of variables, organized into different omic levels. The following step usually aims to select those variables which are more important for explaining the variability found in the system, discarding (or not giving much consideration) those which cannot be related to biological changes of samples. This reduction of complexity is usually tackled by employing multivariate statistical tests such as principal component analysis (PCA), partial least squares (PLS), or K-means clustering, which point to those variables more important to the system, or other less fancy methods like interquartile range (IRQ) or even ANOVA's p-values which point to those variables showing major variations within datasets.

Following initial filtrations, multivariate methods can be applied again in order to analyze either the independent datasets or the entire experiment. PLS, multiple co-inertia analysis, MCIA, or DIABLO can handle omics levels differentially. Random forest or PCA can also be used, but this requires mixing the different datasets in order to be analyzed. The outputs of some of these algorithms can be employed to define correlation or causality networks, employing direct or Bayesian approaches. These networks provide a comprehensive overview of the most important variables of our system and its evolution during the experiment allowing to unequivocally choose those variables more important for our experiment (from a mathematical point of view). However, despite these variables can be perfect for discriminating in our experiment, we cannot forget that correlations not necessarily imply cause, so it is always interesting employing interaction databases such as STRING to curate these mathematical interactions considering also biological interactions. Fusing biological interactions and mathematical networks increases the robustness of the models, providing strong candidate variables and processes which will probably explain the biological basis of the different experimental results. This kind of models is not perfect and should be validated using molecular biology in further experiments, since in most of the cases, these networks and models are built from data obtained in small experiments, from a mathematical perspective, involving a very low number of replicates (3–5), being this number very far from optimal.

In model species, in which most of the pathways and chemical reactions are known, a new layer involving not only interactions but chemical reactions can be added, allowing us to numerically explain environmental inputs, biological response at different omic levels, and phenotypical outputs. Despite this can be considered the final goal of Systems Biology and even biochemistry/molecular biology (explain biological responses by a mathematical formulation), we are very far from it. The complexity of the different omics datasets is incredible; from hundreds of genes, thousands of proteins species can be generated (considering splicing forms and PTMs), which can be in different organelles, with many other proteins and metabolites around leading to not-yet-known processes and pathway balancing. Secondary metabolite pathways are being still discovered, and further interactions between genetic, epigenetic, transcriptional, proteomic, and metabolomics dataset to themselves and each other cannot be easily predicted and should be determined experimentally before we can have a deep, functional model of a plant system.

5 Some Illustrative Investigations

As previously stated, the number of published original papers about the simultaneous use of the integrated three -omics approaches is quite limited in plant biology research (Fig. 1), being almost half of them reviews about this topic. In this chapter, we mention a few illustrative examples that are either highly cited by scientific community or published recently.

Cho et al. (2008) (103 times cited) was one of the first work reported at Web of Science (WoS). The authors studied plant responses to ozone (O3) in leaves of rice seedlings by using transcriptomics (DNA microarrays and differential gene expression validation by RT-PCR), proteomics (2-DE/MS), and metabolomics (capillary electrophoresis coupled to MS, CE-MS). They found 1,535 nonredundant genes showing altered expression levels about fivefold, belonging to 8 main functional categories. The number of differential abundant protein species was much lower, 23, corresponding to 21 different gene products. CE-MS revealed that accumulation of amino acids, GABA, and glutathione occurred in O3-exposed plants. It was a quite descriptive paper, with low number of molecular species identified without a real integration of the data. The main conclusion, as appearing in the abstract itself: "This systematic survey showed that O3 triggers a chain reaction of altered gene, protein and metabolite expressions involved in multiple cellular processes in rice."

One of the applications of the multi-omics approach is the study of variability, food traceability, and substantial equivalence in transgenics (Ricroch et al. 2011). Barros et al. (2010) in a paper with 120 cites (WoS) reported on a multi-omics evaluation of kernels in 2 maize transgenic lines (*Bt* and glyphosate resistant). Transcriptomics, proteomics, and metabolomics were evaluated using microarrays, 2-DE/MS, and NMR spectroscopy and GC-MS, respectively. This was a descriptive paper in which differences among genotypes, environments, and growth seasons were statistically analyzed, and 3,541 transcripts, 714 proteins, and 120 compounds

were included in a principal component analysis (PCA). The variability caused by growth conditions (65 genes, 5 proteins, and 15 metabolites differentially accumulated) and season was higher than to the transgenic event. The figure of the different molecules was increased, although no real integrative effort was carried out on the data. In the same direction, Decourcelle et al. (2015) analyzed seed and endosperm in transgenic maize lines with enhanced carotenoid biosynthesis. Both targeted (carotenoids by high-performance liquid chromatography, HPLC) and non-targeted (GC-MS) metabolite analyses were performed, together with Affymetrix transcriptomics microarray and shotgun proteomics. Besides the amount of data generated, the paper clearly focused on enzymes and intermediates of the central and carotenoid pathways. From the targeted data, "An integrative model is put forward to explain the metabolic regulation for the increased provision of terpenoid and fatty acid precursors, particularly glyceraldehyde 3-phosphate and pyruvate or acetyl-CoA from imported fructose and glucose."

Multi-omics analysis in transgenic plants has also been analyzed in poplar transformed lines with an antisense superoxide dismutase transcript in order to study plant responses to stresses (Srivastava et al. 2013). Data were integrated by applying OnPLS modeling to all the variables.

Mensack et al. (2010; 23 times cited) have also used the three -omics approaches to characterize variability between common bean cultivars from different domestication centers, and Ghan et al. (2015; 13 times cited) employed five different platforms for multi-omics analysis of berries from plants subjected to drought stress. Up to 29,549 (microarrays) and 27,252 (RNA-seq) transcripts were detected, this figure, as in most of the works reported, being much lower for proteins, 2,867 (nlC-MS/MS), and metabolites, 67 (GC-MS), and 42 (LC-MS), but much higher than the reported in earlier papers. PCA analysis showed similar results for the cultivars and treatments analyzed, even though transcriptomics data did not correlate very well with proteomics data.

Amiour et al. (2012; 73 times cited) studied nitrogen (N) use efficiency in maize by using multi-omics with leaf tissue collected at different developmental stages of plant grown at various N doses. They employed the same platforms as indicated for the papers commented above. Seventy one metabolites showing differences between N fertilization regimes were identified, with a decrease of up to 37-fold in the concentration of 18 out of the 22 amino acids identified in N deficiency conditions. Other compounds of the carbohydrate metabolism were also reduced, showing the coordination between C and N pathways. Less changes were observed when the protein profile was analyzed by 2-DE, with only 40 variable spots, of which 31 proteins were identified. With respect to transcripts, 74 and 71 were, respectively, less and more abundant under N limitation, representing quite different functional categories. Plant responses to N starvation resembled those observed under other biotic and abiotic stresses, considering that the most of them reflect the consequences of the stress better than an active response of the plant in order to overcome the restrictions imposed by the environment. The authors concluded that the integration of the omics data is not straightforward since different cellular mechanisms operate

at the different gene expression steps. Thus, it is not always clear to know whether the observed differences among the -omics data are mostly analytical or biological.

Simple experimental systems are valuables for multi-omics approaches. In this regard, Balcke et al. (2017; 19 times cited) have worked on the -omics profile of glandular trichomes and leaves in cultivated and wild tomato species to understand the connection between primary and secondary metabolism. In this work, more potent analytical techniques such as LC-MS for metabolite, shotgun for protein, and qRT-PCR, in addition to microarrays, for transcript analyses were used. The differences between trichome and trichome-free leaves were analyzed independently and correlated between transcriptomics and proteomics (Pearson coefficient). In general, and among the 20,445 transcripts and 4,390 proteins included in the analysis, slightly positive r values were found for both trichomes and leaf tissue (0.34–0.42). Based on the results, a model of the metabolism is showed in this specialized organ.

Multi-omics approach has been employed in studying different biological processes such as responses to light (Liang et al. 2016) and mycorrhizal interactions (Vijayakumar et al. 2016). Liang et al. (2016) worked on the effect of light on *Arabidopsis* growth and development. They found a negative correlation between mRNA and proteins that corresponded to the photosystem elements revealing the importance of posttranscriptional events in gene expression regulation.

Galland et al. (2017) have profiled the embryo and endosperm of rice seeds in an attempt to find parameters of physiological and nutritional quality. From the generated dataset, including 124 unique metabolites (GC-MS); 15,339 and 16,998 transcripts (microarrays) for embryo and endosperm, respectively; and 2,212 proteins (shotgun), the authors discussed on the physiological and metabolic particularities of each tissue, relationships between tissues, and the maturation and desiccation processes. The endosperm is not just a reservoir organ but does play an active role in the seed germination process. As stated in concluding remarks: "In addition, the seed definitely constitute a fascinating plant organ in which post-transcriptional regulations and translational selectivity fine-tune the biological processes that are spatially and temporally regulated within a few hours."

In summary, since the number of molecular entities identified is increasing, the algorithms to integrate all these -omics data should be improved in parallel. This should not be considered as a trivial issue considering the potential of each -omics technique, its properties as analytical techniques, and difference in number of molecular entities revealed. While genome, transcriptome, and proteome are easily integrated, in the case of the metabolome, this is not so obvious, as the result of the enzyme activities cannot be analyzed in a holistic approach. In the meantime, it is important to focus on specific gene products in which the information provided can be handled manually in a hypothesis-driven strategy. This can be feasible for studying a metabolic or signal transduction pathway, but it is still a real challenge for other more complex biological processes such as responses to abiotic or biotic stresses.

6 Concluding Remarks

Beyond the limitations observed in wet classical techniques of protein chemistry (protein extraction and separation by electrophoresis or chromatography), all advancements in MS analyzers and bioinformatics tools have taken proteomics to an unimaginable achievement in terms of number of protein species confidently identified, quantified, and characterized. In the last 20 years, a vast amount of data has been accumulated on protein species detected in different plant systems, specific organs, tissues, cells of subcellular fractions, specific developmental stages, or under different environmental conditions. To lesser extent, we have started to identify different gene products that have been modified by posttranslational modifications and how they interact with other molecules. Even so all the protein species already reported can be considered as the tip of the iceberg. This is because in order to go in depth in the proteome coverage, this should be fractionated at the cellular or subcellular levels or by fractionating proteins based on physicochemical and biological properties. However, this is just the beginning, the descriptive part in which the pieces of the puzzle have been collected that will be used to understand plant biology. Because of the technical constraints and analytical biases, proteomics data should be validated and integrated with other classic and modern (-omics) approaches in the Systems Biology direction. Proteomics should be integrated with other biochemical areas and biological disciplines such as morphometry and phenology, among others. We are only covering a small part of the central dogma of biology, from mRNA to metabolites. The paradox is that it is difficult to perform such a transversal and holistic approach as investigation request reductionism. The most appropriate way is probably to focus on the thousands of data generated by the potent technologies available today. Finally, proteomics scientists should collaborate with experts in other disciplines to interpret the plant biology from the protein language, whose main objectives are to connect the genotype with the phenotype, model, predict, and take advantage of such a knowledge about proteomics.

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Membrane Trafficking and Plant Signaling

Robert Kendle and Marisa S. Otegui

Contents

Abstract Endocytosis and endosomal trafficking pathways control the composition and surface area of the plasma membrane. These pathways are regulated by protein complexes that recognize specific proteins (cargo), mediate their sorting into vesicles, and specify the identity of the acceptor organelles to which they fuse. Under abiotic stress, plants need to rapidly adjust the composition of their plasma membrane by inducing changes in endocytic and endosomal membrane fluxes. In this chapter, we discuss the molecular mechanisms that mediate the recognition and

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R. Kendle
Department of Botany, University of Wisconsin-Madison, Madison, WI, USA

Laboratory of Molecular and Cellular Biology, University of Wisconsin-Madison, Madison, WI, USA

M. S. Otegui (✉)
Department of Botany, University of Wisconsin-Madison, Madison, WI, USA

Laboratory of Molecular and Cellular Biology, University of Wisconsin-Madison, Madison, WI, USA

Department of Genetics, University of Wisconsin-Madison, Madison, WI, USA
e-mail: otegui@wisc.edu

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sorting of protein cargo along the endocytic and endosomal pathways and their connection to development and physiological responses to abiotic stress in plants.

Keywords Endocytosis, Endosomal sorting, Membrane remodeling, Plasma membrane

1 Introduction

Plants are exposed to a wide range of stressful environmental conditions. As they cannot escape stress by moving to more benign conditions, they have evolved complex adaptive stress responses that often involve extensive membrane remodeling and endomembrane trafficking. Some of these membrane trafficking events include the removal or incorporation of protein and signaling molecules at the plasma membrane, changes in the composition of membrane lipids, changes in the protein composition of the vacuolar membrane (tonoplast) to favor sequestration of specific molecules (e.g., ions), and the activation of catalytic pathways that lead to the degradation of plasma membrane proteins. Of special relevance are the endocytic/endosomal pathways that modulate proteins and organelle homeostasis during stress responses. Endocytosis and endosomal sorting regulate removal, recycling, and degradation of plasma membrane proteins. In plants, as plasma membrane proteins are internalized in vesicles through endocytosis, they are delivered to the trans-Golgi network (TGN), which acts as an early endosome (Dettmer et al. 2006; Lam et al. 2007). From this location, proteins can recycle back to the plasma membrane or be sorted into endosomal intralumenal vesicles in multivesicular endosomes. When multivesicular endosomes fuse with vacuoles, their internal vesicles containing plasma membrane proteins are released into the vacuolar lumen and degraded by hydrolases (Fig. 1).

In this chapter, we discuss the major roles and regulatory mechanisms of membrane trafficking pathways, with emphasis on endocytosis and endosomal sorting in response to abiotic stress in plants.

2 Endocytosis

Plant cells continuously sense external and internal signals through proteins and other molecules located on their plasma membrane. Here, receptors, transporters, enzymes, and lipids are at the direct interphase with external environmental signals. The ability to regulate the composition of the plasma membrane is critical for survival and physiological responses. Endocytosis is the sequestration of plasma membrane components into vesicles. This process requires several regulated steps,

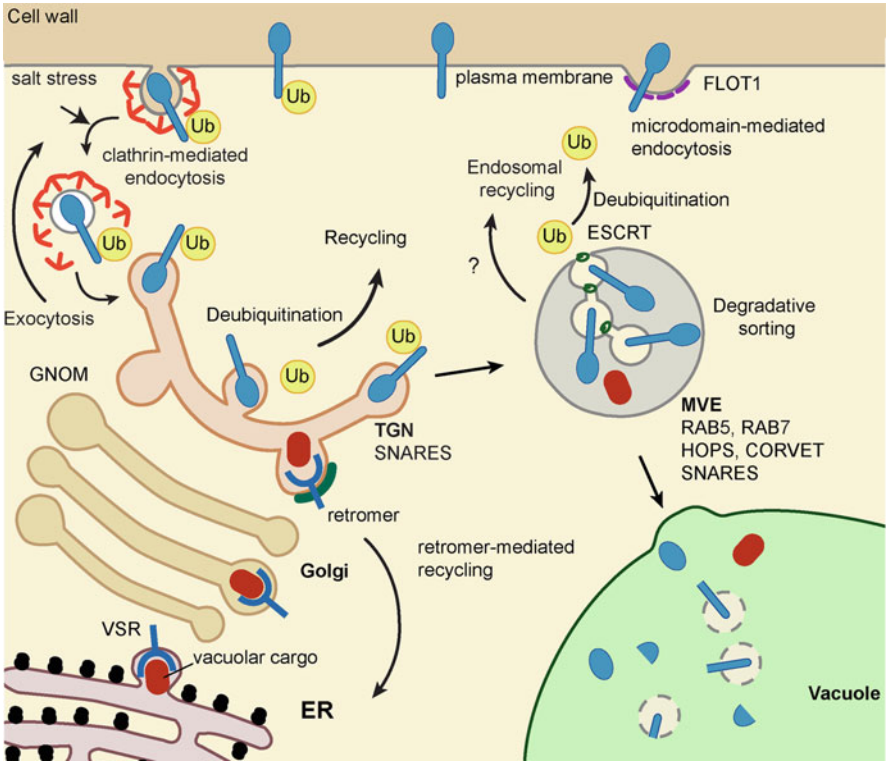

Fig. 1 Overview of the main steps in the endocytic and endosomal trafficking pathways in plants. *ESCRT* endosomal sorting complex required for transport, *MVE* multivesicular endosome, *Ub* ubiquitin

including the selection of proteins to be sequestered (cargo), and the formation and release of the vesicle into the cytoplasm. The sequestration of cargo into vesicles requires their concentration in defined areas of the donor membrane. During this process, cargo proteins can interact directly or indirectly through adaptor proteins with other factors capable of bending membranes, leading to the assembly of a coat that can induce the formation of a vesicle bud at the donor membrane. Besides assisting with cargo recognition, retention, and concentration, adaptor proteins can also interact with specific phosphoinositides, providing some level of membrane specificity (Paczkowski et al. 2015). The forming vesicle is finally released from the donor membrane through neck scission. Once released into the cytoplasm, vesicles lose their coat and are ready to fuse with the acceptor membrane/organelle.

2.1 Clathrin-Mediated Endocytosis

The best understood endocytic mechanism is mediated by clathrin, also called clathrin-mediated endocytosis (CME). In plants, CME was first shown to be operational in the constitutive internalization of PIN auxin efflux facilitators (Dhonukshe et al. 2007). During CME, adaptor proteins play a critical role by engaging cargo proteins, interacting with membrane lipids, and recruiting clathrin from the cytoplasm. Heterotetrameric adaptor protein (APs) complexes are involved in clathrin-mediated vesiculation. The *Arabidopsis thaliana* genome encodes subunits for five AP complexes, AP-1 to AP-5, but only AP-2 has been shown to be involved in CME (Bashline et al. 2013; Di Rubbo et al. 2013; Kim et al. 2013; Yamaoka et al. 2013; Fan et al. 2013). AP-2 simultaneously binds clathrin, specific sorting motifs in cargo proteins, and PtdIns(4,5)P_2 (phosphatidylinositol 4,5-bisphosphate) at the plasma membrane.

The AP complex and other accessory proteins recognize cargo through sorting motifs in the cytosolic domains of plasma membrane proteins. These signals consist of linear amino acid sequences, conformational motifs, and posttranslational modifications, most commonly phosphorylation and ubiquitination (Traub and Bonifacino 2013). In many cases, the recognition of endocytic cargo involves the binding of adaptor proteins to more than one sorting signal. An increasing number of plant plasma membrane proteins are known to be internalized by CME in response to environmental and signaling cues (Liu et al. 2018). However, the mechanisms by which they engage adaptor proteins are known for only of subset of them.

In animals, AP-2 binds two sorting motifs in cargo proteins, di-Leu-based [DE] xxxL[LI] and Tyr-based YxxΦ (where Φ is a bulky hydrophobic amino acid) motifs (Kelly and Owen 2011; Ohno et al. 1995). In plants, endocytosis of the pathogen-related receptor leucine-rich repeat receptor-like protein LeEix (ETHYLENE-INDUCING XYLANASE RECEPTOR) in tomato (*Lycopersicon esculentum*) and the borate exporter BOR1 (BORON TRANSPORTER 1) in *Arabidopsis* relies on the presence of Tyr-based sorting signals in their cytoplasmic domains (Bar and Avni 2009; Takano et al. 2010). Under low boron concentration, the Tyr-based motifs mediate the constitutive endocytosis of BOR1 molecules that are then recycled back to the plasma membrane from the endosomes. In addition, the brassinosteroid receptor BRI1 (BRASSINOSTEROID INSENSITIVE1) interacts with AP-2 to mediate its endocytic internalization, although the motifs responsible for the interaction between BRI1 and AP-2 remain unknown (Di Rubbo et al. 2013). PIN1 is also internalized by CME and interacts with AP-2 both through Tyr- and Phe-based motifs (Fan et al. 2013; Sancho-Andres et al. 2016). The Phe-based motif in PIN1 shows similarity to a sorting signal of the mammalian mannose-6-P receptor (Sancho-Andres et al. 2016).

Plants also use an ancestral adaptor complex called TPLATE during CME. The TPLATE complex consists of eight core subunits (TPLATE, TASH3, LOLITA, TWD40-1, TWD40-2, TML, AtEH1, and AtEH2) and assembles at CME initiation sites before the recruitment of AP-2 (Gadeyne et al. 2014). TPLATE interacts with

clathrin, AP-2, accessory proteins, and dynamin-related proteins (Gadeyne et al. 2014). Interestingly, whereas plants can survive without a functional AP-2 complex, the TPLATE complex is essential for plant development (van Damme et al. 2006).

Posttranslational modifications are also important to drive endocytosis of specific cargo proteins. Ubiquitination is a posttranslational modification that consists in the conjugation of a ubiquitin molecule to a target protein, usually on Lys residues. Ubiquitination can occur as a single modification (monoubiquitination), as a conjugation of single ubiquitin moieties at multiple sites of the substrate protein (multi-monoubiquitination), or by attachment of ubiquitin chains with different configurations depending on the type of ubiquitin-ubiquitin linkages (polyubiquitination). The most common type of polyubiquitinated configurations in *Arabidopsis* are Lys-48 (K48)-, Lys-63 (K63)-, and Lys-11 (K11)-linked chains (Callis 2014). Both membrane and soluble proteins can be ubiquitinated. In fact, ubiquitination serves as recognition signal for several catalytic pathways such as degradation of soluble proteins via the 26S proteasome, degradation of plasma membrane proteins through endosomal sorting, and turnover of protein aggregates, protein complexes, and whole organelles through autophagy. The conjugation of ubiquitin moieties is mediated by the sequential action of three enzymes: ubiquitin-activating (E1), ubiquitin-conjugating (E2), and ubiquitin ligase (E3) enzymes. The *Arabidopsis* genome encodes for 2 E1s (Callis 2014), 37 E2s (Kraft et al. 2005), and more than 1,000 E3 ubiquitin ligases (Mazzucotelli et al. 2006), providing a glimpse of the complex regulation and substrate specificity underlying protein ubiquitination in plants.

In plants, both mono- and polyubiquitination of plasma membrane proteins are important for their endocytic internalization and subsequent endosomal trafficking. For example, the ubiquitination of the pathogen-related receptor FLS2 (FLAGELLIN-SENSING 2) by the plant U-box (PUB) E3 ubiquitin ligases PUB12 and 13 is required for its internalization upon ligand binding (Lu et al. 2011; Zhou and Zeng 2018). Another receptor complex involved in pathogen recognition, LYK5-CERK1 (LYSM RECEPTOR KINASE5-CHITIN ELICITOR RECEPTOR KINASE 1), also depends on posttranslational modifications for its trafficking and turnover. In the absence of chitin, both CERK1 and LYK5 localize to the plasma membrane, and LYK5 interacts with the E3 ligase PUB13 that mediates its continuous turnover by ubiquitination (Dehua et al. 2017). However, upon exposure to chitin, the two receptors undergo different fates; LYK5 is phosphorylated by CERK1, which leads to its endocytic internalization, whereas CERK1 remains at the plasma membrane (Jan et al. 2017). BRI1 and the auxin efflux facilitator PIN2 (PIN-FORMED 2) are modified by K63 polyubiquitination to regulate endocytosis and endosomal sorting (Martins et al. 2015; Leitner et al. 2012) by yet unidentified E3 ligases.

The homeostasis of ion transporters at the plasma membrane is also regulated by ubiquitination. For example, exposure to high concentration of boron in *Arabidopsis* seedlings leads to BOR1 mono- or di-ubiquitination and its subsequent internalization and degradation through the endosomal pathway (Kasai et al. 2011). In addition, the iron transporter IRT1 (IRON-REGULATED TRANSPORTER 1) which

localizes at the outer domain of root epidermal cells is monoubiquitinated on Lys residues at its cytosolic loop (Barberon et al. 2011) by the IDF (IRT1 Degradation Factor) E3 ligase (Shin et al. 2013) to mediate its constitutive internalization and recycling. Besides iron, IRT1 also transports zinc, manganese, cobalt, and other toxic metals into the root. Iron concentration does not seem to affect IRT1 abundance or polar localization at the plasma membrane (Barberon et al. 2011). However, high concentration of other metal substrates affects the partition of IRT1 between the plasma membrane and TGN in root epidermal cells. Under these potentially toxic conditions, IRT1 seems to be retained in the TGN to reduce the absorption of toxic metals (Barberon et al. 2014).

Another important group of plasma membrane proteins whose abundance is tightly regulated is aquaporins. Aquaporins are channels that allow diffusion of water across a membrane (Johanson et al. 2001). PIP2;1 (PLASMA MEMBRANE INTRINSIC PROTEIN 2;1), as many other plasma membrane proteins undergo constitutive internalization and recycling but under high-salt conditions, is removed from the cell surface and relocated to endosomes, restricting the flow of water across the plasma membrane (Ueda et al. 2016; Prak et al. 2008). The phosphorylation state of PIP2;1 seems to be important for its trafficking since, under resting conditions, the phosphorylation of Ser^{283} is necessary to target AtPIP2;1 to the plasma membrane, whereas the salt-induced internalization of PIP2;1 requires a non-phosphorylated Ser^{283} residue. The endocytic internalization of PIP2;1 triggered by salt stress seems to depend on both CME and membrane microdomain-associated pathways (Li et al. 2011). Treatment with H_2O_2 also seems to trigger internalization of PIP2;1 into endosomal compartments. Interestingly, however, PIP2;1 molecules do not seem to be degraded under these conditions but just preferentially retained in endosomal compartments, likely ready to be cycled back to the plasma membrane (Wudick Michael et al. 2015).

Clathrin subunits interact with activated adaptor protein complexes and polymerize on the membrane surface, deforming it into a clathrin-coated pit. The mechanochemical GTPase dynamin is then recruited to the neck of the clathrin-coated pit. Dynamin polymerizes around the neck of the budding vesicle and hydrolyzes GTP. Hydrolysis of GTP causes a conformational change in the dynamin polymer (Antonny et al. 2016) that results in constriction of the neck, membrane scission, and ultimately the release of a clathrin-coated vesicle. After vesicle release, the clathrin coat disassembles freeing the vesicle to fuse with downstream compartments.

Arabidopsis has six subfamilies of dynamin-related proteins (DRPs 1–6) that participate in several membrane scission processes (Hong et al. 2003), but only DRP1 and DRP2 participate in endocytosis (Fujimoto et al. 2010). There are major structural differences between DRP1 and DRP2. DRP2 proteins contain a GTPase domain, a coiled-coil domain that facilitates dynamin oligomerization, a pleckstrin homology (PH) domain that binds PtdIns(4,5)P_2, a GTPase effector domain, and a proline-rich domain (PRD), which is important for recruitment to CME sites (Fujimoto et al. 2010; Yan et al. 2011). DRP1 proteins, however, lack the PH and PRD domains and, in contrast to other dynamins, are unable to tubulate liposomes

(Backues and Bednarek 2010). Despite these structural differences, DRP1 and DRP2 appear to work together during CME (Fujimoto et al. 2010). Expression of a dominant negative (DN) form of DRP1A interferes with the polar localization of BOR1 at the plasma membrane of epidermal cells but not in the columella cells, suggesting a cell type-specific role for DRP1A (Yoshinari et al. 2016). There is also evidence for specialized roles within the DRP subfamilies. For example, endocytosis of FLS2 appears to be partially dependent on DRP2B, but not DRP2A (Smith et al. 2014). Binding of FLS2 to its ligand results in the phosphorylation of DRP2B and subsequent endocytosis of activated FLS2 receptors.

The regulation of CME also relies on lipid signals, especially phosphoinositides. The AP-2 complex is brought to the plasma membrane through interactions with PtdIns(4,5)P_2. Lipid-modifying enzymes acting on the plasma membrane regulate the steady-state levels of PtdIns(4,5)P_2 exerting an effect on CME rates. The increase in plasma membrane PtdIns(4,5)P_2 correlates with increased colocalization of clathrin and PtdIns(4,5)P_2 and a several-fold increase in endocytosis (Konig et al. 2008).

Signaling lipids have an important regulatory function of CME during osmotic stress. Changes in osmotic conditions alter the cell volume, leading to shifts in exocytosis and endocytosis rates to accommodate plasma membrane surface area and transport activity (Zwiewka et al. 2015). Thus, for example, salt and hyperosmotic stress lead to a reduction if cell volume increases production of PtdIns(4,5)P_2 and endocytosis (Pical et al. 1999). Another important lipid in signaling and membrane dynamics is phosphatidic acid (PA). In both plants and animals, PA is an important positive regulator of CME (Antonescu et al. 2010; Li and Xue 2007). During osmotic stress, PA is produced through two major phospholipase pathways (Testerink and Munnik 2005). Cleavage of PtdIns(4,5)P_2 by phospholipase C results in the production of inositol-1,4,5-trisphosphate, which moves into the cytoplasm and promotes release of Ca^{2+} from internal stores, and diacylglycerol, which is rapidly converted into PA by a diacylglycerol kinase. The cleavage of structural phospholipids (e.g., phosphatidylcholine or phosphatidylethanolamine) by phospholipase D (PLD) is also a major source of cellular PA. Hyperosmotic stress induces the activity of PLDs and increases endocytosis, possibly by increased production of PA (Galvan-Ampudia et al. 2013). Salt stress results in the enhanced recruitment of clathrin to the plasma membrane. Under conditions of salt stress, the clathrin heavy chain and other accessory proteins required for clathrin coat assembly were found to bind PA (McLoughlin et al. 2013).

Ca^{2+} release into the cytoplasm is a common response to many different types of biotic and abiotic signals and has an important regulatory function on endomembrane trafficking under stress conditions. For example, Ca^{2+} modulates lipid binding and phospholipase activity of PLD by different mechanisms. Ca^{2+} binds both the catalytic site and the C2-lipid-binding domain of plant PLDs (Rahier et al. 2016; Pappan et al. 2004). Whereas Ca^{2+} binding to the catalytic site reduces PLD affinity for its substrate phosphatidylcholine, Ca^{2+} binding to the C2 domain increases PLD affinity for phosphatidylcholine and its association with membranes.

The salt-induced stimulation of PLD is also critical for halotropism, or the movement away from saline environments (Galvan-Ampudia et al. 2013). During halotropism, a salinity gradient in the environment stimulates PLDζ2 activity unevenly across the plasma membrane. Activated PLDζ2 produces PA, which stimulates endocytosis. Auxin efflux facilitators, such as PIN1 and PIN2 are more frequently endocytosed from regions of the plasma membrane with higher PA content, leading to a change in the directional movement of auxin across cells. Auxin accumulates on the side of the root tip facing away from the higher-salt concentration inhibiting the growth of those cells. The different growth rates across the root tip cause the root to bend away from areas of higher-salt concentration (Galvan-Ampudia et al. 2013). PA derived from PLDs may also contribute to downstream sorting of PIN proteins. Inhibition of PLD results in reduction of the endosomal PA pool, which is critical for the recruitment of the protein phosphatase 2A (PP2A). Dephosphorylation of PIN2 by PP2As influences the normal trafficking of PIN2, and mutants of PLDζ2 show altered PIN2 localization (Gao et al. 2013).

Another salt-responsive signaling pathway that depends on endocytosis is the RGS1 (Regulator of G Signaling protein 1) heterotrimeric G-protein pathway. G-protein signaling pathways operate in both plants and animals albeit with some major mechanistic differences. In animals, G-protein signaling involves G-protein-coupled receptors (GPCR) that upon binding of a ligand stimulate the exchange of GDP for GTP in the α-subunit of the heterotrimeric G-protein. The GTP-bound α-subunit then detaches from the β- and γ-subunits, and the released subunits bind to signaling effectors such as adenosine cyclase. In plants, the G α-subunit spontaneously exchanges GTP for GDP without the aid of a receptor and is thus active in its default state (Stateczny et al. 2016). Interaction with the GTPase-activating protein RGS1 stimulates the hydrolysis of GTP and maintains the G-protein complex in the inactive state (Colaneri et al. 2014). This pathway is regulated by the protein kinase WNK8 (WITH NO LYSINE KINASE 8), whose expression is rapidly induced upon hyperosmotic stress (Zhang et al. 2013). WNK8 specifically phosphorylates RGS1 at threonine residues in its C-terminal tail, which triggers RGS1 rapid internalization into endocytic vesicles (Urano et al. 2012). The endocytosis of RGS1 reduces the abundance of RGS1 in the plasma membrane, leading to the autoactivation of the G α-subunit and stimulation of downstream signaling effectors that modulate the hyperosmotic stress responses (Colaneri et al. 2014; Urano et al. 2012).

Endocytosis is also regulated by hormone signaling. The phytohormone abscisic acid (ABA) is produced in response to abiotic stress and has profound effects on membrane trafficking. This is most clearly demonstrated in guard cells where production of ABA results in stomatal closure. During stomatal closure, up to 40% of the plasma membrane surface area is removed by endocytosis (Shope et al. 2003). One class of plasma membrane proteins that undergo ABA-regulated endocytic turnover are ion channels, such as the potassium channel KAT1. The internalization and gating of KAT1 in response to ABA promotes the efflux of solutes and loss of turgor pressure in guard cells during ABA-triggered stomatal closure (Sutter et al. 2007). Intriguingly, KAT1 does not seem to follow the canonical endosomal pathway. Once endocytosed, internalized KAT1-GFP signal

fails to colocalize with TGN and endosomal markers. After ABA washout, KAT1 is recycled back to the plasma membrane (Sutter et al. 2007).

In addition to modulating the endocytosis of ion channels, ABA also regulates the endocytosis of ABA transporters. ABA efflux and influx transporters fine-tune the cellular levels of ABA in response to abiotic stress (Park et al. 2016). ABCG25 is an ABA efflux transporter whose levels at the plasma membrane are regulated by ABA and environmental stresses. Abiotic stress leads to the endocytosis of ABCG25 and the increase of intracellular levels of ABA and ABA signaling. In the presence of exogenous ABA, ABCG25 is recycled from endocytic pools to the plasma membrane, promoting efflux of ABA and reducing intracellular ABA levels (Park et al. 2016).

The phytohormone auxin negatively regulates CME of plasma membrane-localized PIN auxin carriers, such as PIN1 and PIN2, but promotes their endosomal recycling, resulting in an increase of PIN proteins at the plasma membrane (Paciorek et al. 2005). However, auxin has some specificity in terms of the CME cargo affected. For example, the internalization of the aquaporin PIP2 and auxin efflux carrier AUX1 do not seem to be dependent on auxin (Pan et al. 2009). Although how exactly auxin controls CME of PIN proteins is not completely clear, it does require the presence of membrane sterols (Pan et al. 2009) and the activation of the small GTPase ROP6 (Lin et al. 2012). Auxin also decreases the association of clathrin with the plasma membrane (Wang et al. 2013). In addition, an unanticipated connection between plastidial retrograde signaling and auxin-controlled PIN endocytosis has been recently revealed. Methylerythritol cyclodiphosphate (MEcPP), a plastidial metabolite that acts as retrograde signal to communicate environmental perturbations sensed by plastids to the nucleus, reduces auxin abundance and promote PIN endocytosis (Jiang et al. 2018).

Salicylic acid, a phytohormone involved in pathogen-related responses, negatively regulates endocytosis of PIN1, PIN2, PIP2, and FM4-64 in a mechanism that is independent of gene transcription and seems to affect the association of clathrin with the plasma membrane (Du et al. 2013).

In contrast to auxin and salicylic acid, strigolactones promote PIN1 endocytic removal from the plasma membrane (Shinohara et al. 2013).

2.2 Other Forms of Endocytosis

Clathrin-independent endocytosis is much less understood than CME. Some forms of clathrin-independent endocytosis rely on flotillin proteins, which are membrane-associated proteins that form microdomains in the plasma membrane. Hydrophobic loops in the flotillin protein can partially insert into the plasma membrane, deforming membranes and creating local invaginations that can be internalized upon membrane scission. The *Arabidopsis* flotillin protein FLOT1 is known to participate in microdomain endocytosis independent of clathrin. In addition, vesicles produced

by FLOT1 have a diameter of 100 nm, whereas clathrin-coated vesicles are on average approximately 30 nm in diameter (Li et al. 2012).

Many proteins can be internalized by both CME and clathrin-independent endocytosis. For example, RbohD (RESPIRATORY BURST OXIDASE HOMOLOG D), which is an important signaling protein in several biotic and abiotic stress responses, is endocytosed through both clathrin-dependent and clathrin-independent pathways under salt stress (Hao et al. 2014).

3 Endosomal Trafficking

Endocytosis is followed by a complex and highly regulated set of trafficking pathways that determine the fate of the cargo proteins, either to recycling or to degradation. The TGN acts as an early endosome in plants and is a hub for the recycling and catalytic endosomal pathways. The TGN is a transient dynamic structure that matures from the trans-Golgi and, as it detaches from the trans side of the Golgi, it gives rise to secretory vesicles and late endosomes (Fig. 2). Constitutive endocytosis followed by sorting and recycling from endosomes back to the plasma membrane is important for cells to maintain a dynamic pool of proteins that can rapidly be removed or sent back to the plasma membrane in response to environmental or developmental cues. Endosomal recycling is also important to maintain the polarized localization of PIN proteins (Ambrose et al. 2013) and for the rapid changes in ion fluxes in stomatal guard cells (Sutter et al. 2007). Those plasma membrane cargo proteins that remained ubiquitinated are not recycled and are instead sorted into intralumenal vesicles of multivesicular endosomes for degradation in the vacuole.

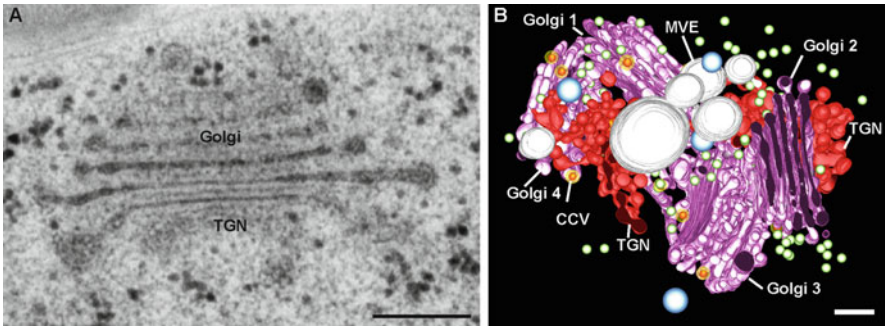

Fig. 2 The plant trans-Golgi network (TGN). (**a**) Transmission electron micrograph of a Golgi stack and associated TGN. (**b**) Tomographic reconstruction of a cluster of four Golgi stacks, TGNs, clathrin-coated vesicles (CCV), other secretory vesicles, and multivesicular endosomes (MVE) (modified from Otegui et al. 2006) (Copyright ©2006 American Society of Plant Biologists). Bar = 200 nm. Scale bars = 200 nm

3.1 Early Endosomal Sorting at the TGN

Endocytic vesicles fuse to TGN as they enter into the endosomal pathway. The TGN serves both as a sorting station for newly synthesized vacuolar cargo progressing through the Golgi and as an early endosome; both functions are tightly interconnected. The specific fusion of vesicles to an acceptor compartment requires SNARE proteins in both membranes. SNARE proteins are classified based on their structure in R-SNAREs and Q-SNAREs. Three Q-SNAREs and one R-SNARE assemble into a tetrameric SNARE complex, along with regulatory proteins (Antonin et al. 2002). The tetrameric SNARE complex undergoes a conformational change that brings the opposing membranes close enough to trigger fusion. The R-SNARE YKT6 and the Q-SNAREs SYP61, SYP41, and VTI12 are important for TGN function (Chen et al. 2005; Zouhar et al. 2009).

Endosomal recycling requires different protein complexes that recognize cargo and induce membrane deformation to generate vesicles that travel back to the plasma membrane. Small GTPases of the ARF family have been shown to participate in vesicle formation during endosomal recycling. For their activation, ARF GTPases employ ARF guanine exchange factors (ARF-GEFs) that facilitate the GDP to GTP exchange. One of the best-characterized ARF-GEF in plants is GNOM. GNOM is required for the dynamic trafficking, polarized localization of the PIN auxin efflux facilitators, and normal auxin distribution (Steinmann et al. 1999). GNOM was originally thought to localize to endosomes and act directly on endosomal cargo recycling. However, GNOM was later shown to localize to the Golgi (Naramoto et al. 2014) and function in endoplasmic reticulum-to-Golgi transport (Doyle et al. 2015), suggesting that GNOM involvement in endosomal recycling is indirect. Another ARF-GEF in *Arabidopsis* called BIG5/BEN1 (BFA-VISUALIZED ENDOCYTIC TRAFFICKING DEFECTIVE 1) localizes to the TGN and affects the endosomal recycling of PIN1 (Tanaka et al. 2009).

Another important recycling route at endosomes is controlled by the retromer complex, which is known to affect the recycling of both biosynthetic and endocytic cargo. In yeast, the retromer consists of two subcomplexes, the cargo recognition Vps35p-Vps26p-Vps29p heterotrimer (VPS subcomplex) and a heterodimer of two sorting nexins, Vps5p and Vps17p. The sorting nexins bind PtdIns(3)P and can tubulate membranes (van Weering et al. 2012). The VPS35 subunit engages the cargo based on a recognition motif characterized by hydrophobic and aromatic amino acids. In mammals, Sorting nexin 1 (SNX1) and SNX2 are the counterparts of yeast Vsp5p and form heterodimers with either SNX5 or SNX6, giving rise to four SNX subcomplex combinations (Bonifacino and Hurley 2008). The retromer complex coats endosomal membranes enriched in cargo and mediates their tubulation. At least in yeast, the fission of these tubular domains generated by the retromer and containing recycling cargo is mediated by the dynamin-related protein Vps1 (Chi et al. 2014). *Arabidopsis* contains three *VPS35* genes, two *VPS26* genes, one *VPS29* gene encoding, and three *SNX* genes (SNX1, SNX2a, and SNX2b) (Oliviusson et al. 2006; Jaillais et al. 2007; Yamazaki et al. 2008; Jha et al. 2018).

Among the biosynthetic cargo recycled by the retromer are the mammalian cation-independent mannose 6-phosphate (Seaman 2007) receptor, the yeast vacuolar hydrolase receptor Vps10p (Nothwehr et al. 2000), and the VSR1/BP80 receptor in *Arabidopsis* that sort storage proteins to the vacuole (Yamazaki et al. 2008; Zelazny et al. 2013; Shimada et al. 2006; Niemes et al. 2010). The retromer is also necessary for the normal trafficking of PIN1 and PIN2 (Jaillais et al. 2006, 2007; Kleine-Vehn et al. 2008) and the plasma membrane-localized ceramide-1-phosphate transfer protein ACD11 (accelerated cell death11), which regulates phytoceramide levels during programmed cell death (Munch et al. 2015). However, whether the retromer directly targets these proteins or affects their trafficking in a more indirect fashion is currently unclear. The balance between the degradative and recycling functions performed by sorting nexins in plants has been recently shown to be influenced by levels of the phytohormone gibberellic acid (Salanenka et al. 2018).

3.2 Late Endosomal Sorting for Degradation

The main sorting signal at endosomes for cargo degradation is ubiquitin. Whether the cargo has remained ubiquitinated during its journey from the plasma membrane or has been ubiquitinated at the TGN, it is recognized by a group of protein complexes called ESCRT (Endosomal Sorting Complex Required for Transport). Besides ubiquitin on cargo proteins, several ESCRT components bind Ptdns(3)P, which is actively synthesized at endosomal membranes by the activity of lipid kinases such as phosphatidylinositol 3-kinases. As ESCRT complexes begin to assemble on the endosomal membrane, they concentrate the cargo and induce membrane deformation, ultimately leading to the formation of intralumenal vesicles that accumulate inside multivesicular endosomes and contain the cargo proteins. Unlike CME, the coordinated action of ESCRTs promote negative bending of membranes and budding away from the cytosol. Proteins sorted into intralumenal vesicles by the ESCRT machinery reside inside the lumen of multivesicular endosomes, inaccessible to the cytosol. Actively signaling receptors that are sorted into intralumenal vesicles loose contact with cytosolic effectors, abolishing continued signaling. When ESCRT-mediating sorting fails, then receptors may remain active on the endosomal-limiting membrane and tonoplast leading to improper and prolonged signaling with profound physiological consequences.

ESCRT-dependent intralumenal vesicle formation comprises the sequential recruitment of five complexes, ESCRT-0, I, II, III, and the VPS4/SKD1-LIP5 complex. ESCRT-0 engages ubiquitinated cargo proteins, likely leading to cargo clustering. A canonical ESCRT-0 complex with ubiquitin-binding capabilities has only been identified in Opisthokonta (fungi and metazoans), whereas in other eukaryotes, TOM1 and TOM1-like (TOL) proteins seem to play this role (Blanc et al. 2009). *Arabidopsis* contains nine TOL proteins that bind ubiquitin and are necessary for the endocytic internalization of PIN2 (Korbei et al. 2013), suggesting that ESCRT-0 may associate with its cargo as early as at the plasma membrane.

In Opisthokonta, there are four ESCRT-I subunits: Vps23p/Tsg101 (Tumor Susceptibility Gene 101), Vps28, Vps37, and Mvb12 (Multivesicular Body 12) or the Mvb12-like protein UBAP1 (Ubiquitin Associated Protein 1) (Schuh and Audhya 2014). Plants contain two isoforms of only three ESCRT-I subunits, ELCH/VPS23A (Spitzer et al. 2006) and VPS23B, VPS28-1 and VPS28-2, VPS37-1 and VPS37-2, but lack an MVB12 homologue. Interestingly, a plant-specific ESCRT component called FREE1 (FYVE DOMAIN PROTEIN REQUIRED FOR ENDOSOMAL SORTING 1) may form part of the plant ESCRT-I complex (Gao et al. 2014). FREE1 is essential for endosomal vesiculation, PIN2 turnover, and polar localization of the iron transporter IRT1 (Barberon et al. 2014; Gao et al. 2014; Kolb et al. 2015).

The ESCRT-II complex is a tetrameric complex made of two subunits of VPS25, one subunit of VPS22, and one subunit of VPS36, which binds ubiquitin. In vitro studies showed that ESCRT-I and II promote membrane bending (Wollert and Hurley 2010; Fyfe et al. 2011), likely initiating the process of vesicle budding. Once ESCRT-I and II complexes have assembled on the endosomal membrane, the ESCRT-II subunit VPS25 recruits ESCRT-III proteins to the site of vesicle budding (Teis et al. 2010). ESCRT-III subunits polymerize, forming spiral filaments that further deform the endosomal membrane and constrict the neck of the budding vesicle (Shen et al. 2014; Adell et al. 2017; Chiaruttini et al. 2015). The ESCRT-III complex consists of five core proteins (VPS20, SNF7, VPS32, VPS24, and VPS2) and several accessory proteins (CHMP1, VPS60, and IST1). In plants, loss of core ESCRT-III subunits is lethal and leads to embryo development arrest. Mutations in the ESCRT-III accessory protein CHMP1A and B in *Arabidopsis* cause severe trafficking defects and seedling lethality (Spitzer et al. 2009). ESCRT-III proteins share many structural similarities and contain an N-terminal core domain that is responsible for the assembly of filaments on membranes. While ESCRT-III proteins are in the cytoplasm, they are thought to exist in an auto-inhibited state that prevent them from binding ESCRT-II and activating other ESCRT-III proteins ectopically (Bajorek et al. 2009). At their C-terminus, ESCRT-III proteins contain MIM (MIT Interacting Motif) domains that bind MIT domains of other ESCRT proteins such as the AAA ATPase VPS4/SKD1, LIP5, and AMSH3 (Associated Molecule with the SH3 domain of STAM 3). For example, in plants, the ESCRT-III associated protein ALIX binds the MIT domain of AMSH3, a deubiquitinating enzyme that removes the ubiquitin from ESCRT cargo proteins (Kalinowska et al. 2015; Katsiarimpa et al. 2011). The interaction between the MIM domain of core and accessory ESCRT-III proteins and the MIT domains of SKD1 is critical for membrane scission and vesicle release. The MIM domain of ESCRT-III proteins recruits VPS4/SKD1, which assembles with its cofactor LIP5 at the site of ESCRT assembly (Shestakova et al. 2010). Hydrolysis of ATP by VPS4/SKD1 results in the remodeling of the ESCRT coat, its recycling, and formation of intralumenal vesicles. In plants, LIP5 is a positive regulator of SKD1 activity, but it is not essential for plant development (Haas et al. 2007). However, LIP5 seems to be important during the increased membrane flux through the endosomal pathway during abiotic stress as evidenced by the hypersensitivity of *lip5* mutant plants to heat and salt stress (Wang et al. 2015).

Using electron tomography and mathematical simulation tools, we have recently reported that successive rounds of ESCRT assembly result in the formation of concatenated intralumenal vesicles in plants. ESCRT-III proteins are trapped in the necks of the concatenated vesicles and do not seem to be recycled back to the cytoplasm but rather degraded inside the vacuole together with the intralumenal vesicles and their cargo (Fig. 3). The retention of ESCRT proteins at inter-vesicle bridges during endosomal sorting could provide diffusion barriers that aid in the sequestration of cargo proteins (Buono et al. 2017; Otegui 2018). Evidence of incomplete separation of intralumenal vesicles during ESCRT-mediated sorting have also been reported in *Caenorhabditis elegans* (Frankel et al. 2017).

Besides its well-known role in the degradation of plasma membrane proteins, the ESCRT pathway is also important for the turnover of cytosolic ABA signaling components in plants. The phytohormone ABA is detected by PYR, PYL, and RCAR proteins that act as an ABA receptor complex. This ABA receptor complex then binds and inhibits protein phosphatase 2Cs (PP2Cs), which are negative regulators of ABA signaling (Melcher et al. 2009). The ABA receptors PYR1 and PYL4 are degraded through ESCRT-dependent sorting. PYL4 is ubiquitinated by

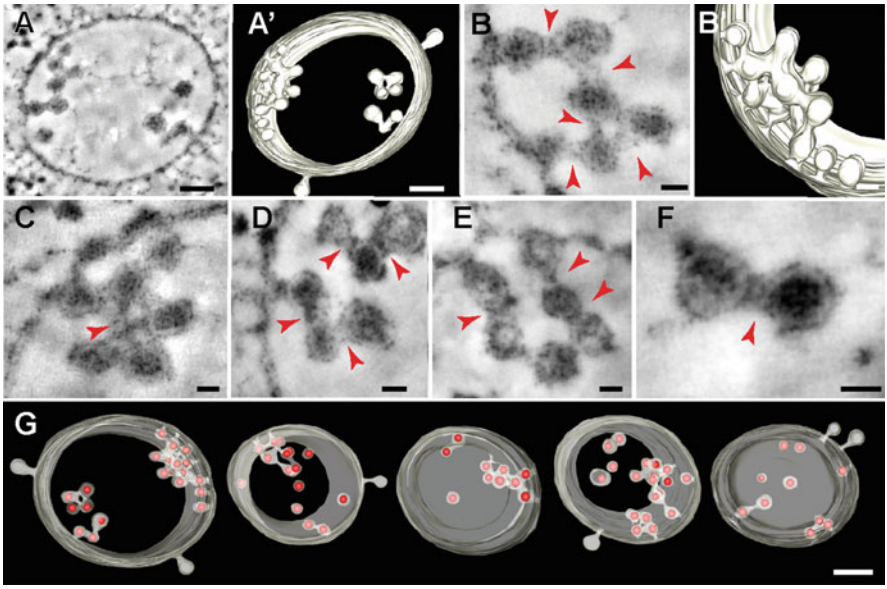

Fig. 3 Vesicle concatenation in multivesicular endosomes (MVE). (**a**, **b**) Tomographic slices and (**a′**, **b′**) corresponding tomographic reconstructions of an MVE from *Arabidopsis* root cells. Note the network of vesicle buds interconnected by narrow membrane bridges (arrowheads). (**c–f**) Tomographic slices from several wild-type MVEs with concatenated vesicle bud networks connected to the endosomal-limiting membrane. Membrane bridges linking vesicle buds are indicated by red arrows. (**g**) Gallery of wild-type MVEs from *Arabidopsis*. For easy visualization of the number and position of intralumenal vesicles within endosomes, a red sphere was placed at the center of each vesicle/vesicle bud. Scale bars = 50 nm in (**a**, **a′**, **g**); 20 nm in (**b–f**). From Buono et al. (2017)

RSL1, a plasma membrane-localized E3 ubiquitin ligase (Bueso et al. 2014). Ubiquitinated PYR1/PYL4-containing complexes interact with FYVE1/FREE1, which selects them to be incorporated into intralumenal vesicles (Belda-Palazon et al. 2016). As indicated above for the other pathways, membrane trafficking in connection to abiotic stress is tightly connected to Ca^{2+}-mediated signaling. The activity of PP2Cs is suppressed by both ABA and Ca^{2+} signaling. ROP-GEF1 activates ROP GTPases, which promote the activity of PP2Cs. Phosphorylation of ROP-GEF1 induces its degradative sorting into MVEs, whereas PP2C phosphatase activity stabilizes ROP-GEF1 (Li et al. 2016). Cytosolic Ca^{2+} activates PLDs, which as explained above produce PA, a negative regulator of PP2C activity (Zhang et al. 2004). Cytosolic Ca^{2+} also stimulates the activity of Ca^{2+}-dependent protein kinases, which phosphorylate ROP-GEF1, causing it to localize to multivesicular endosomes and be trafficked to the vacuole for degradation (Li et al. 2018) (Fig. 1).

3.3 Endosome Maturation and Fusion to Vacuoles

As multivesicular endosomes mature, they gain competency to fuse with the vacuole. In plants, the maturation and vacuolar fusion of endosomes is controlled by a number of regulatory proteins that include small GTPases of the RAB family, the tethering factors HOPS (HOMOTYPIC FUSION AND PROTEIN SORTING) and CORVET (CLASS C CORE VACUOLE/ENDOSOME TETHERING), and SNARE complexes (Takemoto et al. 2018). Interestingly, RAB5 and RAB7 GTPases act on different membrane tethering and fusion modules to promote fusion between endosomes and vacuoles. Thus, whereas RAB7 proteins recruit the tethering complex HOPS to endosomes, promoting the formation of SNARE complexes with the R-SNARE VAM713, RAB5 GTPases interact with the CORVET tethering complex, promoting the assembly of complexes containing the R-SNARE VAMP727 (Takemoto et al. 2018).

Overexpression of the RAB7 protein AtRABG3e promotes endosomal flow and enhances salt tolerance (Mazel et al. 2004). Plants overexpressing AtRabG3e show accelerated endocytosis and higher accumulation of Na^+ in their vacuoles (Mazel et al. 2004). In contrast, plants with reduced expression of the SNARE VAMP7C show increased resistance to salt stress. Under salt stress conditions, these plants hyperaccumulate endosomal-like compartments with H_2O_2, likely unable to fuse with the vacuole (Leshem et al. 2006).

When multivesicular endosomes fuse with vacuoles, the endosomal-limiting membrane becomes part of the tonoplast, whereas the intralumenal vesicles with their cargo are released inside the vacuole and degraded by vacuolar hydrolases.

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Molecular Aspects of Iron Nutrition in Plants

Carla S. Santos, Teresa Deuchande, and Marta W. Vasconcelos

Contents

Abstract The plant kingdom comprises 766 gymnosperms and ~350,000 angiosperms, for which iron (Fe) is an essential and highly demanded nutrient. Iron is necessary for plant growth and development, being involved in a multitude of functions within the plant, including chlorophyll biosynthesis. The understanding of the mechanisms that govern Fe uptake, transport and storage has been the subject of numerous studies since the middle of the twentieth century, but it was only in the 1990s, with the advent of molecular genetics, cheaper genome sequencing and associated bioinformatic techniques, that scientists began to really unveil the detailed molecular networks responsible for regulating iron homeostasis within the plant. Homeostasis must be guaranteed in order to prevent Fe overload and toxicity but also to assure sufficient levels within the plant to exert its numerous roles, since the

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C. S. Santos, T. Deuchande, and M. W. Vasconcelos (✉)
CBQF – Centro de Biotecnologia e Química Fina Laboratório Associado, Escola Superior de Biotecnologia, Universidade Católica Portuguesa, Porto, Portugal
e-mail: mvasconcelos@porto.ucp.pt

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unalike consequences of both deficiency and toxicity are equally adverse. In this chapter we explore the current knowledge on the different molecular aspects that regulate Fe metabolism in higher plants, looking at Fe uptake and distribution mechanisms, the known signalling molecules and Fe sensing mechanisms, the part of Fe in plant-bacteria symbiosis (including nodulated and non-nodulated plants) and finally, how the molecular aspects of Fe metabolism impact and are impacted by other metals.

1 Introduction

Dietary iron (Fe) deficiency is a worldwide public health problem affecting more than two billion people, whose primary dietary sources are legumes and grains (Myers et al. 2014). Therefore, any enhancement of Fe concentrations in these primary crops would be valuable. Iron (Fe) is also an essential nutrient for plants, with intermediate phloem mobility, whose concentration within a plant system must be tightly regulated. Under Fe deficiency, plants usually develop yellowing of leaves and reduced growth, since this nutrient is necessary in the electron transport chains of photosynthesis and respiration (Prasad 2003); on the other hand, if accumulated in high levels, Fe can generate hydroxyl radicals, which damage lipids, proteins and DNA (Kampfenkel et al. 1995). Hence, plants must respond to Fe levels in terms of both Fe deficiency and Fe overload (Connolly and Guerinot 2002).

The ascension of the omics era has contributed with vast information on Fe metabolism response and associated mechanisms, essential for the development of plant genotype improvement programmes (Vasconcelos et al. 2017). As of today, the genomes of at least 225 plant species have been completely sequenced, providing a plethora of data which allows scientists to understand the commonalities and particularities of the genes underlying mineral uptake, transport and storage. Most species with whole-genome sequences are crops, with food crops comprising the biggest fraction (57.7%), and include not only cereals but other important crop species grown for food, such as fruits, legume grains and vegetables (Leitch et al. 2017). Whole-genome sequencing and gene functional studies nowadays are not only targeting yield and disease resistance but also are targeting the understanding of the key genes involved in enhancing nutrient concentrations, so that better breeding and plant improvement techniques may be applied. Still, although Fe uptake, transport and accumulation mechanisms have been studied for long and applied in different types of biofortification strategies, from the point of view of Fe relationship with enzymatic activities (Weinstein and Robbins 1955), Fe translocation within the plant (Tiffin 1966) or the complexity of the plant-soil interactions (Chaney and Bell 1987), the genetic data gathered in recent years, still postulates assumptions that require further investigation, such as the classical division of plants according to their Fe uptake mechanism.

Soil properties, such as pH, amount of organic matter, clay minerals or carbonate content, greatly impact Fe availability for plant utilization (Bech et al. 2008). However, as soil is a complex matrix composed by several other nutrients, which are also affected by these properties, it is important to account for nutrient crosstalk and the net formed between them. Further in this chapter, the common genes regulated by Fe and other metals (copper, zinc, manganese, boron, molybdenum and cobalt), as well as between Fe and two non-metal nutrients (phosphorus and sulphur), will be scrutinized.

Fe is also essential for the establishment of plant-bacteria symbiosis. When growing in nitrogen-deficient soils, plants develop symbiotic interactions with nitrogen-fixing microorganism developing a new organ named root nodule. This newly formed plant organ, tightly regulated by the host plant, confers plants' ability to fix atmospheric nitrogen (N_2). In this chapter the importance of iron uptake and mobilization within legume plants for nodulation and establishment of effective symbiotic interactions with soil bacteria at a molecular level are reviewed and discussed. Although a brief summary is presented considering differences between legume and nonlegume root nodules, this section is focused on legume-rhizobia symbiosis. Legumes represent one third of primary crop produced in the world being an important dietary source of protein and minerals for both livestock and humans. On a global scale, biological nitrogen fixation in the legume-rhizobia symbiosis accounts for roughly 200 million tons of fixed nitrogen per year (Graham and Vance 2003; Ferguson et al. 2010) with symbiotic crops requiring 35–60% less fossil-based energy than conventional, *N*-fertilized crops (Jensen et al. 2012). In order to potentiate intensive and sustainable agriculture, the production of nitrogen-fixing legumes should be promoted. In parallel, efforts should also be made to develop biofortified legume crops with increasing levels of iron. Still, to integrate the beneficial environmental and dietary effects resulting from legume nodulation, it is crucial to understand how iron is transported to nodules, utilized by the nodule and mobilized towards the plant. Despite the importance of understanding the mechanisms involved on Fe uptake throughout the plant for future modulation towards increasing grain Fe concentrations in these environmentally friendly nodulated plants, little is known about these processes. In this chapter an overview of current knowledge is given. In the context of current climate changes, understanding these mechanisms is even more urgent. Increasing atmospheric CO_2 levels (predicted to double by the end of the century reaching 800 ppm) are foreseen to negatively impact the nutritional status of several crops (Loladze 2014). The reduced Fe levels found were purported to be related with mechanisms involved on Fe transportation within the plant. Under eCO_2 whereas photosynthesis is generally induced leading to a generally higher sugar and biomass accumulation and better yields (Högy et al. 2009; Köehler et al. 2019), it also reduces stomatal conductance which may decrease Fe uptake through the xylem (Ainsworth and Rogers 2007; Xu et al. 2016; He et al. 2018). Recently, it has been reported that increasing air temperature (foreseen to increase 6°C by 2050, relative to 1961–1990) counteracts the negative effect of eCO_2 in the nutritional quality of soybean grains (Köehler et al. 2019), and

this opposite effect was attributed to differences in stomatal conductance regulation (Urban et al. 2017).

After discussing the recent knowledge on Fe uptake mechanisms, in this chapter, the essential genetic factors involved in Fe transport and signalling within the plant will be reviewed.

2 Iron Uptake Mechanisms

Although Fe is present in great abundance in the soils, it exists mainly as Fe^{3+}, which forms insoluble hydroxides and becomes unavailable for plant absorption. Therefore, when the conditions are adverse, e.g. alkaline or upland soils, plants are unable to uptake Fe from the soils. To overcome this problem, plants developed strategies to acquire Fe from the rhizosphere, which are classically divided into two. Strategy I, also referred to as 'reduction strategy', is utilized by all dicotyledonous and non-graminaceous plants. The first engaged step consists of proton release via H^+-ATPases in order to decrease rhizosphere's pH and, consequently, increase Fe solubility (Colangelo and Guerinot 2004). After the acidification step, Fe^{3+} is reduced to Fe^{2+} by a root ferric chelate reductase. In *Arabidopsis*, this enzyme is encoded by ferric reductase oxidase 2 (AtFRO2), which is composed of two intramembrane haem groups, and is induced in the root epidermis to transfer electrons across the plasma membrane (using NAD(P)H as an electron donor), performing the reduction step (Robinson et al. 1999). Genes encoding the FRO enzyme include eight members that are differentially expressed at the tissue levels, being not only important for metal acquisition from soil but also for intracellular distribution of Fe (Jain et al. 2014). More specifically, FRO1 was characterized in pea to have 74% of overall similarity to AtFRO2 (Waters et al. 2002); AtFRO5, AtFRO7 and AtFRO8 do not seem to be Fe-regulated, and AtFRO3 is expressed in Fe-deficient leaves (Jeong and Connolly 2009); AtFRO6 overexpression in tobacco plants enhanced ferric reductase activity in the leaves (Li et al. 2011). Alongside with FRO, other compounds have been proposed to have a key role in the reducing step, such as phenolics, organic acids, sugars and flavins (López-Millán et al. 2000; Rodríguez-Celma et al. 2011), and recent reports identified scopoletins, a class of phenolic-type compounds, to be secreted under Fe-deficient conditions and have an important role in plant Fe nutrition (Fourcroy et al. 2014; Schmid et al. 2014; Tsai et al. 2018).

After Fe^{3+} is reduced, Fe^{2+} is transported into the root by iron-regulated transporter 1 (IRT1), which belongs to the zinc-regulated transporter/IRT-like protein (ZIP) family (Guerinot 2000). IRT1 was described to be expressed only under Fe-deficient conditions (Connolly et al. 2002), but it can also transport other divalent metals, and it has been shown that the overexpression of AtIRT1 induces metal overload (Barberon et al. 2011). Other studies showed that when the peanut AhIRT1 gene was introduced in tobacco and rice, it had a dual function: besides being responsible for Fe absorption, it could also be responsible for Fe translocation, as

the transgenic plants increased their tolerance to Fe deficiency and, even under Fe sufficiency, Fe concentration was enhanced in roots and shoots (Xiong et al. 2014).

Graminaceous plants, like barley, rice and maize, utilize Strategy II (a 'chelation strategy') for Fe uptake. In order to increase uptake, plants release phytosiderophores (PSs) to the rhizosphere which act as chelators with high affinity for Fe^{3+}. The primary member of the PSs family is deoxymugineic acid (DMA), and nicotianamine (NA) is the main precursor for its synthesis (Morrissey and Guerinot 2009). Constitutive expression of the rice OsNAS2 gene in bread wheat increased the biosynthesis of NA and DMA leading to higher concentrations of grain Fe, Zn, NA and DMA and increased Fe bioavailability of the resulting flours (Beasley et al. 2019). Two transcription factors seem to have an essential role in DMA and NA synthesis, namely, IRO2 that regulates their synthesis by influencing DMA and NA synthase (DMAS and NAS) expression (Ogo et al. 2007) and an Fe deficiency-responsive cis-acting element-binding factor 1 (IDEF1) that intervenes in this synthesis by positively regulating the expression of IRO2 (Kobayashi et al. 2009). Phytosiderophores are effluxed to the rhizosphere via TOM1, a transporter whose expression levels augment under Fe-deficient conditions (Nozoye et al. 2011). Once in the rhizosphere, the complex Fe^{3+}-PS is formed and is taken up into the root cells by transmembrane proteins of the yellow stripe1 (YS1) family (Curie et al. 2001). YS1 transporters have been identified in several grass species, and, interestingly, non-graminaceous plants also have YS1-like (YSL) genes that encode proteins essential in metal-NA complex transporting Fe (Inoue et al. 2009).

Although this classic division is mostly true, there are few studies showing that some Strategy II plants could use Strategy I mechanisms, as is the example of rice (Bughio et al. 2002; Ricachenevsky and Sperotto 2014; Pereira et al. 2014). Evidences suggest the use of a 'combined strategy', where rice plants besides absorbing Fe(III) via the chelation strategy also take up Fe(II) directly by the induction of Strategy I transmembrane transporters IRT1/IRT2 (Ishimaru et al. 2006; Sperotto et al. 2012; Pereira et al. 2014). Alike rice, homologs for Strategy I-related genes have been found in maize, such as *ZmNRAMP1* (Zanin et al. 2017); however the physiological role and putative functions of these genes in maize are yet to be explored (Li et al. 2018).

3 Molecular Factors Involved in Fe Distribution Within the Plants

After entering the root cells, Fe can be transported to the aboveground organs via the xylem (Broadley et al. 2012). This transport has for long been associated to the formation of complexes between Fe and citrate, which seemed to be the preferential form for Fe loading in the xylem (Tiffin 1966). In the meantime, studies confirmed this theory (Green and Rogers 2004), and a ferric reductase defective 3 (FRD3)

protein, belonging to the multidrug and toxic compound extrusion (MATE) family, has been described to be necessary for efficient Fe translocation (Durrett et al. 2007).

Despite being predominantly transported through the xylem (López-Millán et al. 2000), Fe can also be transported through the phloem, complexed with NA, as this metabolite, although not secreted by non-graminaceous plants, is synthetized and chelates Fe (Stephan and Scholz 1993; Takahashi et al. 2003). In apple, NAS1 expression was shown to increase under Fe deficiency and to facilitate redistribution of Fe in plants (Sun et al. 2018). When Fe reaches the leaves, it is putatively unloaded in the apoplastic space via the YSL transporters (Waters et al. 2006).

Free Fe is toxic; therefore, it must be incorporated in storage structures. Ferritins, for example, store Fe in excess for detoxification and maintain the mineral available for protein synthesis (Briat et al. 2010). Ferritins can be found in most of the cellular compartments, but the main storage organelle is the chloroplast (Briat et al. 2010). It is generally established that under Fe supply, genes of the ferritin family are usually overexpressed (Lescure et al. 1991; Wu et al. 2016). The majority of the Fe pool is mainly located in chloroplasts (Roschzttardtz et al. 2013), and, although the method for influx is still not well described (López-Millán et al. 2016), it is thought to require a reduction-based mechanism, mediated by a member of the FRO family, probably FRO7, both in Strategy I and Strategy II plants (Solti et al. 2014). The other major reservoir for inactive Fe is the vacuole, and Fe is imported via a vacuolar membrane transporter, VIT1, and remobilized by the NRAMP3 and NRAMP4 transporters (Lanquar et al. 2005). Moreover, these proteins have been shown to have a conserved role in Fe transportation and homeostasis in different crops, as is the case of VIT expression in rapeseed (Zhu et al. 2016) and of *Arabidopsis* VIT1 expression in cassava that showed promising results for biofortification programme development (Narayanan et al. 2015). The later results were further improved in 2019 by combining the overexpression of VIT1 with IRT1 and FER1, with cassava plants accumulating iron levels 7–18 times higher and zinc levels 3–10 times higher than those in non-transgenic controls in the field (Narayanan et al. 2019). Other examples include AhNRAMP1 in peanut (Xiong et al. 2012) and MxNRAMP1 in apple (Pan et al. 2015) leading to increased Fe accumulation.

4 Signalling Molecules and Fe Sensing Mechanisms

In plants, the complete network behind Fe sensing and signalling is yet to be described (Kobayashi et al. 2018). However, it is known that Fe homeostasis requires different signals and regulators, having an ultimate implication on photoassimilate partitioning, due to its source-sink control (Marschner et al. 1996; Lemoine et al. 2013). Shoots have always been pointed as the main responsible organ for signalling the need for increased Fe uptake at the root level (Brown et al. 1961; Schmidt 2003). A negative feedback control for Fe uptake has been proposed, where Fe sufficiency represses the synthesis of the ferric chelate reduction system (Maas et al. 1988); however, a positive regulation has also been proven to exist,

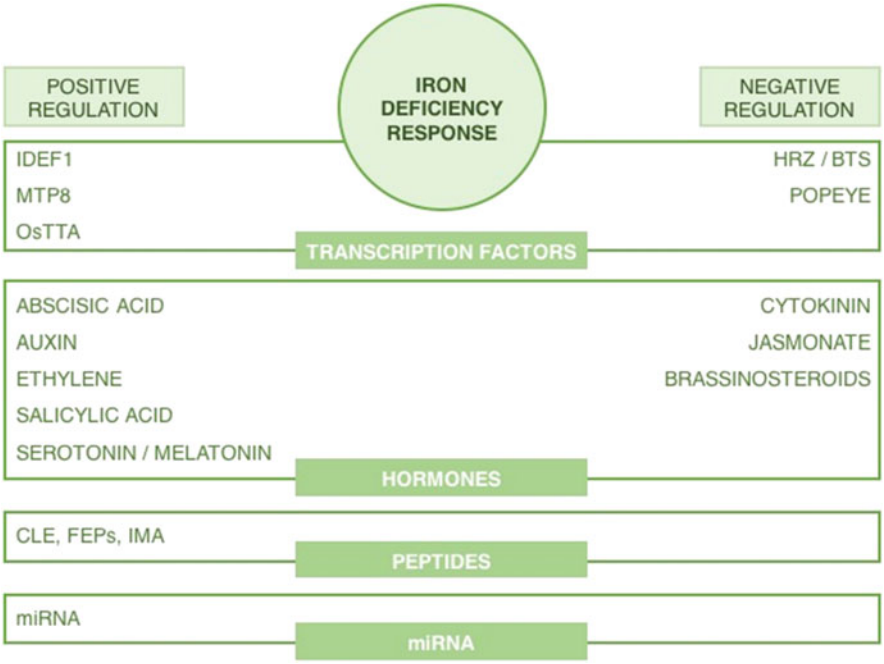

Fig. 1 Positive and negative regulators involved in iron deficiency signalling in plants

where a long-distance signal for Fe deficiency in the shoots induces Fe uptake in the roots (Enomoto et al. 2007). A combined network system for the activation of physiological Fe stress responses has also been suggested (García-Mina et al. 2013). In this model, together with a predominant root-to-shoot signal, which is dependent not on the Fe conditions at the root level, but on the development of Fe stress symptoms in the leaves, a local Fe sensing is also present in the roots, which corresponds to the triggering of FRO and IRT1 genes in response to Fe stress at the root level, independently of the Fe conditions presented by the leaves (Fig. 1).

Molecules with the potential to regulate Fe accumulation have been identified. These include the transcription factor *IDEF1* that positively regulates IRO2 gene in graminaceous plants under Fe deficiency (Kobayashi and Nishizawa 2014); hemerythrin motif-containing really interesting new gene (RING) and zinc-finger proteins (*ZNFs*)/BRUTUS (*BTS*) ubiquitin ligases, which negatively regulate Fe deficiency responses in both graminaceous and non-graminaceous plants, controlling Fe uptake and translocation under Fe sufficiency to prevent Fe excess caused damage (Kobayashi et al. 2013; Matthiadis and Long 2016); a bHLH protein, *POPEYE*, which downregulates genes related to metal translocation (FRO3, NAS4 and ZIF1) under Fe stress conditions (Long et al. 2010); metal tolerance proteins (*MTP*), identified in wheat grains, with a role in divalent metal effluxing out of the cytoplasm

and involvement in metal tolerance under Fe deficiency stress (Eroglu et al. 2017; Vatansever et al. 2017); and a putative *Arabidopsis thaliana TITANIA1* ortholog in rice (*OsTTA*) that was shown to be a positive transcriptional regulator of Fe (Tanaka et al. 2018).

Besides the abovementioned proteins and cofactors, phytohormones have been pinpointed for their preponderant role in Fe homeostasis. This is because the concentration and transport of hormones inside the plants seem to dictate shoot responses to nutrient availability in the soil (Kudoyarova et al. 2015). *Cytokinins* act both as root-to-shoot long-distance signal and as a local signal, and they repress IRT1, FRO2 and FIT gene expression (Kiba et al. 2011); *jasmonic acid* also impacts negatively on the expression of Fe uptake genes, IRT1 and FRO2 (Cui et al. 2018; Kobayashi et al. 2016), although some research show that methyl jasmonate foliar application might increase Fe concentration in plant tissues (Ghassemi-Golezani and Farhangi-Abriz 2018; Li et al. 2017); and finally, *brassinosteroids* seem to aggravate Fe deficiency responses by repressing Strategy I response genes, FRO1 and IRT1 (Wang et al. 2012), and phytosiderophore synthesis genes, NAS1, NAS2 and YSL2, in rice (Wang et al. 2015), and by modulating Fe accumulation via a feedback loop (Singh et al. 2018).

Among the phytohormones that positively regulate Fe deficiency responses, it has been demonstrated that *abscisic acid* (ABA) promotes Fe transportation from root-to-shoot and phenolic compound secretion from the roots (Lei et al. 2014) and regulates the maintenance of Fe levels by inducing ferritin expression, as shown in rose petals (Liu et al. 2017a, b); there is a crosstalk between *auxin* and Fe, especially in Strategy II utilizing plants (Garnica et al. 2018; Kabir et al. 2016; Liu et al. 2015; Shen et al. 2016), where auxin application regulates Fe-related genes mainly implicated in phytosiderophore release (NAS, YSL and DMAS), but also in Fe uptake (IRT and FRO), demonstrating its role in root-to-shoot signalling; *ethylene* is an important regulator of Fe deficiency responses (Li and Lan 2017; García et al. 2018) and, more recently, the role of ethylene response factors (ERFs) has been studied (Liu et al. 2017a, b, 2018); *salicylic acid* improves oxidative damage caused by Fe deficiency by affecting the oxidation reaction at the transcriptional level (Kong et al. 2014; Shen et al. 2016), and elevated endogenous levels of this hormone induce other hormone-response genes, such as auxin and ethylene (Shen et al. 2016), implying an interplay in the hormonal signalling; and supplementation with *melatonin* and its precursor *serotonin* alleviates Fe deficiency response by inducing the expression of bHLH protein-encoding genes, FRO2 and IRT1 (Wan et al. 2018), and also by interplaying with abscisic and salicylic acids (Yoon et al. 2019).

However, other molecules are known to also significantly intervene in Fe signalling. Different peptide families have a role in response to Fe stress in plants, for example, the CLAVATA3/ENDOSPERM SURROUNDING REGION (*CLE*) peptides (Araya et al. 2016; Gutiérrez-Alanís et al. 2017) and the FE-UPTAKE-INDUCING PEPTIDEs (*FEPs*) that are induced under Fe-deficient conditions (Hirayama et al. 2018) or a short C-terminal amino-acid sequence consensus motif (IRON MAN; *IMA*) that positively regulates Fe uptake in roots (Grillet et al. 2018). Few studies also point out the role of microRNA (*miRNA*) in Fe homeostasis,

whether by up-regulating the expression of Fe deficiency inducible transcription factors and auxin signalling pathways (Kong and Yang 2010) or by post-transcriptionally regulating photosynthetic machinery-related genes (Checovich et al. 2016; Patel et al. 2017) (Fig. 1).

5 The Role of Iron in Plant-Bacteria Symbiosis

Plants are botanically sub-divided into the graminaceous and non-graminaceous families. As mentioned before, these two groups use different pathways to mine iron from the soil. The non-graminaceous plants including the legumes use Strategy I, whereas graminaceous including cereals such as wheat or maize use Strategy II (see Sect. 1). In this section the importance of iron uptake and mobilization within legume plants for nodulation and establishment of effective symbiotic interactions with soil bacteria are reviewed and discussed. A brief summary is presented considering differences between legume and nonlegume root nodules, but considering the importance of legumes for human nutrition, this section is focused on legume-rhizobia symbiosis.

6 Legume and Nonlegume Root Nodules

Root nodules are specialized lateral root organs with 2–5 mm diameter which look like white dots and result from the establishment of an effective mutually beneficial interaction between a plant (the host) and selected soil microorganisms. When plants grow in nitrogen-deficient soils, the formation of root nodules is promoted in order to fulfil the plant nutritional needs. Although nitrogen gas (N_2) is the most abundant gas in the Earth's atmosphere, this molecular form cannot be used by most organisms. Only few bacteria and archaea are capable of fixing N_2 into ammonia that can be assimilated. Nevertheless, many legumes have evolved to establish a symbiosis with gram-negative nitrogen-fixing soil bacteria collectively known as rhizobia. These bacteria belong to the *Rhizobiaceae* family and spread to the alpha- and beta-proteobacteria classes (Moulin et al. 2001) including the genera *Azorhizobium*, *Allorhizobium*, *Bradyrhizobium* (Jordan and Bacteriology 1982), *Mesorhizobium* (Jarvis et al. 1997; Laranjo et al. 2014), *Rhizobium* and *Sinorhizobium* (Chen et al. 1988), among others. *Rhizobia* bacteria grow in the rhizosphere, the narrow soil region in contact with the root, where, due to the organic carbon provided by plant roots in the form of root exudates, the number of microorganism is largely higher (10–100 higher) than in bulk soil (Pii et al. 2015).

Though the majority of plants able to establish this symbiotic relation are in the legume family Fabaceae, there are few exceptions. In the order Rosales, *Parasponia* species belonging to the Cannabaceae family can also establish a symbiotic

interaction with *Rhizobia*, constituting the unique nonlegume specie known to be nodulated by *Rhizobia* (Hirsch 1992; Sytsma et al. 2002).

The filamentous gram-positive actinomycete *Frankia* also forms actinorhizal root nodules on a variety of woody shrubs, trees and some herbaceous plants, fixing approximately the same amount of nitrogen as rhizobial symbioses. All actinorhizal species, about 200, belong to the Rosid I clade sharing a common ancestor with legumes (Fabaceae) but differing from them in their wide distribution in numerous botanical families. These species belong to three plant orders, the Fagales which include the Betulaceae, Casuarinaceae and Myricaceae families; the Rosales with the Rosaceae, Elaeagnaceae and Rhamnaceae families; and the Cucurbitales including the Coriariaceae and Datiscaceae families (Santi et al. 2013). Differently from legume nodules which have a peripheral vasculature, the nodules formed by these nonlegume plants possess a central vascular system like roots (Santi et al. 2013; Downie 2014), and bacteria are never released from the infection thread. According to Downie (2014), it may signify that in legumes the oxygen is available for energy production, while in nonlegume the haemoglobin in the cells surrounding the vasculature would tend to bind most of the available oxygen slowing respiration and reducing ATP levels making this symbiosis less efficient than that formed by legume-rhizobia.

7 Legume Root Nodule Formation

Nodulation begins when *Rhizobia* contact the root hairs of the host plant and the roots release flavonoids which are recognized by specific *Rhizobia* bacteria inducing the expression of rhizobial genes encoding enzymes involved in the synthesis and secretion of Nod factors. Nod factors are lipochito-oligosaccharide signalling molecules sensed by the root hair cells through membrane receptor-like kinases, triggering a series of biochemical and morphological changes involved on nodule development (Desbrosses and Stougaard 2011). Root hair deformation (curl) is induced leading to the complete encapsulation of one or more bacteria which multiply, being then transported through an infection thread which grows from the root hair into the basal part of the epidermis cell extending to the root cortex (Hirsch 1992). In the cortical cells *Rhizobia* bacteria are surrounded by a plant-derived membrane named symbiosome membrane forming the symbiosome, an organelle-like structure separated from the plant cell (Verma and Hong 1996). In mature symbiosomes, rhizobia differentiate into a nitrogen-fixing form named bacteroid, which has the ability to absorb dinitrogen gas (N_2) from the atmosphere and convert it to ammonia (NH_3) which is incorporated into organic form before being exported from nodules. For nitrogen fixation the bacteroid requires a constant supply of energy and carbon from the host plant in the form of organic acids. In the nodules, sugars translocated from the shoot are converted to phosphoenolpyruvate through glycolysis which is converted to malate by the action of phosphoenolpyruvate carboxylase (PEPC) and malate dehydrogenase activities. Malate is considered to

be the primary source of carbon transported to bacteroids where it is converted to CO_2 and water to produce ATP and reducing power required for nitrogen fixation (Vance and Gantt 1992; Colebatch et al. 2004; Udvardi and Poole 2013).

Legume nodules formed through these symbiotic interactions can be determinate or indeterminate. Determinate nodules lose meristematic activity shortly after initiation, and nodule growth is due to cell expansion resulting in mature spherical nodules. These nodules develop in tropical legume, such as those of the genera *Glycine* (soybean), *Phaseolus* (bean), *Arachis* (peanut) and *Vigna* (cowpea), and on some temperate legumes such as *Lotus* (e.g. *Lotus japonicus*). Indeterminate nodules maintain an active apical meristem that produces new cells through cell division at the distal end of the nodule becoming elongated. Therefore, these nodules form four zones: meristem (zone I); invasion zone (II) in which rhizobia colonize plant cells, proliferate, and differentiate; the nitrogen fixation zone (III) where bacteroids produce ammonia for the plant; and the senescence zone (IV), which is present only in older nodules and may serve to recycle nutrients (Hirsch 1992). Examples of plants developing indeterminate nodules include temperate legumes such as those from the genera *Pisum* (pea), *Medicago* (alfalfa) and *Trifolium* (clover) (Mao et al. 2015).

8 Impact of Soil Iron Deficiency in Nodulation

Nodulation is regulated by external (heat, soil pH and composition, drought, nitrate) and internal factors (autoregulation of nodulation, ethylene). Autoregulation of nodulation controls nodule numbers per plant through a systemic process involving the leaf (Ferguson et al. 2010). Several studies have shown that Fe starvation limits root nodule bacterial survival and multiplication, as well as host plant growth, nodule initiation and development and nitrogen fixation (Tang et al. 1991a; Slatni et al. 2011, 2014).

Lupinus angustifolius plants supplied with mineral nitrogen have been reported to have lower shoot weight than nodulated plants supplied with increasing Fe levels (Tang et al. 1992). These results demonstrated that nodule formation and function require more Fe than the growth of the host plant.

Nitrogen concentrations and contents in nodulated *Phaseolus vulg*aris L. (Hemantaranjan 1988) and *Arachis hypogaea* L. (Tang et al. 1991b) were reported to decrease under Fe deficiency. In addition, in several legumes, Fe deficiency decreases nodule number and bacteroid nitrogenase activity (Tang et al. 1992). In the nodules of iron-deficient *A. hypogaea*, nitrogenase activity has been shown to be reduced by 10–20% with nitrogen fixation being further delayed in these plants as compared to plants grown under normal conditions (O'Hara et al. 1988; Tang et al. 1991b). The reduced activity may be partly caused by reduced synthesis of leghemoglobin and bacteroid proliferation (Tang et al. 1992). In *L. angustifolius* nodule initiation is impaired by Fe deficiency (Tang et al. 1991a, 1992). Due to the inhibition of rhizobia proliferation in roots, the division of root

cortical cells required to form nodule meristems is limited causing the nodulation impairment (Tang et al. 1992).

9 Iron Requirements for Legume-Rhizobia Symbiosis

The establishment of a symbiotic relation between the host plant and the bacteroid requires increased uptake of micronutrients by the plant, since the bacteroids in root nodules are dependent on the plant for all micronutrients (Clarke et al. 2014). Iron is a critical micronutrient for the symbiosis because several symbiotic proteins incorporate iron including the bacterial nitrogen-fixing enzyme (nitrogenase), cytochromes required for phosphorylation in the plant and bacteria, plant leghemoglobin which transports oxygen, ferredoxins involved in transferring electrons and reducing the Fe component of nitrogenase and a variety of other Fe proteins such as hydrogenases (Guerinot 1991; Broadley et al. 2012; Brear et al. 2013). Leghemoglobins are the most abundant plant proteins in nodules representing as much as 25–40% of the total soluble protein in the infected plant cell (Kozlov 2014) and containing about 24% of the soluble iron within the nodule (Ragland and Theil 1993). These proteins are composed of an apoprotein and a haem moiety both synthesized by the plant (O'Brian 1996). Since the bacteroids do not have contact with the pool of leghemoglobin in the cytoplasm of infected cells (Wittenberg et al. 1996), leghemoglobins may not constitute a major source of Fe during symbiosis. However, they have been shown, via reverse genetics, to have a crucial role in symbiotic nitrogen fixation (Ott et al. 2005). Suppression of the expression of the gene encoding leghemoglobin in *Lotus japonicus* led to loss of leghemoglobin in nodules, higher steady-state levels of free oxygen but lower ATP/ADP ratios and a complete absence of nitrogenase activity (Ott et al. 2005). The bacteroid nitrogenase complex consists of six protein subunits (two each of NifH, NifD and NifK), four iron-sulphur clusters (two each of [4Fe–4S] and Fe_8S_7) and two iron-molybdenum cofactors (Fe_7MoS_9N) named FeMoco (Downie 2014). This enzyme is very sensitive to oxygen; therefore, in order to avoid nitrogenase degradation and provide the necessary oxygen for bacterial respiration, the legume host maintains microaerobic oxygen concentrations by controlling the permeability of the nodule cells to oxygen through changes in the proportion of gas and water in the inner cortex of the nodule (Wei and Layzell 2006) or by increasing the expression of leghemoglobin in the infected nodule cells. Leghemoglobin buffers free oxygen content in the nitrogen-fixing zone of the plant nodule reducing it to the nanomolar range avoiding the irreversible inactivation of the oxygen-labile nitrogenase while providing adequate oxygen levels for bacterial respiration (Ott et al. 2005).

10 Iron Uptake to the Nodule and Remobilization

Regardless of the importance of understanding the mechanisms involved on iron uptake to the nodule and translocation from the nodule, little is known about these transport mechanisms (Slatni et al. 2011; Brear et al. 2013).

Legumes are Strategy I plants, reducing Fe by activating the plasma membrane ferric chelate reductase enzyme or by exuding organic acids and protons to the rhizosphere and by taking up reduced Fe using specific Fe transporters. In root nodules of *Phaseolus vulgaris* grown under Fe deficiency, H^+-ATPase and ferric chelate reductase activities have been shown to be induced (Slatni et al. 2009, 2011). In addition it was reported that under Fe deficiency there is an over-accumulation of H^+-ATPase and ZIP transporters (IRT1) in the nodules' cortex cells of plants tolerant to Fe deficiency particularly around the cortex cells of nodules (Slatni et al. 2012). These results suggest that Fe would be transported through the apoplast using H^+-ATPases and ZIP family members to uptake apoplastic Fe. In such case nodule epidermis would play a major role in Fe uptake in tolerant cultivars. This hypothesis was further supported in a subsequent study reporting that under Fe deficiency Fe allocation from the root system to the nodules was preferential in cultivars tolerant to Fe deficiency (Slatni et al. 2014). However, in indeterminate nodules of *Medicago truncatula*, Fe was not observed at the epidermis of the nodule appearing to be delivered into nodules via the vascular system and released into the apoplasm of cells in the invasion zone to the rhizobia-infected cells by an NRAMP plasma membrane transporter before being taken up by plant cells and transported to bacteroids (Rodríguez-Haas et al. 2013; Tejada-Jiménez et al. 2015). These contrasting results suggest that nodule Fe uptake directly from the medium may be a secondary route of Fe acquisition, comprising a mechanism developed by specific cultivars to cope with Fe limitation.

Multidrug and toxic compound extrusion (MATE) proteins transport citrate and can form chelates with ferric Fe facilitating Fe mobilization (Takanashi et al. 2013). In *Arabidopsis* a root citrate transporter (*AtFRD3*), a member of MATE family, has been reported to be required for Fe translocation from roots to shoots (Durrett et al. 2007). And two MATE transporters (*GmFRD3* and *GmFRD3b*) have been shown to mediate Fe translocation in soybean (Rogers et al. 2009). Regarding the possible involvement of MATE transporters on Fe regulation in the nodule, Takanashi et al. (2013) reported that in *Lotus japonicus LjMATE1* is induced during nodule formation, mainly in the infection zone, and suppression of its expression leads to reduced Fe accumulation in infected cells. These observations suggest that *LjMATE1* is a nodule-specific transporter providing citrate for Fe translocation from the root to nodules being involved on Fe accumulation in the nodules (Takanashi et al. 2013) (Fig. 2). In a recent study conducted in *Medicago truncatula*, the *MtMATE67* protein which segregates into a clade that includes the citrate efflux transporters *LjMATE1* and *AtFRD3* was found to be located in the plasma membrane of nodule cells and also in the symbiosome membrane surrounding bacteroids in infected cells. *MtMATE67* has been shown to actively transport citrate out the cells when expressed

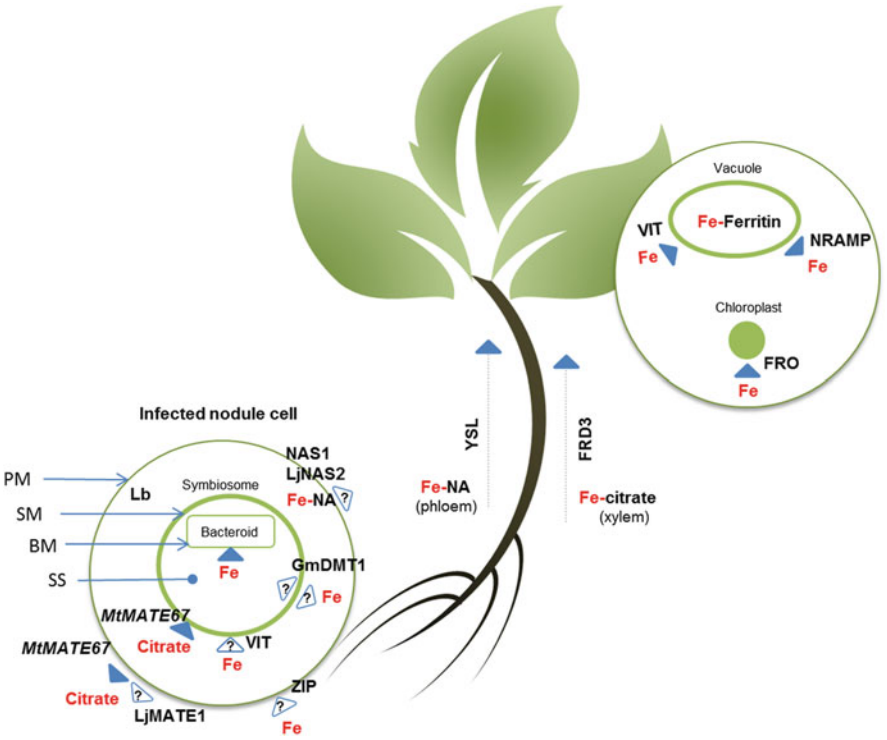

Fig. 2 Main proteins involved in the Fe transport and distribution in the plant and in the nodules. Transport movement is indicated by a blue arrow; all transport proteins and chelators are depicted in black. *PM* plasma membrane, *SM* symbiosome membrane, *SS* symbiosome space, *BM* bacteroid membrane, *Lb* leghemoglobin. Undetermined direction of Fe transport is marked by '?'

in *Xenopus oocytes* with its suppression leading to Fe accumulation in the apoplasm of nodule cells and a significant decrease in nitrogen fixation and plant growth (Kryvoruchko et al. 2018). In this study it became clear that *MtMATE67* plays an important role in the citrate efflux from nodule to cells by increasing Fe(III) solubility and mobility in the apoplasm and uptake into nodule cells. Also at symbiosome level, the citrate transport by *MtMATE67* into the symbiosome space would contribute for pH reduction and increased Fe solubility and availability for bacteroids (Kryvoruchko et al. 2018) (Fig. 2).

In non-graminaceous plants, such as legumes which use Strategy I mechanism for Fe uptake, nicotinamide (NA), a phytosiderophore precursor, is not exuded by the plant to increase Fe uptake, but it chelates metal cations including Fe within the plant. Therefore, NA is believed to play a role as a long-distance signalling molecule (Curie and Briat 2003) possibly acting as a regulator of internal Fe transport, being involved on Fe homeostasis in plants (Takahashi et al. 2003; Hakoyama et al. 2009). When a gene encoding nicotianamine aminotransferase (*HvNAAT*) from barley

(*Hordeum vulgare*, Strategy II plant) was introduced into tobacco plants (Strategy I plants), the plants developed chlorosis symptoms related with NA shortage resulting from the overproduction of the enzyme (NAAT) (Takahashi et al. 2003).

Although in *Lotus japonicus* the suppression of NA synthase 2 (*LjNAS2*) responsible for NA formation did not affect nitrogen fixation, it has been shown to be specifically expressed in the nodules particularly in nodule vascular bundles and further expressed in a later stage of nodule development (maximum reached 24 days after inoculation). In this study the authors hypothesized that *LjNAS2* may be involved on Fe remobilization from the nodules at senescence (Hakoyama et al. 2009) (Fig. 2). During seed filling, the levels of Fe in nodules have been shown to be reduced by about 50%, suggesting that Fe present in nodules may possibly be remobilized at senesce to the seeds as a NA chelate through the phloem (Curie et al. 2009). In soybean plants overexpressing the barley NA synthase 1 (*HvNAS1*) gene, the seeds NA content increased by fourfold and the seeds Fe concentrations by twofold compared with the levels found in the seeds of non-transgenic plants. Furthermore, the transgenic plants showed tolerance to Fe deficiency (Nozoye et al. 2014). In a similar study conducted in sweet potato, transgenic plants overexpressing *HvNAS1* showed 7.9-fold higher levels of leaf NA and accumulated threefold higher levels of Fe than non-transgenic plants. Also, similar to the one observed for soybean, transgenic plants exhibited tolerance to restricted Fe supply which was positively correlated with the *HvNAS1* expression level (Nozoye et al. 2017). These studies further support the hypothesis previously proposed regarding Fe remobilization as Fe-NA chelates from the nodule to the seeds at senescence. If so, this transport would require the involvement of YSL (yellow stripe-like) transporters (Fig. 2).

11 Iron Transport Within the Nodule

A number of transporter families have been implicated in Fe movement across the symbiosome membrane including natural resistance-associated macrophage protein (NRAMP) (Kaiser et al. 2003; Jeong et al. 2017), vacuolar iron transporter (VIT) (Kim et al. 2006; Jeong and Guerinot 2009; Jeong et al. 2017), yellow stripe-like (YSL) (Curie et al. 2009), zinc-iron permease (ZIP) (Moreau et al. 2002; Abreu et al. 2017) and multidrug and toxic compound extrusion (MATE) (Takanashi et al. 2013; Kryvoruchko et al. 2018). The symbiosome membrane is derived from the host plasma membrane but shares properties with the vacuolar membrane (Verma and Hong 1996). It acts as a regulation point under plant control which contains transporters and channels to facilitate the movement of nutrients and metabolites between the host and the symbiosome space (Brear et al. 2013).

The transport of Fe through the symbiosome membrane of soybean has been shown to be faster than Fe transport across the bacteroid membrane (LeVier et al. 1996). Accordingly, ferric Fe chelates are believed to be mainly located in the symbiosome space rather than in the bacteroid. The lower pH of the symbiosome

space as compared with that of plant cytosol would promote the stabilization of ferric chelates such as citrate for subsequent uptake by the bacteroid (Moreau et al. 1995). Recent studies suggest that the symbiosome membrane may possess ferric chelate reductase activity (Slatni et al. 2009, 2011). This property would be inherited by the plasma membrane of the infected plant cell of the host plant from which it was formed and would be involved in the reduction of ferric Fe to ferrous Fe for subsequent uptake by the symbiosome. The symbiosome has the ability to uptake ferric and ferrous Fe, but the import of ferrous Fe is much efficient (Moreau et al. 1995; LeVier et al. 1996). Other findings support the hypothesis of ferric chelate accumulation in the symbiosome space. Whereas in the symbiosome space of isolated soybean symbiosomes the levels of leghemoglobin are extremely low, Fe bound to siderophores (compounds binding ferric Fe with high affinity) are present in high concentration in the symbiosome space (Wittenberg et al. 1996).

The ZIP transporter IRT1 was detected in nodule cortex cells of *Phaseolus vulgaris* (Slatni et al. 2012), but the only ZIP family member characterized in this organ, *GmZIP1*, seems to be localized in the symbiosome and has been shown to have a role in Zn uptake (Moreau et al. 2002). Also, a member of the NRAMP family of transporters, the divalent metal transporter 1 (DMT1), has been identified as a ferrous Fe transporter on the soybean symbiosome membrane (Kaiser et al. 2003). However, it is not clear if it transports ferrous Fe across the symbiosome membrane to the symbiosome space or to the cytosol of the infected plant cell or even if this is a bidirectional transporter.

In *Lotus japonicus* a vacuolar iron transporter 1 (VIT1), SEN1, has been shown to be expressed in nodule-infected cells being essential for nitrogen fixation in this host (Hakoyama et al. 2012). It suggests that SEN1 may have a role in the symbiosome membrane as a Fe importer. In *Arabidopsis* VIT1 transports ferrous Fe into the vacuole (Kim et al. 2006), and the symbiosome membrane is known to possess vacuolar membrane properties (Verma and Hong 1996).

YSL transporters belonging to the oligopeptide (OPT) superfamily are mainly located in plasma membrane, transporting ferric and ferrous Fe chelated to nicotinamide (NA) into the phloem or sink cells (Curie et al. 2009). These transporters are mainly found in monocots since they mediate the uptake of Fe(III)-PS complexes from the rhizosphere, but they also exists in dicots where they are thought to be specialized in Fe(II)-NA long-distance transport (Curie and Briat 2003; Nozoye et al. 2011; Nozoye 2018). In a transcriptome study conducted in soybean including the analysis of nodule tissue, it has been shown that 15 YSL transporters were encoded in the genome with one of these transporters (Glyma 11g31870) presenting nodule-specific expression, whereas eight others were also detected (Libault et al. 2010; Severin et al. 2010).

Figure 1 summarizes the main players in the Fe trafficking pathways described above.

Table 1 Common gene regulation and mineral interactions between iron and other metals

Iron interaction with	Common gene regulation	References
Copper	FRO2, IRT1, SOD, oxidoreductases, *redox-responsive transcription factor 1* (*RRTF1*), ethylene response factor (ERF)	Ramamurthy et al. (2018) and Santos et al. (2013)
Zinc	ZIP-like transporters, metallothioneins, metal ion transport and binding, defence response genes	Darbani et al. (2015), Khokhar et al. (2018), Ma et al. (2017), Santos et al. (2013) and Zeng et al. (2018)
Manganese	NRAMP1, NRAMP6, IRT1, MTP8	Cailliatte et al. (2010), Connolly et al. (2002), Eroglu et al. (2017), Long et al. (2018) and Peris-Peris et al. (2017)
Cobalt	IRT1, IREG1/FPN1, IREG2/FPN2	Korshunova et al. (1999), Lange et al. (2017) and Morrissey et al. (2009)
Molybdenum	MOT1, CNX2, CNX3, ABA3, FRO6, FRO7, IREG1	Bittner (2014), Morrissey et al. (2009), Tomatsu et al. (2007) and Vigani et al. (2016)
Nickel	IRT1, IREG2, MTP3, NRAMP1, FRO2	Meier et al. (2018), Mihucz et al. (2012), Nishida et al. (2011) and Schaaf et al. (2006)

12 Molecular Interactions of Fe with Other Nutrients

Since the regulation of Fe homeostasis implies significant responses by plants, other metals essential in the maintenance of optimal plant growth might also be impacted. However, information on the potential interaction and crosstalk among different metal ions and their effect on plant mineral metabolism are still limited (Anjum et al. 2015).

When comparing ionome data from three different plant systems, namely, Strategy I *Glycine max* (Santos et al. 2016), Strategy II *Brassica napus* (Maillard et al. 2016) and *Oryza sativa* (Pereira et al. 2014), that utilizes a combined strategy system, the uptake of micronutrients such as Cu, Zn, Mn, B, Mo and Co was consistently increased by Fe depletion in all plant systems. Hence, it is clear that Fe homeostasis has a serious implication in plants' metal ion regulation, and potential interactions and uptake/transport mechanisms are commonly triggered (Table 1).

Plants utilize the same reduction strategy described for Fe to reduce *Cu*, where FRO2 reduces Cu^{2+} to Cu^{+} at the root surface, although it has been hypothesized that Cu^{2+} could be directly taken up via IRT-like transporters (Jain et al. 2014). Since under Fe deficiency this FRO2/IRT1 system is usually up-regulated (Robinson et al. 1999), it seems probable that, in the absence of Fe substrate and having high affinity for Cu ions, plants increase Cu uptake. A positive correlation between leaf Cu and Fe concentrations was found (Stein et al. 2016), and, in a recent work, a crosstalk between Fe and Cu has been demonstrated (Ramamurthy et al. 2018). Furthermore,

this metal is essential for oxidation and reduction reactions, is a constituent of several enzymes with oxidase function and is also a cofactor of Cu/Zn superoxide dismutase (SOD) (Broadley et al. 2012). At a molecular level, Cu deficiency leads to similar gene regulation as Fe, with a particular impact on oxidative stress-related genes, namely, oxidoreductases, *redox-responsive transcription factor 1* (*RRTF1*) and ethylene response factor (ERF) (Ramamurthy et al. 2018; Santos et al. 2013).

On the other hand, *Zn*, which also shares ZIP-like transporters like IRT1, is sometimes found decreased in Fe-deficient roots (Roriz et al. 2014; Santos et al. 2015) but is generally increased in the shoots of different plants. Studies show that the transfer of Zn from the root to the shoot is very fast (Page and Feller 2005); hence, the increase of Zn in the shoot could be representative of increased Zn root uptake by the plants. Low levels of Zn supply are also related to increased Cu concentration in plant shoots (Zeng et al. 2018). This metal is the second most abundant metal in living organisms after Fe, and, in cereals, Zn deficiency is one of the most serious micronutrient deficiencies on calcareous soils, deeply impacting other nutrient accumulation (Broadley et al. 2012; Khokhar et al. 2018). Like Fe, it is typically taken up as a divalent cation (Broadley et al. 2012) and is required for structural and functional activities of several essential proteins (Fox and Guerinot 1998). Molecular studies show that both Fe and Zn homeostasis require the regulation of metallothioneins, metal ion transport and binding and defence response genes (Darbani et al. 2015; Santos et al. 2013; Zeng et al. 2018). With the identification of QTL for levels of Fe and Zn, common markers for these two minerals have been further identified (Ma et al. 2017).

Despite having a wide range of oxidation states, Mn^{2+} is the most soluble form in the soil and consequently the most accumulated form in plants (Broadley et al. 2012). Mn has a major role in activation of enzymes involved in lignin biosynthesis, photosynthesis and detoxification of O_2 free radicals through Mn SOD activity (Page et al. 2006). Its uptake occurs via active diffusion through root epidermal cells, and, like Cu, Mn competes for common transporters and ligands of Fe, namely, NRAMP1 (Cailliatte et al. 2010), NRAMP6 (Peris-Peris et al. 2017) and metal transporters from the ZIP family (IRT1), both in Strategy I plants, like *Arabidopsis* (Connolly et al. 2002), and in Strategy II plants, like barley (Long et al. 2018). Metal tolerance protein 8 (MTP8) was also found to be able to transport both Fe and Mn in *Arabidopsis* plants, with a particular role in regulating Fe and Mn homeostasis in the seeds (Eroglu et al. 2017). Hence, when Fe is present, it has been found a positive correlation between these two metals, particularly regarding root concentrations (Alagic et al. 2018).

Required by legumes for N_2 fixation, *Co* is mainly accumulated in the root system (Page et al. 2006). Reports on the crosstalk between Co and Fe hypothesize that the molecular mechanisms behind Co accumulation might have evolved from Fe homeostasis mechanisms (Lange et al. 2017). Like Fe, Co uptake is mediated by IRT1 (Korshunova et al. 1999), and Co excess is correlated to the decrease of photosynthetic pigments due to the prevention of the incorporation of Fe in the protoporphyrin molecule, the precursor for chlorophyll (Jayakumar et al. 2009). Also in common with Fe metabolism, ferroportins Iron Regulated1 (IREG1/FPN1)

and IREG2/FPN2 seem to be important in Co detoxification and translocation to the shoot (Morrissey et al. 2009).

For long, *Mo* and Fe have been correlated with each other (Kannan and Ramani 1978; Bittner 2014), and recent evidence has proven that Fe nutritional status greatly impacts Mo homeostasis in plants (Vigani et al. 2016). Plants absorb Mo essentially as molybdate (MoO_4^{2-}), and this element is a constituent of nitrate reductase enzyme, consequently having an important role in plant metabolism, especially in legumes (Duan et al. 2017). This mineral only has biological activity when complexed by a pterin compound, forming the molybdenum cofactor (Moco) and active Mo enzymes depend on the presence of Fe and Cu (Mendel 2007). Hence, Fe is essential for plant Mo regulatory metabolism since Moco biosynthesis is dependent on Fe availability. Its bioavailability is hindered under acidic conditions (Kaiser et al. 2005), and, given the fact that under Fe deficiency plants usually acidify the rhizosphere, Mo concentration is usually decreased in the leaves (Baxter 2009; Roriz et al. 2014; Vigani et al. 2016) and increased in the roots (Santos et al. 2016; Vigani et al. 2016). Few Mo metabolism-related genes have been identified in plants, but the genes MOT1 (Tomatsu et al. 2007) and MOT2 (Gasber et al. 2011) have been shown to have key role on Mo intracellular and interorgan transport. The MOT1 gene, in particular, was shown to be downregulated under Fe deficiency, while three genes essential for Moco biosynthesis in plants, CNX2, CNX3 and ABA3, seem to be up-regulated (Bittner 2014). On the other hand, Mo deficiency does not impact the main players in Fe reduction, uptake and accumulation (FRO2, IRT1 and ferritin) but impacts other isoforms of the FRO gene, FRO6 and FRO7, as well as the iron-regulated protein-encoding gene IREG1 Morrissey et al. (2009).

Abundantly available in the soils, *Ni* predominant oxidation state is +2, which is the most available for plant uptake through both passive diffusion and active transport (Seregin and Kozhevnikova 2006). This metal is utilized in several physiological processes, being an important component of many metalloenzymes and having a vital role in nitrogen metabolism, mainly in root nodule growth and hydrogenase activation (Yusuf et al. 2011). Studies show that high dosages of Ni increase relative transcription level of AtIRT1 in roots (Nishida et al. 2011) and that Fe and Ni share similar uptake mechanisms (Mihucz et al. 2012), putatively competing for absorption (Boostani et al. 2019). After uptake by IRT1, Ni seems to be internalized in the vacuole by iron-regulated protein 2 (IREG2) (Schaaf et al. 2006). Other Fe-related genes were found to be up-regulated in the presence of Ni, namely, Fe deficiency-induced transcription factor (FIT); two metal transporters, MTP3 and NRAMP1; as well as FRO2 (Meier et al. 2018).

Other, non-metal, nutrients are also correlated to Fe metabolism (Table 2). For example, the concentration of available phosphorus (*P*) modulates Fe^{2+} uptake system (Ward et al. 2008), and P deficiency seems to lead to a common regulation of 579 genes in *Arabidopsis* (Li and Lan 2015). Among this pool of genes, the ones with stronger induction and highly correlated to Fe mechanisms were FRO2 and

Table 2 Common gene regulation and mineral interactions between iron and other non-metal minerals

Fe interaction with	Common gene regulation	References
Phosphorus	FRO2, IRT1, AT3G12900, CYP82C4, AT5G38820, COPT2, AT1G30560, AtOCT1, NRAMP1, PHT1, PHO1, FRO3, IRT1, IRT2, NAS1, FRO6, PHR1	Briat et al. (2015), Li and Lan (2015), Santos et al. (2013) and Zanin et al. (2017)
Sulphur	FRO1, IRT1, SIST1;1, SIST1;2, IRO2, APR, SIR	Astolfi et al. (2006), Ciaffi et al. (2013), Forieri et al. (2013), Grewal et al. (2018), Hantzis et al. (2018), Paolacci et al. (2014) and Zuchi et al. (2009)

IRT1 and AT3G12900, CYP82C4, AT5G38820, COPT2, AT1G30560 and AtOCT1. On a different study, also in *Arabidopsis*, P deficiency decreased the expression of FRO3, IRT1, IRT2, NAS1 and FRO6, but both Fe and P homeostasis were found to be impacted by the regulation of a MYB-like transcription factor, phosphate starvation response 1 (PHR1) (Briat et al. 2015). Moreover, as seen before in soybean plants under Fe deficiency (Santos et al. 2013), P deficiency induces the regulation of flavonoid biosynthetic process (Li and Lan 2015). Strategy II utilizing plants, like maize, also showed up-regulation of transporters for Fe^{2+}, like NRAMP1, PHT1 and PHO1, which resulted in higher accumulation of P in both roots and shoots (Zanin et al. 2017).

Sulphur (*S*) and Fe interaction has been well described from the perspective of the formation of Fe-S clusters, which are essential in photosynthesis, respiration and other cellular reactions (Broadley et al. 2012; Lu 2018). However, the crosstalk between these two minerals at a molecular level is yet to be fully understood. In the last decade, it has been shown that FRO1 and IRT1 gene expression is repressed under S deficiency (Zuchi et al. 2009; Forieri et al. 2013) and that Fe deficiency also modulates S-related gene expression by inducing the up-regulation of S transporters, such as SIST1;1 and SIST1;2 (Paolacci et al. 2014). In Strategy II utilizing plants, S uptake is also related to Fe uptake mechanisms since, when S is deficient, the release of phytosiderophores to the rhizosphere is repressed (Astolfi et al. 2006) and, for example, the expression of Fe-related transcription factor IRO2 is increased (Grewal et al. 2018); on the other hand, when Fe is deficient, S deficiency response is triggered at a molecular level, particularly S transporters and the genes encoding ATP sulphurylase (Ciaffi et al. 2013), adenosine 5′-phosphosulphate reductase (APR) and sulphite reductase (SIR) (Hantzis et al. 2018). It is also interesting to note that S application to the soil acidifies the pH (Ramzani et al. 2017), turning the conditions more appropriate for Fe reduction and uptake mechanisms activation.

13 Conclusion

Since Fe homeostasis regulation is a complex system, much is yet to decipher. Some molecules have been demonstrated to be key in the process of Fe sensing within the plant; however, the positive and negative feedback signals described are not enough to explain this regulation. Local sensing and a long-distance sensing system contribute for the homeostasis maintenance that is strongly supported by the equilibrium of the Fe uptake systems and rapidly triggered/repressed, depending on plant's necessities. Furthermore, since it is difficult to mimic, under controlled conditions, the interactions between various nutrients, studies where two or three different nutrients are assayed at the same time are more frequent. The study of the crosstalk between different nutrients is crucial to understand the variation and plant distribution of different nutrient pools that can impact plants' vigour and health.

Fe is an essential micronutrient for the establishment of effective legume-rhizobia symbiosis, but Fe transport from the root to the nodule and within the nodule of symbiotic legumes is very complex, and little is known on how Fe is transported across a number of membrane layers until reaching the bacteroid. Despite some efforts that have been done to identify Fe transporters involved on Fe uptake by the nodules, it has been challenging to recognize their exact location and their precise function within the nodule.

Considering the importance of nodulation for increasing sustainable nitrogen fixation concerning environmentally friendly agricultural practices and the crucial role of Fe nutrition in the effectiveness of these mutually beneficial associations, it is of utmost importance to further understand the molecular mechanisms involved on Fe uptake in nodulated legumes, and we believe this is an area that warrants further exploration in the future. In the context of current climate changes, understanding these mechanisms is even more urgent. Increasing atmospheric CO_2 levels are foreseen to decrease Fe accumulation in several crops (Loladze 2014), and it is believed to be related with Fe transportation throughout the plant particularly through the xylem since stomatal conductance is generally reduced under eCO2 (Ainsworth and Rogers 2007; Xu et al. 2016; He et al. 2018). Increasing temperature on the contrary induces stomatal conductance (Urban et al. 2017) and has recently been reported to counteract nutritional losses induced by eCO2 (Köehler et al. 2019).

In the frame of current (1) climate changes possibly inducing nutritional losses is several crops, (2) necessity for highly nutritious enhanced food crops capable of coping current Fe deficiencies in populations worldwide, and (3) need of intensive and sustainable agricultural practices to feed an increasing human population, it is crucial to understand the molecular features of Fe uptake and movement within the plant.

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Urea in Plants: Metabolic Aspects and Ecological Implications

Alejandra Matiz, Paulo Tamaso Mioto, and Helenice Mercier

Contents

Abstract Urea is widely used as a crop fertilizer because of its low cost. However, how this organic compound is absorbed and assimilated by plants is still poorly characterized. This chapter gives a general overview of how plants deal with urea and use it to support growth, covering the high- and low-affinity transport systems, which accounts for external urea uptake, intracellular remobilization and transport, as well as urea assimilation through the action of plant ureases. In addition, the ecological relevance of urea is discussed through some examples of organisms that live in habitats in which urea may be a preferential source of nitrogen. We believe that it is very important to study organisms that have a high potential for efficiently

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A. Matiz and H. Mercier (✉)
Department of Botany, Institute of Biosciences, University of São Paulo, São Paulo, SP, Brazil
e-mail: alematiz@usp.br; hmercier@usp.br

P. T. Mioto
Department of Botany, Biological Sciences Center, Universidade Federal de Santa Catarina, Florianópolis, SC, Brazil

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metabolizing urea in order to apply this knowledge to improve the nitrogen use efficiency (NUE) in plants, which is the topic of the last section of this chapter.

Abbreviations

AA	Amino acids
AMT	Ammonium transporter
AQP	Aquaporins
ASN	Asparagine synthetase
DUR3	High-affinity urea transporter
GIPs	GlpF-like intrinsic proteins
GOGAT	Glutamate synthase
GS	Glutamine synthetase
HIPs	Hybrid intrinsic proteins
MIPs	Major intrinsic proteins
NIPs	Nodulin-26-like intrinsic proteins
NUE	Nitrogen use efficiency
PEPC	Phosphoenolpyruvate carboxylase
PIPs	Plasma membrane intrinsic proteins
Rubisco	Ribulose-1,5-bisphosphate carboxylase/oxygenase
SIPs	Small basic intrinsic proteins
TIPs	Tonoplast intrinsic proteins
XIPs	X intrinsic proteins

1 Introduction

Urea is a small, neutral organic compound formed by two ammonia (NH_3) and one carbon dioxide (CO_2) molecules. In the environment, urea can originate as a degradation product from organic matter or from the urine of animals, such as mammals, as a key detoxification product of nitrogenous compounds (Wang et al. 2008). Additionally, the low cost of industrial manufacture and the high nitrogen content of urea (46% N of total urea molecular mass) make this organic compound a frequent fertilizer used in arable soils (Kojima et al. 2006; Pinton et al. 2016). As a result, the addition of nitrogen to the soil has increased through the application of urea fertilizers, which is one of the main strategies for improving crop production. In the coming years, urea demand is expected to increase at an average annual rate of 3.2% (http://www.ceresana.com/en/market-studies/chemicals/urea/). Unfortunately, more than half of the nitrogen applied in agriculture is not absorbed by plants, resulting in the polluting of water bodies and the atmosphere by ammonia and nitrogen oxides. The significant losses of urea applied as fertilizer in certain soils are due to the high urease activity of the microorganisms (Dawar et al. 2011; Krajewska 2009; Zanin et al. 2016) and/or the secretion of ureases by plants to the

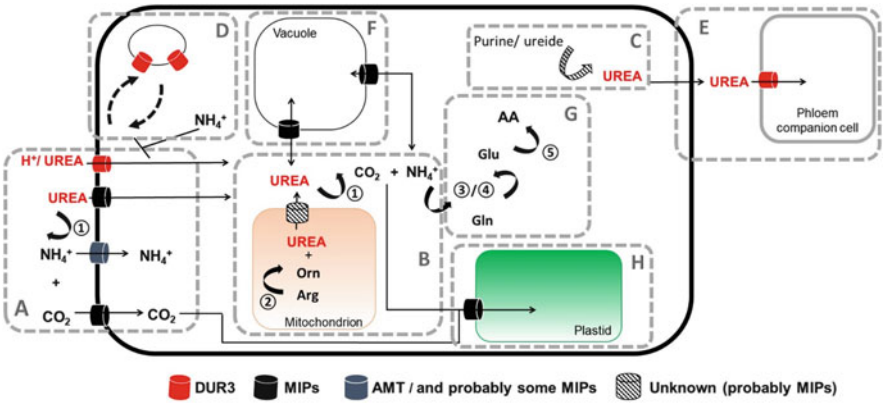

Fig. 1 Processes of urea absorption and metabolism in plant cells. (**a**) Extracellular urea can be absorbed in its intact form either through the low-affinity system (mediated by MIPs) or though proton symport (mediated by DUR3). Alternatively, extracellular urease (①) may generate NH_4^+, which can be absorbed mainly by AMT transporters and CO_2 that may diffuse into the plastids through MIPs. (**b**) Intracellular urea can also be originated from arginine catabolism in the mitochondria by arginase (②) or (**c**) through ureide degradation (see text for details). (**d**) DUR3 can be recycled into the endomembrane system, a process that seems to be related to intracellular NH_4^+ concentrations. (**e**) Urea may be exported from source tissues, mainly senescent leaves through phloem to other organs, possibly entering in the vascular system through DUR3. (**f**) Intracellular urea can be stored inside the vacuole through TIPs to avoid toxicity and be remobilized whenever needed. (**g**) Upon urease action, the resulting NH_4^+ is assimilated by GS and GOGAT (③ and ④, respectively), forming glutamate, and through transaminases (⑤), originating the other amino acids (AA), while (**h**) the resulting CO_2 may diffuse

external environment (Inselsbacher et al. 2007; Cambuí et al. 2009), which quickly causes the conversion of urea to ammonia (Fig. 1a), a form of nitrogen that is normally lost through volatilization.

In addition to external sources of urea (such as fertilizers, degradation of organic matter, and animal excreta), inside plant cells, urea is an important metabolite generated mainly by the catabolism of the amino acid arginine through the enzyme arginase (Fig. 1b) (Polacco et al. 2013; Winter et al. 2015). Arginine has the highest nitrogen-to-carbon ratio (4:6), which makes it an outstanding compound during protein mobilization for seed germination since it is an important constituent of storage proteins (Zonia et al. 1995). In source tissues, such as senescent leaves and seeds during germination, urea can accumulate; the nitrogen from this organic source is, subsequently, remobilized through urease activity to support the growth of the plant (Polacco and Holland 1993; Zonia et al. 1995; Bohner et al. 2015). In addition to arginine degradation, other endogenous sources of urea in plant cells, such as the degradation of ureides (which are intermediates in purine degradation pathways), are still controversial since it seems that this process does not necessarily lead to the generation of urea in all plants (Fig. 1c) (Werner et al. 2010; Werner and Witte 2011).

Despite the importance of urea as a metabolite in plant cells and its extensive use as a nitrogen-releasing fertilizer in arable soils, little attention has been given to the study of its absorption and assimilation compared to its inorganic counterparts,

ammonium and nitrate. Moreover, few studies have examined the ecological relevance of urea as a nitrogen and carbon source for plants.

This review highlights the main metabolic aspects of urea regarding its absorption and assimilation as well as the ecological relevance and potential use of urea transporters in plants that are naturally exposed to this organic source as a strategy to improve nitrogen efficiency in crops.

2 General Features of Urea Absorption and Assimilation in Plants

Urea is a widespread molecule in nature with different physiological roles. In mammals, urea is a waste product from protein catabolism, but at the same time, its physiological role as osmolyte is essential for water reabsorption in the kidneys (Bagnasco 2005). Moreover, for enteric pathogenic bacteria, urea can serve as a buffer to survive the acidic conditions of the stomach (Weeks et al. 2000). Also, urea is a nitrogen source for bacteria, algae, fungi, and plants (McCarthy 1972; Yamanaka 1999; Endres and Mercier 2001; Valladares et al. 2002). Despite the many physiological roles of urea in different organisms, its importance as a nutrient in plants has been little investigated, in part due to the misconception about whether plants were capable of absorbing this organic nitrogen source directly. Since most arable soils possess relatively high urea-hydrolyzing activity from microorganisms, it has been suggested that the nitrogen derived from urea enters the root cells mainly in the form of ammonium (Watson et al. 1994; Marschner 1995). Therefore, it had long been thought that plants were able to uptake only inorganic sources, such as ammonium and nitrate; as a consequence, most of the studies of nitrogen plant nutrition focused precisely on these forms (Paungfoo-Lonhienne et al. 2012). Nowadays, more attention has been given to the importance of organic nitrogen sources, such as amino acids, peptides, and urea, in plant nutrition (Lipson and Näsholm 2001; Persson and Näsholm 2001; Jones et al. 2005; Mérigout et al. 2008; Ganeteg et al. 2017), and physiological evidence has demonstrated that urea is absorbed by plants in its intact form (Syrett and Bekheet 1977; Wilson and Walker 1988; Krogmeier et al. 1989; Liu et al. 2003a, b; Mérigout et al. 2008). Furthermore, urea can be translocated from the roots to other parts of the plant, as evidenced by significant amounts of this compound in the shoots or xylem sap of plants treated with urea (Hine and Sprent 1988; Gerendás et al. 1998; Mérigout et al. 2008). Moreover, urea can be transported to sink organs after foliar fertilization (Witte et al. 2002), indicating that intact urea is highly mobile throughout the vascular system of the plant.

It has been around 53 years since it was first suggested that protein transporters facilitated the transmembrane passage of urea in biological systems (Hunter et al. 1965; Wieth et al. 1974), as opposed to the low diffusion rates observed in artificial lipid bilayers (Galluci et al. 1971). Later on, it was shown that facilitated and active transporters in both eukaryotes and prokaryotes played a role in urea transport (ElBerry et al. 1993; Sands et al. 1997; Weeks et al. 2000; Liu et al. 2003a, b). In

plants, one of the first pieces of evidence that corroborated the involvement of urea transporters came through the analysis of ^{14}C-labeled urea absorption by the alga *Chara australis* R. Brown, in which electrophysiological measurements and concentration-dependent uptake analysis demonstrated the existence of two urea transport systems (Wilson and Walker 1988; Wilson et al. 1988). The first one is a high-affinity system characterized by the transportation of urea against its concentration gradient, which makes it the main route of urea entry at extracellular concentrations <0.25 mM. As a result, this active transport is sensitive to energy metabolism inhibitors since it uses the electrochemical gradient across membranes to energize urea transport (Liu et al. 2003a). On the other hand, low-affinity systems are the predominant mode of urea entry at extracellular concentrations above 0.5–1 mM and do not require a proton-dependent mechanism to transport urea (ElBerry et al. 1993; Liu et al. 2003b). In higher plants, it was only possible to identify and functionally characterize low- and high-affinity urea transporters at the molecular level in the late 1990s and early 2000s (Gerbeau et al. 1999; Eckert et al. 1999; Liu et al. 2003a, b).

Urea transporters are present in almost all organisms ranging from bacteria and animals to fungi and plants but are encoded by different gene classes. For example, the urea transporter (UT) family present in animals, the H^+-activated urea channel (UreI), and the urea transporter gene (Yut) present in bacteria, such as *Helicobacter pylori* Marshall & Warren and *Yersinia pseudotuberculosis* Pfeiffer, respectively, encode for low-affinity urea channels that appear to be absent in plant genomes. In higher plants, orthologs of the urea active transporter DUR3 of *Saccharomyces cerevisiae* Meyen ex E.C. Hansen and members of the aquaporin family have been identified as high- and low-affinity urea transporters, respectively (Gerbeau et al. 1999; Eckert et al. 1999; Gaspar et al. 2003; Liu et al. 2003a, b; Soto et al. 2008; Gu et al. 2012; Azad et al. 2012). These findings have brought new insights into the physiological and molecular basis of urea absorption in plants.

2.1 *DUR3: High-Affinity Absorption Transporter of Urea*

High-affinity urea transporters have been physiologically described in bacteria, fungi, algae, and plants (Cooper and Sumrada 1975; Syrett and Bekheet 1977; Pateman et al. 1982; Valladares et al. 2002; Liu et al. 2003a; Beckers et al. 2004). In plants, they have been identified as orthologs of ScDUR3 of *S. cerevisiae*, a member of the sodium-solute symporter (SSS) family responsible for transporting several solutes, including urea. However, to date, this kind of transporters in plants has been functionally characterized at the molecular level only in *Arabidopsis*, maize, and rice (Liu et al. 2003a, 2015; Kojima et al. 2007; Wang et al. 2012; Zanin et al. 2014).

In yeast, ElBerry et al. (1993) showed that the uptake of ^{14}C-labeled urea was reestablished in a urea uptake-defective yeast mutant (*dur3*) through its complementation with the functional *ScDUR3* gene. A decade later, Liu et al. (2003a) showed that AtDUR3 from *Arabidopsis* complemented the growth of a yeast *dur3* deletion

mutant in urea concentrations below 5 mM as a sole nitrogen source. Additionally, in order to characterize the urea transport mechanism, AtDUR3 was heterologously expressed in *Xenopus laevis* Daudin oocytes (a biological system that expresses no endogenous urea transporters), and it was demonstrated that AtDUR3 mediated the active transport of urea in a proton- and concentration-dependent way. By using similar approaches, OsDUR3 and ZmDUR3 were described as high-affinity transporters responsible for absorbing urea at low concentrations in rice and maize plants, respectively (Wang et al. 2012; Zanin et al. 2014; Liu et al. 2015). In addition, the DUR3 protein family in plants appears to consist of only one member since in both rice and maize genomes *OsDUR3* and *ZmDUR3* are the only genes that show significant homology to *AtDUR3* (Wang et al. 2012; Zanin et al. 2014).

Regarding the physiological role of DUR3, both OsDUR3 and ZmDUR3 were independently capable of complementing a T-DNA insertion line of *Arabidopsis* that could not grow at concentrations below 1 mM of urea as a sole nitrogen source, confirming the key role of DUR3 in active urea acquisition (Zanin et al. 2014; Liu et al. 2015). *Arabidopsis DUR3* encodes a putative hydrophobic protein with 14 transmembrane-spanning domains (while in maize, DUR3 is predicted to have 15 transmembrane domains), and its expression is linked to the N status since it was observed that under N deficiency DUR3 is upregulated in plant root cells (Liu et al. 2003a, 2015; Kojima et al. 2007; Zanin et al. 2014; Bohner et al. 2015). Moreover, it appears that the upregulation of *DUR3* expression under N starvation is more likely due to the derepression from catabolic products of urea, such as ammonium (Fig. 1d) (Kojima et al. 2006, 2007). In addition, after supplying urea at low concentrations to N-deficient plants of rice and *Arabidopsis*, an upregulation of *DUR3* expression levels was observed in roots in a short time span, indicating that DUR3 expression is stimulated by its own substrate and reflecting its involvement in urea acquisition from the external medium (Kojima et al. 2007; Mérigout et al. 2008; Wang et al. 2012). However, in maize plants a downregulation of DUR3 expression together with an increase in urea absorption by the roots was observed (Zanin et al. 2014, 2015a; Liu et al. 2015). These results suggest that after urea is supplied, DUR3 might be regulated by other mechanisms rather than exclusively by transcriptional control. In agreement with this, some researchers have suggested that DUR3 proteins are under control of a trafficking/recycling pathway since this transporter appears to be localized in the plasma membrane (Kojima et al. 2007; Liu et al. 2015) as well as in the endomembranes (Zanin et al. 2014), indicating that the cellular localization can regulate its activity as a urea transporter (Fig. 1d).

Although it has been observed that in rice and *Arabidopsis* DUR3 increases its expression in roots after a short time of urea resupply, it is difficult to know the internal metabolic status of the plant and, therefore, to understand which elements are involved with the regulation of DUR3 activity. Moreover, DUR3 transcript levels seem to be organ/tissue specific (differing in roots, shoots, and leaves) and dependent on the ontogenetic stage of the plant (Liu et al. 2015; Bohner et al. 2015). During leaf senescence, N is remobilized to younger parts of the plant to allow growth. Along this process, urea leaks to the apoplast, while DUR3 appears to be responsible for retrieving or taking up urea from the apoplast of senescent leaves,

contributing to the translocation of N (Fig. 1e). In older/senescent leaves, both the increase in *DUR3* transcript levels in phloem cells and urea concentrations in the apoplast when DUR3 function is lost show that DUR3 plays an important role in endogenous urea remobilization and vascular loading during leaf senescence (Bohner et al. 2015; Liu et al. 2015).

Thus far, there is little doubt that DUR3 represents the major active urea transporter in plants. However, the present view was restricted to the findings related only to three model plants: *Arabidopsis*, rice, and maize. Despite the obvious importance of DUR3 as a strategy to use available diluted urea sources from soils and translocate endogenous urea during senescence, little is known about its physiological relevance in other plant systems, as well as the key elements responsible for its regulation since the studies carried out in other plants have only predicted putative DUR3 orthologs by bioinformatics.

2.2 *Aquaporins as Low-Affinity Urea Transporters*

In addition to active urea transport mediated by DUR3, facilitated diffusion of urea can be mediated by aquaporins, whose structure and transport mechanisms are completely different. Aquaporins are channels of the major intrinsic protein family (MIPs) that exhibit specificity for small, low-weight molecules, such as urea. Since water and several neutral solutes are capable of crossing the lipid bilayer of membranes, it was initially thought that no transporters were needed to facilitate their diffusion. Later on, it was shown that aquaporins were responsible for increasing the passive flow rate of water and some small solutes through the membranes in biological systems (Maurel et al. 1993; Chrispeels and Maurel 1994; Biela et al. 1999). MIPs are present in archaea, bacteria, protozoa, yeasts, plants, and animals. In plants, based on sequence homology and subcellular localization, the aquaporins were classified into seven subfamilies (PIPs, TIPs, NIPs, SIPs, XIPs, HIPs, and GIPs) (Wang et al. 2016), with the PIPs and TIPs being the most abundant subgroups and generally localized in the plasma and vacuolar membrane, respectively (for further information about structure and classification of plant aquaporins, see Luang and Hrmova 2017).

Some of the first aquaporins identified in plants were described as facilitators of the transport of small molecules in the peribacteroid membrane of nitrogen-fixing symbiotic nodules in soybean (Sandal and Marcker 1988) and as water stress-induced proteins (Guerrero et al. 1990), and only later were they functionally characterized as water-permeable channels. In light of this finding, aquaporins have been associated with various physiological processes in plants, such as cell growth, stomatal movement, germination, and abiotic stress responses, among others (Chaumont and Tyerman 2014; Li et al. 2014; Reddy et al. 2015). However, interactions between aquaporins and plant nutrition remain poorly understood. PIPs, NIPs (present in the plasma membrane), and TIPs have been shown to play an important role in the uptake and mobilization of nitrogen in the plant (Gerbeau

et al. 1999; Liu et al. 2003b; Loqué et al. 2005; Hwang et al. 2010, Bárzana et al. 2014). Accordingly, the expression of some aquaporin genes varies according to the availability of nitrogen in the environment. For example, AtTIP2;1 of *Arabidopsis* can be induced either by the presence of ammonium or the lack of nitrogen in the environment (Loqué et al. 2005), and it has been shown to be permeable to urea (Liu et al. 2003b). In tomato, analyses carried out in a root cDNA library showed the induction of several aquaporin genes by nitrate (Wang et al. 2001), with the *ZmPIP1;5b* gene of maize also induced by this nitrogen source (Gaspar et al. 2003). To date, not a single aquaporin has been functionally characterized as a nitrate transporter; however, its induction in response to this nitrogen source may be related to the increase of hydraulic conductivity in roots (Gorska et al. 2008; Li et al. 2016).

On the other hand, the permeability of MIPs to urea and ammonia has already been demonstrated. As a result of its neutrality and low molecular weight, urea can easily be transported by aquaporins. Through stopped-flow spectrofluorimetry of purified vacuolar membrane vesicles and heterologous expression in *Xenopus* oocytes, it was possible to functionally characterize the first urea-permeable aquaporins in plants: NtTIPa (TIP) (Gerbeau et al. 1999) and NtAQP1 (PIP) of tobacco (Eckert et al. 1999). Since then, some NIPs (in *Arabidopsis*, maize, tobacco, zucchini, and cucumber), PIPs (in maize), and TIPs (in *Arabidopsis* and maize) have been shown to be permeable to urea, suggesting that the potential urea transport mediated by aquaporins may occur through the plasma membrane as well as across the vacuolar membrane in plants (Fig. 1a, f) (Gerbeau et al. 1999; Eckert et al. 1999; Gaspar et al. 2003; Kleb et al. 2003; Liu et al. 2003b; Soto et al. 2008; Gu et al. 2012; Yang et al. 2015; Zhang et al. 2016). The TIP aquaporin subfamily constitutes approximately 40% of the total vacuolar membrane protein (Maeshima 2001) and may be involved with the remobilization of N into and out of the vacuole in order to avoid toxicity and maintain nitrogen balance in the cytosol (Fig. 1f) (Liu et al. 2003b; Jahn et al. 2004; Loqué et al. 2005).

It is worth pointing out that arginine hydrolysis, which takes place at the mitochondria, is an important source of endogenous urea in plant cells. However, N release from urea occurs mainly in the cytosol via urease, indicating the essential requirement of a mitochondrial transporter for urea (Polacco and Holland 1993; Goldraij and Polacco 2000). Although several TIPs have already been characterized in tonoplast as urea carriers, others, such as AtTIP5;1, have been found in the mitochondria, likely facilitating the passage of urea into the cytosol (Fig. 1b) (Soto et al. 2008, 2010), which makes TIPs important elements in urea metabolic pathways. Interestingly, AtTIP1;3, which also facilitates urea diffusion, has been found in the plasma membrane of seed embryos (Gattolin et al. 2011) and in endomembranes of pollen tubes of *Arabidopsis* (Soto et al. 2010), while AtTIP2;1 has been found in tonoplasts and apparently in chloroplast membranes, as well (Liu et al. 2003b; Loqué et al. 2005; Ferro et al. 2003, 2010). Taken together, these findings highlight both the existence of different trafficking mechanisms for MIPs according to organ type and ontogenetic stages (since it is possible to find the same aquaporin subfamily in different types of membranes) and the relevance of MIPs in physiological processes, such as nitrogen mobilization.

Despite the high number of genes identified as encoders of MIP family members (between 30 and 71 depending on the species) (Chaumont et al. 2001; Park et al. 2012), channel selectivity and exact membrane location are difficult to predict based only on its protein sequence, which makes experimental confirmation necessary. Nevertheless, the NPA and ar/R selection filters (located in the aquaporin pore) have been widely discussed in the literature as key points in their selectivity (Froger et al. 1998; Fu et al. 2000; Sui et al. 2001; Savage et al. 2010; Hove and Bhave 2011) but not the only control points. Channel diameter, gating control by pH, binding of divalent cations (probably Ca^{2+}), phosphorylation, and the length and movement of the cytoplasmic MIP loops are important points in the selectivity and activity of aquaporins (Daniels et al. 1999; Kukulski et al. 2005; Törnroth-Horsefield et al. 2006; Frick et al. 2013). Likewise, homo- or heterotetramerization are key regulator mechanisms of aquaporin activity since, in part, they control the targeting of MIPs to specific plant membranes. Related to this, it has been observed that heterotetramerization of two different PIP subclasses (PIP1s and PIP2s) had a synergistic effect on water transport compared to the PIP homotetramerization (Fetter et al. 2004; Zelazny et al. 2007; Besserer et al. 2012). In a similar way, the establishment of aquaporin heterotetramers might regulate the trafficking of urea aquaporin transporters to the plasma membranes and, subsequently, control the entry of urea into the cells.

In spite of the large amount of data about the molecular components regulating plant aquaporins, it remains unclear how the urea transport through MIPs is regulated and what its physiological significance in plants is.

2.3 Urease as a Key Enzyme in Urea Assimilation

Two different types of enzymes are capable of degrading urea: urea amidolyase and urease. Urease is a metalloenzyme that uses nickel (Ni) ions at its active site, which is key for its activity (Dixon et al. 1975). This enzyme is found in plants, fungi, and bacteria (Mobley and Hausinger 1989; Witte and Medina-Escobar 2001) and catalyzes the hydrolysis of urea to generate ammonia and carbamate, which at physiological pH is spontaneously hydrolyzed to form CO_2 and a second ammonium molecule (Mobley et al. 1995).

It is estimated that spontaneous degradation of urea has a half-life of 520 years to generate ammonia and carbamate, while the urease-catalyzed half-life of urea is 20 ms (Callahan et al. 2005). Thus, urease is one of the fastest known hydrolases. In 1926, *Canavalia ensiformis* (L.) D.C. (jack bean) urease was the first enzyme to be crystallized (Sumner 1926); however, it was not possible to determine its structure until 83 years later (Balasubramanian and Ponnuraj 2010). To date, this is the only urease whose structure has been determined in plants. Plant ureases consist of a dimer of homotrimers in which each active site has two nickel ions, totaling six active sites per active protein (Dixon et al. 1975; Balasubramanian and Ponnuraj 2010; Zambelli et al. 2011).

With the exception of *Utricularia gibba L.*, a carnivorous plant with no identified ureases in its genome (Ibarra-Laclette et al. 2013), in several sequenced plant genomes, urease appears to be encoded by a single gene (Witte et al. 2005a; Ligabue-Braun et al. 2013). However, in soybean three urease isoforms have been described: the embryo-specific (Eu1), expressed highly in embryos (Polacco and Holland 1993); the ubiquitous (Eu4), expressed in all organs of the plant (Polacco et al. 1985); and the SBU-III (Eu5), expressed in the first moments of root development (Polacco et al. 2013; Wiebke-Strohm et al. 2016). It should be noted that, despite the high activity of the embryo-specific urease in soybean, this activity does not seem to be related to its ureolytic function (Stebbins et al. 1991). Instead, the involvement of this enzyme has been suggested in defense mechanisms. Nowadays, it is known that in addition to its ureolytic function, soybean and jack bean ureases have insecticidal and fungicidal properties (Follmer et al. 2004; Becker-Ritt et al. 2007; Carlini and Ligabue-Braun 2016). Despite the great similarity of plant ureases to those of bacteria, only those of plants have insecticidal properties, and, even more importantly, this characteristic is independent of its ureolytic capacity (Follmer et al. 2004; Menegassi et al. 2017; Kappaun et al. 2018).

Although the first urease was discovered in plants, its ureolytic activation process is still poorly characterized. To date, bacterial ureases (such as those from *Helicobacter pylori* Marshal & Warren) have received more attention since they are related to human and animal diseases and are better characterized than plant ureases (Farrugia et al. 2013; Burne and Chen 2000). However, due to the great similarity of the protein sequences of plant and bacterial ureases, it is believed that the urease activation and catalytic mechanisms are highly conserved (Carlini and Polacco 2008; Follmer 2008).

In *Arabidopsis*, urease activation is mediated by the accessory proteins UreD, UreF, and UreG, which form an activation complex that interacts with the inactive urease (apo-urease) (Witte et al. 2005b; Myrach et al. 2017). It is believed that UreD binds to the apo-urease and serves as a scaffold for interaction with UreF, which in turn interacts with UreG (Witte 2011), which is responsible for delivering nickel ions to the metallocenter to create functional active sites (Witte and Medina-Escobar 2001; Witte et al. 2005b; Cao et al. 2010; Myrach et al. 2017). UreG-deficient soybean mutant plants showed no ureolytic activity in either its ubiquitous isoform (present in all tissues) or its embryo-specific isoform, demonstrating that the action of UreG is vital for the activation of ureases (Freyermuth et al. 2000). Once active, urease is highly stable and insensitive to the loss of nickel ions at its active site (Park and Hausinger 1993; Balasubramanian and Ponnuraj 2010). However, some specific residues in its structure (such as Cys592) are involved in many reactions that cause its inhibition (Pearson et al. 1997; Kot and Zaborska 2006; Balasubramanian and Ponnuraj 2010).

Moreover, although the formation of the apo-urease is not compromised by the lack of nickel ions, the activity of the holo-urease (active urease) decreases as the nickel concentration reduces (Winkler et al. 1983); thus, the activation of the urease is dependent on the availability of nickel (Dixon et al. 1975; Polacco 1977; Myrach et al. 2017). The discovery of Ni as an essential component in plant urease activity has brought new interest in Ni nutrition and its role in urea metabolism in plants.

Consistent with this, it has been reported that when plants are grown under Ni-depleted conditions and supplied with urea, large amounts of urea accumulate in the leaves causing leaf tip necrosis due to urea toxicity (Shimada and Ando 1980; Eskew et al. 1983; Walker et al. 1985), failures in the production of viable grains (Brown et al. 1987), and reduced grain yield (Freitas et al. 2018). Interestingly, under natural conditions and due to a relatively constitutive activity and expression of urease in almost all plant cells (Polacco and Holland 1993; Witte 2011), large amounts of urea in the cytoplasm should be unexpected. However, under conditions in which urease is inactive due to the action of an inhibitor, nickel deficiency, or mutations in the accessory protein-encoding genes (such as UreG), significant urea content was observed (Krogmeier et al. 1989; Stebbins et al. 1991; Polacco and Holland 1993; Gerendás and Sattelmacher 1997; Gerendás et al. 1998; Zanin et al. 2016), indicating that transient compartmentalization of urea into the vacuole through TIPs (as mentioned above – Sect. 2.2) could be an important process to detoxify urea excesses in the cytoplasm.

Since urease is localized mainly in the cytoplasm, vacuolar storage of urea keeps this nitrogen source out of its hydrolysis and assimilation pathway (Witte et al. 2002; Cao et al. 2010). Therefore, urease activity is essential to make N available for plant metabolism during urea absorption as well as being a key component in developmental processes, such as germination (when a large flux of nitrogen from arginine pools is remobilized through arginase and urease activity), pollen tube growth, and senescence (when N remobilization is required) (Zonia et al. 1995; Witte et al. 2002; Soto et al. 2010; Polacco et al. 2013; Rechenmacher et al. 2017).

When urea is supplied, the UreG-encoding gene appears to be upregulated (Mérigout et al. 2008). However, due to the constitutive expression and high activity of urease, it has been suggested that this enzyme is not a limiting step in N-derived urea assimilation or remobilization (Cao et al. 2010; Wang et al. 2012, 2013) and an upregulation of either its activity or gene expression is not required. Indeed, in several studies in which potato and *Arabidopsis* plants were supplied with urea, no increases in either activity or expression of urease were observed (Witte et al. 2002; Mérigout et al. 2008; Cao et al. 2010), suggesting that urease is not inducible by its substrate. Nevertheless, in other studies significant increases in urease activity by urea have been reported (Matsumoto et al. 1966, 1968; Chen and Ching 1988; Lakkineni et al. 1995; Takahashi and Mercier 2011; Zanin et al. 2016; Matiz et al. 2017).

Taken together, these findings make it difficult to define whether or not urease is inducible by urea and raise other questions about the involvement of other variables, such as endogenous nickel levels in the plant, time of urease activation, organ type, ontogenetic stage of the plant, availability of carbohydrates for N assimilation, and/or even the N preference of the plant. In *Arabidopsis*, it was observed that with urea as the sole nitrogen source, clear N starvation symptoms develop, even with an active urease (Mérigout et al. 2008), reflecting the low preference that this species has for urea. In addition, these observations make us wonder what other processes are limiting urea utilization in plants.

2.4 N and Carbon Assimilation After Urea Hydrolysis by Urease

Although urea assimilation seems to occur primarily via GS/GOGAT, Walker (1952) noted that the alga *Chlorella pyrenoidosa* H. Chick grown in urea had a high arginine content and it was not possible to detect urease activity, hypothesizing that the incorporation pathway followed a reverse Krebs urea cycle. This idea was also proposed by Thomas and Krauss (1955), who found that urea stimulated the production of arginine in the green alga *Scenedesmus obliquus* (Turpin) Kützing. Hattori (1958) also reported having found arginine as an intermediate in the assimilation of urea in *Chlorella ellipsoidea* Gerneck and, as in the previous study by Walker (1952), was unable to detect urease activity. Baker and Thompson (1962) failed to find ureolytic activity in *Chlorella vulgaris* Beyerinck, concluding from their amino acid profile results that urea incorporation did not involve urease-catalyzed hydrolysis. Although all these studies suggested a path of direct urea assimilation without previous hydrolysis, to date it has not been possible to characterize it biochemically in living organisms. Thus, it would be interesting to test whether a direct assimilation of urea might occur in plants that are naturally exposed to this organic nitrogen source. In addition, it would be very interesting to assess how urea is metabolized in the carnivorous plant *U. gibba* L., which, as previously mentioned, shows no urease-like sequence in its genome.

Several studies conducted in crop plants in which urease had low activity (e.g., by using a urease inhibitor) have shown an increase in urea accumulation and a reduction in ammonium, amino acid, and/or N contents (Witte et al. 2002; Artola et al. 2011; Cruchaga et al. 2011; Zanin et al. 2016), indicating impairments in N-urea assimilation. In addition, by using ^{15}N-labeled urea and $^{15}NH_4^+$, it was observed that these two N sources shared similar assimilation pathways in *Arabidopsis* plants (Mérigout et al. 2008). Taken together, these results indicate that the main assimilatory pathway of NH_4^+ derived from urea follows the Gln synthetase (GS)-Glu synthase (GOGAT) cycle with previous hydrolysis by urease.

GS may be located in the cytosol (GS1 isoform) or plastids (GS2 isoform) (Miflin and Lea 1980; Miflin and Habash 2002) and incorporate an ammonium molecule into glutamate (Glu), giving rise to a glutamine (Gln) molecule. Subsequently, GOGAT transfers the amide group of Gln to 2-oxoglutarate, thereby generating two molecules of Glu (Bowsher et al. 2007), which allows a part of the Glu pool to be the substrate of GS, while the other part may be used to generate other amino acids through the action of aminotransferase enzymes (Fig. 1g) (Forde and Lea 2007).

In plants treated with urea as the sole nitrogen source, it was observed that the expression of the encoding cytosolic GS, GOGAT, and asparagine synthetase (ASN) genes was upregulated (Mérigout et al. 2008; Zanin et al. 2015a, b), reinforcing the idea that urea-derived N is assimilated through urease and GS/GOGAT pathways.

In addition, ammonium can be temporally stored in the vacuole and, whenever required, can be remobilized through TIPs (Fig. 1f) (Liu et al. 2003b; Kirscht et al.

2016). Related with this, Kirscht and collaborators (2016), based on simulations and the crystallographic structure of AtTIP2;1 (a known ammonia transporter aquaporin), suggested that NH_4^+ is ultimately transported through aquaporins (mainly TIPs) in the form of NH_3 due to its deprotonation in a side pore present in one of the aquaporin loops.

Interestingly, less attention has been given to the assimilation of CO_2 derived from urea. One of the few studies that analyzed the incorporation of urea-derived C into organic molecules was performed by Webster et al. (1955), in which after 6 hours of ^{14}C-labeled urea incubation, the incorporation of ^{14}C into free amino acids, such as aspartate, glutamine, and glutamate, and protein-bound amino acids in pea leaves was observed. Lakkineni et al. (1995) found significant radioactivity in wheat leaves treated with ^{14}C-labeled urea after 24 h. Taken together, these findings indicate that after urea hydrolysis, CO_2 is assimilated. In order to form ^{14}C-labeled amino acids from ^{14}C-urea as claimed by Webster et al. (1955), the formation of carbon skeletons is required through the incorporation of CO_2 by phosphoenolpyruvate carboxylase (PEPC) or by ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) in chloroplasts. Little is known about CO_2 conductance into the chloroplast; however, it has been demonstrated that several PIPs mediate its conductance in leaves (Hanba et al. 2004; Uehlein et al. 2008, 2012; Mori et al. 2014; Heinen et al. 2014). Interestingly, the tobacco aquaporin NtAQP1, which is permeable to urea (as mentioned above), also regulates CO_2 diffusion through membranes (Uehlein et al. 2003; Otto et al. 2010). By altering expression of *NtAQP1* or its ortholog in *Arabidopsis AtPIP1;2*, it was observed that both play a significant role in photosynthesis (Uehlein et al. 2003; Flexas et al. 2006; Heckwolf et al. 2011). The transport of CO_2 by aquaporins is apparently mediated by the formation of only homotetramers instead of heterotetramers, which build an additional central pore in the middle of the four units. In plants, this central pore is lined with hydrophobic residues, which do not allow either solutes or water to permeate, only CO_2 (Wang et al. 2007; Uehlein et al. 2008; Otto et al. 2010). These studies indicate that MIPs may mediate the incorporation of CO_2, and some may play an important role in carbon assimilation from urea. Thus, due to the different trafficking mechanisms of MIPs that allow its localization into diverse plant membranes (plasmatic, mitochondrial, plastidial, and vacuolar), not only might urea be transported into the cell and/or stored in the vacuoles, but its catabolic products, ammonium and CO_2, might also be remobilized from/to the plant organelles via aquaporins (Fig. 1f–h).

In addition, it has been suggested that urea may play an important role as a carbon source for bromeliads (plants with a high capacity to absorb urea), especially under conditions that limit atmospheric CO_2 uptake, such as during drought conditions (Matiz et al. 2017). In the same study, through a cytochemical localization technique, it was possible to detect a high production of urea-generated CO_2 near chloroplasts under drought conditions, and by using ^{13}C-labeled urea, it was shown that part of the $^{13}CO_2$ originating from urea hydrolysis was fixated into malate molecules (Matiz et al. 2017).

Even if the relative contribution of C urea to the C total pool of plants remains uncertain, urea as well as other organic N sources may have a positive effect on C/N

plant ratios. Thus, it is worthy considering urea not only as a N source but also as a supplier of carbon to plant cells, as well as a form of N and C for nutrient retranslocation out of senescing leaves.

3 Ecological Relevance of Urea

All plants studied so far, mostly crop or model plants, appear to have the capacity to obtain and metabolize urea. However, the physiological relevance of urea in plant nutrition and developmental processes is still unclear. Therefore, in order to understand the different aspects of urea metabolism and its physiological importance in plants, it would be helpful to examine other systems in which urea might play a major role, for example, plants that are naturally exposed to urea in their natural habitats.

Information regarding organic N use in non-crop or model plants comes from plants that inhabit inorganic nitrogen-limited environments, such as the canopy of tropical forests, or places where there are low rates of microorganisms acting on N cycling, such as in temperate forests (e.g., tundra plants) and coastal lands (e.g., restinga).

It is reasonable to assume that these plants are adapted to preferentially absorb the most available N form (Scott and Rothstein 2011). As an example, it has been shown that *Nepenthes hemsleyana* Macfarl, a pitcher-forming carnivorous, coprophagous-like plant, takes advantage of its mutualistic relationship with the bat *Kerivoula hardwickii* Horsfield in order to use the urea from its feces (Yilamujiang et al. 2017). By adding ^{15}N-labeled urea into the pitchers of *Nepenthes* plants, it was suggested that exogenous urea is absorbed in its intact form and metabolized inside the tissues via urease since no urease activity was detected in the pitcher fluid (Yilamujiang et al. 2017). Because these plants must keep the pitcher fluid acidic, the absence of urease in this fluid prevents any urea hydrolysis, which could potentially alkalinize the pitcher solution (Yilamujiang et al. 2017). In contrast, for epiphytic tank-forming bromeliads (Fig. 2), which also establish mutualistic relationships with organisms, it

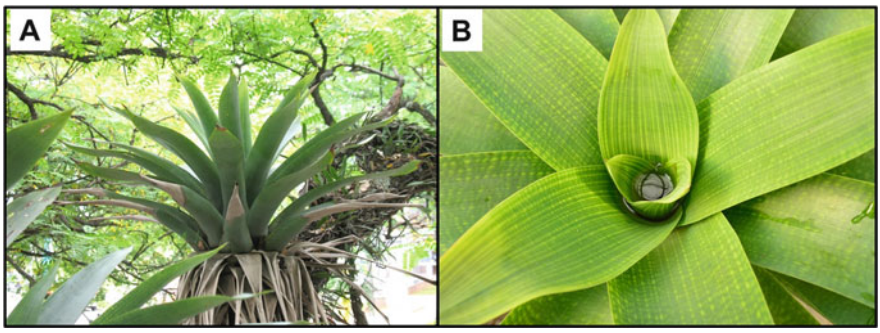

Fig. 2 Epiphytic bromeliad. (**a**) *Vriesea gigantea* Gaudichaud on a host plant. (**b**) Detail of the tank of *Vriesea gigantea* Gaudichaud

has been suggested that ureases could be secreted into the tanks to metabolize the urea deposited into this structure (Cambuí et al. 2009).

All these findings show the importance of mutualistic relationships (also see Sects. 3.1 and 3.2) in order to acquire organic nitrogen sources in environments where inorganic N is scarce and highlight the ecological relevance of urea for plants.

3.1 Urea Utilization by Epiphytic Bromeliads

Epiphytic bromeliads are plants that live in the forest canopy and have no direct contact with the contents of the soil (Benzing 1990). In order to cope with this, they have developed a range of adaptations to acquire and conserve water and nutrients (Laube and Zotz 2003; Givnish et al. 2014; Leroy et al. 2016). One of these adaptations is the tank, a structure typical of some bromeliads, formed by the overlapping of the leaf bases that accumulates water and nutrients (Fig. 2) (Benzing 1990, 2000).

The establishment of the tank is associated with major changes in the bromeliad morphology and physiology, which function to maximize the capacity of the leaf to perform photosynthesis as well as to take up tank contents through specialized absorptive trichomes (Takahashi et al. 2007; Freschi et al. 2010; Takahashi and Mercier 2011; Matiz et al. 2013; Mioto and Mercier 2013; Rodrigues et al. 2016; Pikart et al. 2018; Kleingesinds et al. 2018). Part of the available nutrients comes from precipitation and debris that fall into the tank, but the tank contents may also provide resources or shelter for a wide variety of organisms, forming small ecosystems within the bromeliads (Stewart et al. 1995; Ngai and Srivastava 2006; Cape et al. 2011; Leroy et al. 2016).

If we focus on the association with visiting animals, most of the available nitrogen comes in the form of organic compounds, such as urea. In fact, associations with animals, such as amphibians and spiders, can contribute a high proportion of nitrogen present in the plant (Romero et al. 2006, 2008, 2010). For example, it was estimated that individuals of *Scinax hayii* Barbour, which excrete mainly urea and visit the bromeliads to breed, contribute around 30% of the total plant nitrogen (Romero et al. 2010).

Based on this information, it is not difficult to imagine that organic nitrogen compounds, such as urea excreted by various animals, are a vital source of nitrogen for epiphytic bromeliads and are sometimes more readily available than inorganic sources (Benzing 2000; Romero et al. 2010; Gonçalves et al. 2016). Consistent with this, the overall growth of some bromeliad species is greater when nitrogen is supplied in the form of urea rather than nitrate or ammonium (Mercier et al. 1997; Endres and Mercier 2001). Accordingly, the epiphytic bromeliad *Vriesea gigantea* Gaudichaud showed a high capacity to absorb urea in its intact form through its leaves. Moreover, it has been suggested that this bromeliad may be able to secrete urease in the tank, increasing its rapid utilization (Inselsbacher et al. 2007). Later, the location of urease in *V. gigantea* Gaudichaud was determined to be in the cell walls

and cell membranes in addition to the cytosol, indicating that urease might be secreted into the tank (Cambuí et al. 2009), a very interesting feature considering that most ureases are located exclusively in the cytosol.

When urea was supplied into the tank of *V. gigantea* Gaudichaud, the apical region of the leaf showed the highest GS activity, indicating that urea-derived N assimilation occurs preferably in this leaf portion, while urea hydrolysis seems to occur mainly in the basal portion of the leaf, as evidenced by the high urease activity (Takahashi and Mercier 2011). Interestingly, even if urease activity is constitutive in all leaf tissues of *V. gigantea* Gaudichaud, its activity is inducible and considerably greater in the basal part of the leaves. Moreover, despite the elevated urease activity, urea accumulates in this leaf portion probably in the vacuoles (Takahashi and Mercier 2011; Matiz et al. 2017). However, what at first might be considered as a limitation of N-urea assimilation seems to be a very common strategy in epiphytic bromeliads, which demonstrates "luxury consumption." This process consists of the rapid absorption of nutrients when available and their stockpiling for later use in times of nutritional deficit (Lin and Yeh 2008; Winkler and Zotz 2010; Gonçalves et al. 2016). Strategies such as these may increase the chances of survival in the epiphytic habitat.

Urea absorption in epiphytic bromeliads appears to be mostly mediated by aquaporins since exposure to $HgCl_2$, an aquaporin inhibitor, reduced the absorption of urea by 78% (Inselsbacher et al. 2007). Recently, it was possible to confirm the involvement of aquaporins in nitrogen-acquisition strategies in these plants (H. Mercier, personal communication). Taken together, the increased, inducible activities of urease (with its potential secretion into the tanks) and aquaporin participation in urea absorption and/or accumulation (in the vacuoles) might be important elements that lead epiphytic bromeliads to prefer this organic nitrogen source.

Since one of the most studied species in terms of urea nutrition has been *Arabidopsis* and this species appears not to use urea in an efficient way (Kojima et al. 2007; Mérigout et al. 2008), it would be interesting to compare the affinities for urea (Km) of urease and urea transporters of plants that are naturally exposed to this nitrogen source with their orthologs in crop and model plants. These data could bring new insights into the key elements that make some plants prefer and metabolize urea efficiently.

3.2 Mycorrhization as Facilitator of N-Urea Absorption in Plants

Another possibility to increase the efficiency of urea nutrition in plants could rely on the association with organisms that are capable of absorbing nitrogen from organic compounds, such as fungi. Mycorrhiza refers to a fungus associated with plant roots, which provide carbon compounds to the fungus in exchange for mineral nutrients. It

is estimated that around 80% of angiosperms form some kind of mycorrhizal association, the more common being the arbuscular mycorrhizas and the ectomycorrhizas (ECMs) (Brundrett et al. 1996; Moore et al. 2011).

Since ECMs apparently are capable of absorbing organic nitrogen sources to a much larger extent than arbuscular mycorrhizas (Bago et al. 2001; Smith and Read 2008), they are the focus of this section. In fact, ECMs are dominant in boreal and temperate forests, in which a high proportion of the available nitrogen is in organic form (Wu 2011). Despite this, these associations are not restricted to these biomes and can also be found in the tropics, as highlighted by recent studies (Smith et al. 2011, 2013, 2017; Alvarez-Manjarrez et al. 2017; Corrales et al. 2018). The ECMs form a complex structure with three defined regions: the extraradical mycelium, the mantle, and the Hartig net (Fig. 3) (Smith and Read 2008). The Hartig net develops between the root cortex cells and represents the main site of nutrient exchange between plant and fungus, while the mantle is formed by the wrapping of the hyphae around the roots, which is connected to the extraradical mycelium that extends toward the soil.

The preferred nitrogen form varies among the different ECM symbionts. There are reports of nitrogen use from organic sources, such as amino acids, proteins, and urea (Yamanaka 1999; Guidot et al. 2005; Smith and Read 2008; Shah et al. 2013). In some cases, it has been shown that ECM fungi without the host can grow fairly

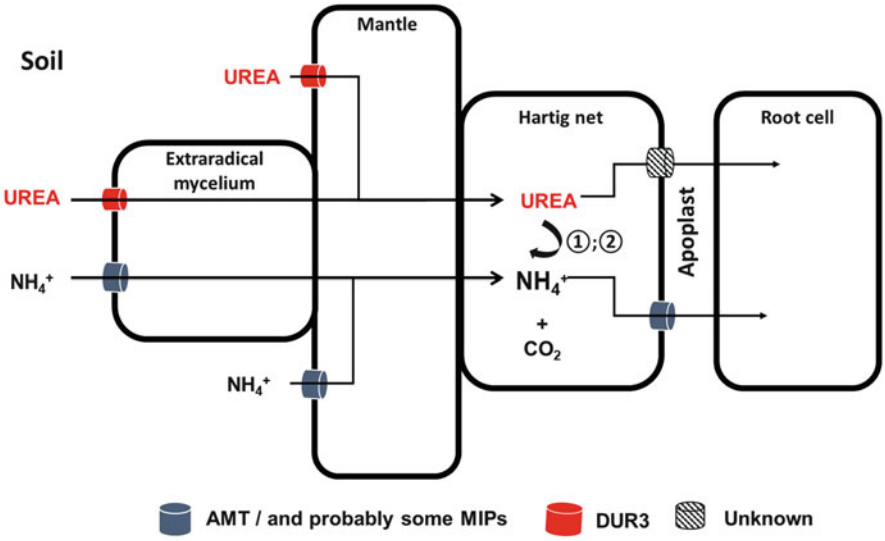

Fig. 3 Proposed model for nitrogen transfer from soil urea to roots through ectomycorrhizal fungi (adapted from Hacquard et al. 2013). Urea is mainly absorbed by the extraradical mycelium and the mantle by DUR3 transporters, while ammonium can be absorbed by AMT or MIPs. After urea is absorbed, it is transferred to the Hartig net and hydrolyzed by urease or urea amidolyase. The resulting ammonium is then excreted in the plant apoplast (possibly by AMTs or MIPs) and absorbed by the root cell through the mechanisms described in Fig. 1. ① = urease ② = urea amidolyase

well on urea as the sole source of nitrogen (Yamanaka 1999; Guidot et al. 2005). There are also a few studies that have investigated whether and how ECMs may absorb this compound and translocate N to the plant, but the data seem somewhat limited, and many questions remain to be answered.

Hacquard et al. (2013) compared microarray data from three different stages of *Tuber melanosporum* Vittad development: free-living mycelium, fruiting body, and ectomycorrhizas. A DUR3-like urea transporter was significantly more expressed when this fungus was forming ECMs. Moreover, microarray data comparing laser-dissected mantle and Hartig net compartments in the same species showed that DUR3-like gene expression was higher in the mantle cells, while urease and urea amidolyase were upregulated in the Hartig net (Hacquard et al. 2013). This suggests that the fungus is capable of absorbing urea from the soil, breaking it down (through urease or urea amidolyase action) and transferring the derived nitrogen to the plant in the form of ammonium (Fig. 3). This is somewhat similar to what happens in arbuscular mycorrhizas, in which the fungus transfers NH_4^+ to the plant, likely originating from internal urea breakdown (Govindarajulu et al. 2005; Pérez-Tienda et al. 2011; Tian et al. 2010).

Morel et al. (2005) quantified nitrogen-containing organic compounds in the ECM *Paxillus involutus* (Batsch ex Fr.) Fr.-*Betula pendula* Roth association, comparing the mycelium directly associated with the roots (mantle + Hartig net) to the extraradical mycelium about 10 cm from the root tips. Strikingly, urea was present in high concentrations in both the extraradical mycelium and the root mycelium. In the same experiment, a microarray analysis of approximately 1200 genes was performed, revealing that the urea transporter *PiDur3* was approximately four times more expressed in the extraradical mycelium than in the ECMs. Again, this points toward a significant participation of urea in the nutrition of ectomycorrhizal plants. Later, *PiDur3* was functionally characterized by complementation growth assays in a urea-defective uptake yeast strain, showing that this transporter is, in fact, capable of transporting urea (Morel et al. 2008). Moreover, these authors showed that *PiDur3* expression was negatively regulated by glutamine and, possibly, intracellular urea and ammonium (Fig. 3).

It is likely that aquaporins also control metabolite transport as well as other processes between host plants and fungi, but there is very little information on the subject. Dietz et al. (2011) showed that three aquaporins are capable of transporting NH_4^+/NH_3 through yeast complementation assay and that one of these (Lacbi1:317173) was highly expressed in the ECM compartment when compared to the extraradical mycelium and the isolated fungus. This could indicate that this MIP may play a role in NH_4^+ transfer to the host plant. On the other hand, Lacbi1:387054 and Lacbi1:391485, which also appear to transport NH_4^+/NH_3, were more expressed in the extraradical mycelium (Dietz et al. 2011).

It is very likely that the role of aquaporins in ECM formation is not fully understood. If we take the example of other symbioses, such as the interaction between plants and nitrogen-fixing bacteria, the NIP class of aquaporins plays an important role not only in metabolite transport but in nodule development as well (Uehlein et al. 2007; Afzal et al. 2016). Therefore, we can assume that a similar role may exist for mycorrhizal associations.

Despite the lack of information, there is evidence that at least some ectomycorrhizal associations can use urea from the environment, but how the nitrogen is transferred to the plants is still unclear. Thus far, it appears that urea is absorbed in the extraradical mycelium and mantle and then transported to the Hartig net, where it is broken down into ammonium and then transferred to the plant (Fig. 3). It is important to remember that there are many fungal species capable of forming ECMs and that, accordingly, the mechanisms of urea absorption and N transfer to the plant may vary. Nevertheless, discovering how these associations function could lead to major breakthroughs in how to make plants more effectively absorb and use urea by focusing not only on the physiology of the plants but also on the ECMs.

4 Biotechnological Aspects and Challenges to Improve Nitrogen Use Efficiency (NUE) in Plants

One of the main goals in agricultural production is the increase of NUE of plants. A logical way to achieve this is either by increasing nutrient availability or by engineering crops with a high capacity to utilize N sources. Since urea is the main N fertilizer used nowadays in arable soils, it is worth investigating how to improve its availability and utilization by crop plants.

Regarding urea availability to cultivated plants, the use of large amounts of urea fertilizers has been the main strategy to sustain high productivity in arable soils. However, as mentioned above, due to the rates of urea hydrolysis by microbial ureases in soils, the use of large amounts of urea-based fertilizers causes nitrogen losses by volatilization, which results in water and atmospheric pollution (Zaman et al. 2008, 2009). Therefore, in order to avoid or at least reduce this process, urea is being supplied to crops in conjunction with urease inhibitors, which increases the persistence of urea (Zaman et al. 2008; Sanz-Cobena et al. 2011; Abalos et al. 2012; Soares et al. 2012; Saggar et al. 2013; Ahmed et al. 2018). As a consequence of a longer permanency of urea in the soils, an improvement in its absorption by plants is expected. However, it has been demonstrated that urease inhibitors, such as N-(n-butyl) thiophosphoric triamide (NBPT), have a negative effect on DUR3 transporters and, consequently, on urea uptake (Zanin et al. 2015b). Additionally, it appears that NBPT can also be absorbed and translocated to other tissues of the plant, affecting the endogenous urease activity of the plant (Cruchaga et al. 2011; Zanin et al. 2015b, 2016). Indeed, under NBPT treatments, urea-derived N assimilation is impaired in plants, as evidenced by a reduction in ammonium and amino acid pools, as well as the activity and gene expression of glutamine synthetase (Artola et al. 2011; Cruchaga et al. 2011; Zanin et al. 2015a, 2016).

On the other hand, in Ni-poor soil, impairments of urea utilization might be caused by a hidden Ni deficiency, which impedes the correct function of plant ureases. Fertilization with Ni has been shown to have a positive effect on plant

growth and development, N assimilation, and grain yield (Roach and Barclay 1946; Gerendás and Sattelmacher 1997; Freitas et al. 2018). Moreover, since Ni is an essential element for urease activation, it has to be translocated to the different tissues of the plant in order to allow urea metabolization, a process that may take several days after its application (Myrach et al. 2017). Therefore, it would be interesting to carry out prior applications of Ni in urea fertilization programs, which may benefit the N metabolism of plants and, consequently, production, particularly in soils with low Ni content.

Significant quantities of mineral (ammonium and nitrate) and dissolved organic N (amino acids, peptides, and urea) are present in fertilized soils; consequently, plants are exposed to this combination of N sources (Garnica et al. 2009; Brackin et al. 2015). Therefore, we have to keep in mind that in order to improve our understanding of urea nutrition in plants, it is necessary to know how interactions with other N sources (inorganic and organic N pools) function in the root uptake since this appears to modify urea uptake and assimilation (Bradley et al. 1989; Mérigout et al. 2008; Garnica et al. 2009; Zanin et al. 2015a; Pinton et al. 2016). For example, nitrate supply in conjunction with urea enhances cytosolic and plastidial N-assimilatory pathways, leading to a positive N status and plant growth in maize plants (Zanin et al. 2015a). However, ammonium and ammonium nitrate caused a reduction in urea absorption in wheat seedlings (Bradley et al. 1989). In addition, in *Arabidopsis AtDUR3* gene expression was induced by urea but only in the absence of other N forms (ammonium nitrate) (Mérigout et al. 2008). These results indicate that the response to a combination of N sources depends on the plant species and may be influenced to some extent by initial N status. Therefore, it would be interesting to consider strategies focusing on synchronizing soil N supply with N status of the plant, as well as focusing nutrition plant research on interactions among N forms in crops to achieve a more sustainable use of fertilizers.

On the other hand, urea uptake and metabolism studies that identify key elements as targets for NUE have not received adequate attention. With respect to urea acquisition by plants, as mentioned above, high- and low-affinity transport systems for urea have been identified only recently. Thus, it makes them an obvious target for improving urea absorption. By analyzing the phenotypic changes of wild-type plants of *Arabidopsis* overexpressing rice or maize DUR3 transporters (OsDUR3 or ZmDUR3, respectively) and exposed to micromolar concentrations of urea, it was possible to show that *Arabidopsis*, a plant with low capacity to use urea as a sole N source, was capable of growing better than the untransformed wild-type plants (Wang et al. 2012; Liu et al. 2015).

Considering that in most agricultural soils the urease activity is high, urea would be present in micromolar concentrations; thus, engineering crops overexpressing DUR3 transporters might be an interest strategy to improve NUE. In contrast, since aquaporins manage millimolar concentrations of urea, they may be interesting targets for improving NUE in foliar fertilization programs. Consistent with this, overexpression of AtTIP4;1, a urea-transporting aquaporin of *Arabidopsis* which is detectable in roots but not in leaf tissues, was capable of increasing the absorption of urea in leaf tissues (Kim et al. 2008). These findings show a clear correlation

between the overexpression of urea transporters and the increase of urea uptake, further reinforcing the potential of engineering crops by modifying the expression of urea transporters to positively affect its acquisition.

In addition, overexpression of NtAQP1, an aquaporin that mediates the transport of CO_2, has led to an increase in net photosynthesis and leaf growth (Uehlein et al. 2003). Interestingly, this same aquaporin has been proven to mediate urea transport when expressed in oocytes (Eckert et al. 1999), indicating that overexpression of aquaporins might improve urea absorption as well as membrane permeability for CO_2.

Moreover, as mentioned above, an additional interesting way to increase N-urea acquisition by plants might be achieved through mycorrhizal interactions; however, this research field has been little explored.

Besides effective urea absorption by plants, an efficient assimilatory pathway is also required. As mentioned above, glutamine synthetase-, glutamate synthase-, and asparagine synthetase-encoding genes are upregulated under urea supply (Mérigout et al. 2008; Zanin et al. 2015a, b, 2016); these, too, might be targets to improve NUE under urea supply.

Due to the scant knowledge about the regulatory elements of urea metabolism in plants, further investigation into the elements involved in urea metabolism in plants that naturally receive this organic source of nitrogen, such as *V. gigantea* Gaudichaud, could potentially allow a better understanding of the importance of urea for plant nitrogen nutrition as well as identify key targets that can be used to modify urea usage in plants of economic interest.

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Biosynthesis and Regulation of Secondary Cell Wall

Ignacio Zarra, Gloria Revilla, Javier Sampedro, and Elene R. Valdivia

Contents

Abstract Secondary plant cell walls are produced by specialized plant cell types required for mechanical support and water transport. They are a microfibril-based composite constituted by celluloses, hemicelluloses, and lignins. Cellulose

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I. Zarra (✉), G. Revilla, J. Sampedro, and E. R. Valdivia
Departamento de Biología Funcional, Facultad de Biología, Universidad de Santiago de Compostela, Campus Vida, Santiago de Compostela, Spain
e-mail: ignacio.zarra@usc.es; mgloria.revilla@usc.es; javier.sampedro@usc.es

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microfibrils are associated with hemicelluloses leading to the formation of a polysaccharide network that is impregnated by lignin. They are thick and un-extensible, unlike primary walls. The formation of secondary cell walls involves a series of overlapping events as changes in the biosynthetic machinery of their components: microtubule reorganization, formation of cellulose synthase complexes that synthesize cellulose microfibrils with different characteristics, a shift from pectin to secondary wall hemicelluloses involving xylans or mannans with different side-chain decoration, and synthesis of monolignols and polymerization for lignin production. All the abovementioned processes require a precise regulation not only of each event on its own but also to achieve the integration and coordination of the whole. The main actors in the current model of secondary wall regulation are the transcription factors that act as master switches. They are organized in a hierarchical way where NAC transcription factors are at the top of the network and MYB and others are downstream in the signaling cascade.

1 Introduction

Plants are constituted by about 35 different cell types, which are different in size, shape, position, and cell walls (Cosgrove 2005). Heterogeneity is found at the tissue and cellular levels, and it is crucial for the changing biological functions of the wall during plant growth and development. The cells produced by cell division begin to expand until they reach their final size, and then they can start their differentiation program. Cell division, cell expansion, and cell differentiation are three processes that take place sequentially. They are precisely regulated by a fine-tuned balance between positive and negative regulators where transcription factors have a central role.

After the cell expansion ceases, in some cell types, the deposition of the secondary cell wall, between the primary wall and the plasmalemma, may start leading to the cell differentiation. These cells assume an increasing structural role, their walls become thick, cellulose microfibrils become laminated, and often walls become encrusted with lignin. Mechanical resistance of the wall increases while the passage of small molecules is restricted. Thus, while the primary walls can extend allowing cell growth but are of enough mechanical strength to withstand the turgor pressure of the protoplast, the secondary walls are thick and un-extensible. The transition from primary wall to secondary wall formation takes place through an overlapping series of events, which include remodeling of cell wall biosynthetic machinery, wall synthesis dominated by polysaccharide production, maturation dominated by lignification, and often programmed cell death (Meents et al. 2018). The changes in the biosynthetic machinery involve microtubule reorganization and the exchange of primary wall cellulose synthases for secondary cellulose synthases, a shift from pectins and primary wall hemicelluloses to secondary wall hemicelluloses in the Golgi apparatus, and, later, secretion of monolignols and peroxidases and laccases to the apoplast and their polymerization for lignin production (Fig. 1).

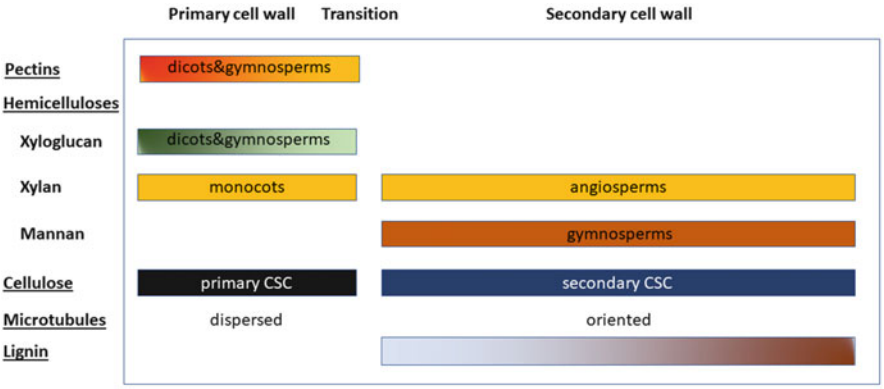

Fig. 1 Main differences between primary and secondary plant cell walls

Both primary and secondary cell walls are microfibril-based composites that show differences in the arrangement, mobility, and structure of matrix polymers, the organization of microfibrils into bundles and lamellae, their rheological and mechanical properties, and their role in the life of the plant. Secondary cell walls are produced only by some specialized cells required either for support of the plant or water transport (Kumar et al. 2016). They are mainly found in fibers and conducting cells of vascular tissues as well as in interfascicular fibers and sclereids. They constitute the raw material for different industrial uses such as fuel, fiber, feed, paper paste, chemicals, and different biomaterials (Harris and DeBolt 2010).

The secondary plant cell walls are mainly constituted by celluloses, hemicelluloses, and lignins. There are compositional and structural differences between primary and secondary walls, cellulose levels being typically higher in secondary walls than in primary ones. Hemicelluloses are also present in secondary walls but in lower amount when compared with primary walls. Glucuronoxylans are the predominant hemicelluloses in dicots, while glucuronoarabinoxylans are more important in grasses and gymnosperms. Moreover, galactoglucomannan is the main hemicellulosic component of gymnosperm wood (Zhong and Ye 2015). The strength and rigidity of plant tissues that have ceased to growth is provided by secondary walls. Stems with bending strength and water-conducting tissues able to support negative pressures are needed for plants to stand up in the terrestrial environment. Therefore, they need both compressive and tensile strength but not extensibility. They contain about 30% water and are thus less hydrated than the primary walls (Cosgrove and Jarvis 2012).

The formation of secondary cell walls is not independent of other processes in plants (Rao and Dixon 2018). First, it is determined by the sugar availability, by the carbon balance, as well as by light and the circadian clock (Rogers et al. 2005). Second, it depends on the differentiation program of the specific cell types under hormonal regulation (Didi et al. 2015). It is also important to note that the establishment of the secondary walls involves the biosynthesis of cellulose as well as hemicelluloses and lignin and these metabolic pathways are closely related not only

among them but also with primary metabolism. Moreover, the lignin pathway has common intermediates with other metabolic pathways such as flavonoids biosynthesis. In the tracheary element differentiation, the secondary wall formation is coupled with programmed cell death (Ohashi-Ito et al. 2010). Thus, coordination of all these processes will be necessary. To achieve that coordination among secondary wall synthesis and other physiological processes, plants employ few transcription factors constituting a complex regulatory network (Rao and Dixon 2018).

2 Cellulose

Cellulose is the most stable polysaccharide in plant cell walls as well as the major structural component and about one-third of plant total mass. It is an unbranched polysaccharide constituted by β-1,4-linked D-glucopyranosyl residues. Although the chemical composition is the same, there are important differences between cellulose in primary and secondary walls (Albersheim et al. 2011). Cellulose is up to 50% in the secondary walls while 20–30% for primary walls. The degree of polymerization of β(1-4)-D-glucan chains of cellulose is higher in the secondary walls. While in primary walls, the degree of polymerization is lower than 5,000, in secondary walls, it is around 10,000–15,000 (Brett 2000). However, virtually nothing is known about how the degree of polymerization is regulated in plants.

2.1 Cellulose Synthase Complex (CSC)

Cellulose synthase genes (*CESA*) in plants were first identified in the 1990s (Arioli et al. 1998; Pear et al. 1996). Plants express multiple cellulose synthases (CESAs) and have a similar number of *CESA* genes, 12 in maize, 10 in rice, 8 in barley, and 7 in poplar (Harris and DeBolt 2010). The CESAs are members of the super family 2 of glycosyltransferases (Lombard et al. 2014). They are distributed in six groups CESA1, 3, 4, 6, 7, and 8 (Kumar and Turner 2015). Recently, a single plant CESA8 protein from hybrid aspen has been heterologously expressed in *Pichia* and reconstituted into proteoliposomes. Under those experimental conditions, it was able to synthesize in vitro linear chains of β-1,4-glucan and form microfibrils. However, if the N-terminal region was suppressed, the synthetic capacity of the system was maintained, but it was not able to form microfibrils (Purushotham et al. 2016).

Cellulose in plants is synthesized at the plasma membrane by a large complex with a diameter of 24–30 nm formed by six lobes, each measuring 8 nm in diameter. This is called the cellulose synthase complex (CSC) and contains at least three different nonredundant cellulose synthase isoforms (CESAs) (Turner and Kumar 2018). The number of cellulose synthases in the CSC has been controversial for many years. It has been suggested that the complex is a hexamer of homo- or

heterotrimers (Vandavasi et al. 2016). The self-assembly of six trimers forming the hexameric rosette has been proposed as model for the cellulose synthase complex (Vandavasi et al. 2016). Thus, each CSC contains 18 CESAs (Turner and Kumar 2018). If each lobe is constituted by three CESA proteins, the whole complex would contain 18 CESA proteins. The large range of size found for the rosettes might be a consequence of different activation stages or altered position of the individual lobes (Turner and Kumar 2018). A previous model postulated that cellulose microfibrils were formed by 36 glucan chains (Delmer 1999). However further studies using wide-angle X-ray and solid-state resonance indicates that each microfibril is composed of 18 or 24 chains. If it is assumed that all CESA polypeptides are active in β(1-4)-glucan synthesis, 18-chain microfibrils are consistent with a cellulose-synthesizing complex containing six particles formed by three cellulose synthase polypeptides (Newman et al. 2013). Plant expresses multiple cellulose synthases organized in large complexes of variable composition that are responsible for cellulose synthesis adding glucosyl residues to the nascent polymer. They are also responsible of the alignment of the new β-glucan chains into microfibrils. Although a 1:1:1 stoichiometry among the different CESA proteins seems to be the most probable situation in secondary walls (Hill et al. 2014), other ratios as 3:2:1 and 8:3:1 for PtCESA8/PtCESA4/PtCESA7 have been found for aspen developing xylem and tension wood, respectively (Zhang et al. 2018b).

Cellulose is synthesized as microfibrils rather than individual chains (Fig. 2). The assembly of the CESA proteins into the cellulose synthase complex as well as its localization in the plasma membrane are required for cellulose biosynthesis in plants. The presence of three different CESAs is necessary for the formation of the multimeric complex. In *Arabidopsis* as well as in most higher plants, it is widely accepted that CESA4, 7, and 8 are the constituents of CSC of secondary plant cell walls (Kumar and Turner 2015). Mutation of any of the three *CESAs*, *AtCESA8* (*irx1*), *AtCESA7* (*irx3*), and *AtCES*4 (*irx5*) genes, results a collapsed vessel phenotype (irregular xylem) caused by a dramatic reduction in cellulose content of secondary walls (Turner and Somerville 1997). They are co-expressed and all form part of the same cellulose synthase complex. Although the 4/7/8 model is widely present, other cell types may use different CESAs for secondary wall synthesis. So, CESA2, 5, and 9 are all necessary for secondary wall synthesis in cells of the seed coat that show thickening of the radial walls suggesting that different cell types require cell-specific CSCs (Mendu et al. 2011).

2.2 Proteins Associated with the CSC

The cellulose synthase complex involves not only the glucosyltransferases elongating the β-glucan chains but also other proteins interacting with microtubules as well as glycosidases putatively editing microfibrils. Interactions of plant CESAs with membrane-integrated or attached proteins as well as cytosolic microtubule-binding proteins have also been shown. Cellulose synthase interactive protein (CSI)

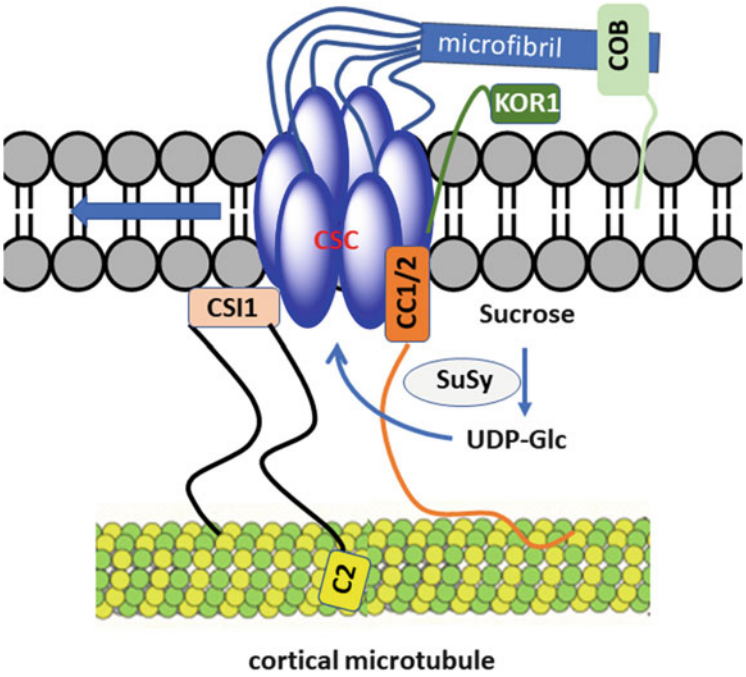

Fig. 2 Complex cellulose synthase. Blue arrow indicates movement of CSC through the plasma membrane. *CSC* cellulose synthase complex, *CSI* cellulose synthase interactive protein, *KOR1* Korrigan, *COB* COBRA, *CC1/2* companion of cellulose synthase, *SuSy* sucrose synthase

establishing a molecular bridge between CSCs and cortical microtubules through its C2 domain drives the directional movement of CSCs through the plasma membrane. In addition, the companion cellulose synthase proteins, CC1 and CC2, bind microtubules and CSC via their N-terminal domain (Li et al. 2012a; Speicher et al. 2018).

Moreover, other proteins that are involved in some way in the microfibril assembly or in providing the substrate for cellulose synthesis may be expected to be associated with the CSC. Other proteins as KORRIGAN and COBRA are involved in cellulose microfibril assembly and crystallinity.

KORRIGAN, a plasma membrane-anchored protein with endo-1,4-β-D-glucanase activity (Nicol et al. 1998), is required for cellulose synthesis in primary and secondary cell walls and during cell plate formation (Szyjanowicz et al. 2004; Turner and Kumar 2018; Vain et al. 2014). The leaky *kor1-1 Arabidopsis* mutant shows a reduction in the cellulose content as well as a reduction in the CSC motility (Vain et al. 2014). *PttCel9A1*, an ortholog of *KOR1*, is more active against noncrystalline cellulose (Takahashi et al. 2009). Different hypotheses have been proposed to explain the role of KOR in cellulose synthesis: (a) proofreading the nascent glucan chains hydrolyzing disordered amorphous cellulose (Molhoj et al. 2001), (b) determining the length of the glucan chains, or (c) releasing the cellulose microfibril from the CSC (Somerville 2006).

COBRA is a GPI-anchored protein at the plasma membrane involved in the orientation of the cellulose microfibril deposition (Taylor 2008). COBRA also contributes in some way to cellulose biosynthesis at least in cells undergoing longitudinal expansion (Schindelman et al. 2001). In *Arabidopsis*, there are 12 *COB* genes, *COB1* and 11 *COB-like* (*COBL*) genes. They are expressed during primary and secondary cell wall formation. AtCOBL4 is necessary for cellulose synthesis in the secondary wall (Brown et al. 2005). A reverse genetic analysis based on genes co-expressed with the secondary cell wall-specific cellulose synthase genes identified five genes as being essential for secondary wall synthesis. The mutation in *COBL4* was the only one that resulted in a severe cellulose-deficient phenotype. Moreover, Brittle Culm1 (BC1), a COBRA-like protein, interacts with crystalline cellulose through a carbohydrate-binding module and participates in the cellulose microfibril assembly (Liu et al. 2013).

Finally, a chitinase-like protein (CTL) that contributes to salt tolerance regulates cellulose assembly and interacts with hemicelluloses via binding to emerging cellulose microfibrils, but its exact function is yet to be defined (Sánchez-Rodríguez et al. 2012).

2.3 Regulation of Cellulose Synthesis

The synthesis and deposition of cellulose, as the other components of plant cell walls, are regulated at the transcriptional as well as at the posttranslational level. Phosphoproteomic studies have revealed a high number of phosphorylation sites within CSCs. CESAs are phosphorylated in numerous sites in the N-terminal region or in the hypervariable region of their catalytic loop (Speicher et al. 2018). An extensive list of phosphorylation sites of CESs and CSC-associated proteins is reviewed in Speicher et al. (2018). For example, AtCESA7, an essential cellulose synthase for secondary wall cellulose synthesis, which interacts with AtCESA8 and AtCESA4 to form CSCs, is phosphorylated in vivo. The phosphorylated sites are two serine residues in the hypervariable region between the CSC catalytic subunits. Phosphorylation of the CESAs may target them for degradation via proteasome, regulating the levels of CESA proteins whose interaction is necessary to constitute an active CSC. Thus, phosphorylation of CESAs may be a regulatory mechanism by which their relative levels are maintained (Taylor 2007). However, phosphorylation not only is important to regulate CESA levels through protein stability but also through changes in CSC motility and catalytic activity. Moreover, other proteins associated with CSCs are extensively phosphorylated in their cytosolic domains (Speicher et al. 2018).

2.4 Assembly of Rosettes and Translocation to Plasma Membrane

CSC proteins are probably synthesized in the endoplasmic reticulum. The complex is believed to be assembled in the Golgi apparatus, since it is the earliest part of the secretory pathway where they have been observed (Bashline et al. 2014), but it does not synthesize cellulose until it is incorporated into the plasma membrane (McFarlane et al. 2014). The knowledge about the CSC assembly in the Golgi apparatus is scarce. Nevertheless, STELLO proteins (STL) that interact with CESAs in the Golgi have been identified (Zhang et al. 2016). The analysis of the predicted catalytic domains of STL proteins indicates that they may be a glycosyltransferase facing the Golgi lumen. In their absence, CSC distribution in the Golgi as well as its secretion and activity are impaired suggesting a role in its assembly. A role of STLs maintaining CSCs inactive until they have localized to the plasma membrane has been proposed. After arrival at plasma membrane, STLs would need to be removed for CSCs to be activated.

The delivery of the CSCs to the plasma membrane by exocytosis is critical for cellulose synthesis. It is mediated by the cooperation of different proteins such as cellulose-interacting protein (CSI), PATROL1 (PTL), and the exocyst complex. The exocyst complex and PTL are necessary for the fusion of the CSC with the membrane. CSI marks the docking site allowing the CSCs containing vesicles to fuse with the plasma membrane through the interaction with microtubules to deliver the CSC to its position into the membrane. PTL1 binds to CSI1 and interacts with the exocyst complex facilitating the fusion of vesicle with the plasma membrane (Zhu et al. 2018). The following sequence of events during CSCs secretion has been proposed. CSI1 appears at the plasma membrane. The exocyst complex associated with CSC-containing vesicles discharges the CSC at the plasma membrane and disappears, probably at the moment of the fusion (Zhu et al. 2018).

2.5 Microfibril Organization

After the β-glucan chains are extruded, they form microfibrils through intra- and intermolecular hydrogen bonds and van der Waals forces. These interactions are not homogenous along the fibers, leading to crystalline zones interspersed with amorphous ones. The contiguous glucosyl residues are positioned at 180° relative to each other and stabilized by hydrogen bonds, causing the β-glucan to adopt a linear conformation. The linear glucan can be associated side by side through hydrogen bonds forming the cellulose microfibrils. Cellulose microfibrils from higher plants are about 3 nm in diameter. They are synthetized by a protein complex containing six units of three β-glucan synthases each. That would give 18 β-glucan microfibrils in accordance with the observed diameter of 3 nm. Partial fusion after synthesis with crystalline continuity or lateral aggregation without crystalline continuity can lead to

single microfibrils containing up to 80 chains being favored in the absence of other polymers. In secondary walls of dicots, microfibrils are 4 nm wide exceeding the ca 3 nm width of primary walls (Jarvis 2018).

Cellulose microfibrils must be deposited in a very specific orientation to confer the physical strength required in secondary walls. The organization of cellulose microfibrils is quite different between primary and secondary walls of higher plants. Secondary walls are typically laid on three distinct layers, S1, S2, and S3. While in primary walls microfibrils are laid down transversally to the elongation axis, in secondary ones, microfibrils are aligned in each layer varying from one layer to the next. Microtubules direct the movement of the cellulose complexes and the direction and in which microfibrils are deposited on the wall (Taylor 2008).

The mechanisms involved in the organization and crystallinity of cellulose microfibrils are not still elucidated although it seems likely to be based on the different cellulose synthases expressed in primary and secondary walls (Ruel et al. 2012). The relative proportion of crystalline to amorphous cellulose undergoes changes during cell development reflecting the differential expression of genes involved in cellulose synthesis. Two crystalline allomorphs, cellulose Iα (triclinic) and Iβ (monoclinic), both with parallel glucan chains in a flat-ribbon conformation exist in the crystalline structure of cellulose but differ in the hydrogen bond patterning between glucan chains (Šturcová et al. 2004). The crystallinity of cellulose is given by the proportion of crystalline to amorphous microfibrils. The degree of crystallinity may differ not only between primary and secondary walls but also among the different layers of the secondary wall. Cellulose in primary walls is mainly formed by the metastable phase Iα, while in the secondary walls, the stable Iβ phase becomes dominant (Kataoka and Kondo 1998; Šturcová et al. 2004).

Cellulose microfibrils formed by CSC are laid down in apoplast close to the plasma membrane. The addition of new glucosyl residues to the nonreducing end of β-glucan chains provides the energy to push the CSC complex along the plasma membrane (Paredez et al. 2006). The disruption of the cortical microtubules by pharmacological drugs such as colchicine leads to alterations in the pattern of wall thickening, localized CSC accumulation, and cellulose microfibril orientation. Thus, the distribution of the cortical microtubules through their linkage with the CSCs drives the orientation in the wall of the cellulose microfibrils (Wightman and Turner 2008).

2.6 Supply of UDP-Glucose

Cellulose synthases use UDP-glucose to synthesize β-1,4-glucan chains. UDP-glucose may be provided directly by hydrolysis of sucrose catalyzed by sucrose synthase (Haigler et al. 2001). It may be also provided by invertase and further conversion of the released glucose to glucose-6-phosphate, glucose-1-phosphate, and UDP-glucose (Barnes and Anderson 2018). Mutation or downregulation of sucrose synthase in *Arabidopsis* and aspen did not have an important effect on

cellulose synthesis (Gerber et al. 2014), while a double mutant of *Arabidopsis* invertases showed a significant decrease in the production of UDP-glucose and cellulose (Barnes and Anderson 2018).

3 Hemicelluloses

At the onset of the secondary cell wall deposition, xylans or mannans become the main hemicellulosic polysaccharides at the wall (Scheller and Ulvskov 2010).

3.1 Xylans

Xylans are the main hemicellulose in the secondary cell walls of angiosperms, but they are also found in primary walls of monocotyledonous. They play an important role in the strengthening of secondary walls as shown by the analysis of xylan-deficient mutants (Scheller and Ulvskov 2010). Xylans are synthesized in the Golgi apparatus and secreted into the cell wall via vesicles. Their biosynthesis requires an important number of glycosyltransferases as well as acetyl and methyl transferases (Fig. 3). They are a diverse group of polysaccharides with a β-(1-4)-linked xylosyl backbone with short sidechains of 2-*O*-linked α-D-glucuronic acid, glucuronoxylan, and also 2-*O*- and/or 3-*O*-linked α-L-arabinopyranosyl residues, in the case of glucuronoarabinoxylan. Glucuronoxylan is characteristic of secondary walls of dicots, where it can reach 25% of total weight in hardwood species and some

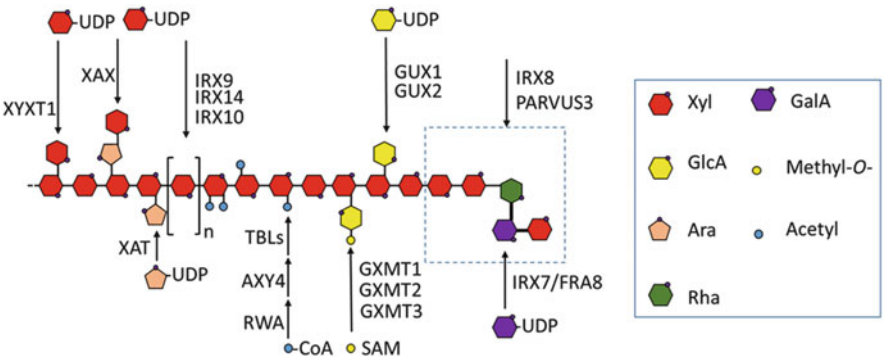

Fig. 3 Proteins involved in xylan biosynthesis. *XAX* xylosyl arabinosyl xylan transferase, *IRX9, IRX14* xylosyl transferases from GT43, *IRX14* xylosyl transferase from GT47 family, *GUX1*, *GUX2* glucuronosyltransferases from GT48 family, *IRX8, PARVUS3* α-glycosyl transferases from GT8 family, *XYXT1* xylan xylosyltransferase, *XAT* xylan arabinosyltransferase, *TBL* xylan acetyltransferase, *AXY4, RWA* putative acetyl-CoA transporters, *GXM1, GXM2, GXM3* glucuronosyl methyltransferases, *IRX7/FRA8* galacturonosyltransferase. Doted box: oligosaccharide at the reducing end

non-grass monocots (Scheller and Ulvskov 2010). Glucuronoarabinoxylan is present in grass species (Peña et al. 2016) and gymnosperm softwood (Scheller and Ulvskov 2010).

Xylose can be *O*-acetylated at C-2 or C-3 position, and glucuronosyl residues can be *O*-methyl substituted at C-4. A unique oligosaccharide in its reducing end, 4-β-D-Xyl-(1-4)-β-D-Xyl-(1-3)-α-L-Rha-(1-2)-α-D-GalA-(1-4)-D-Xyl, has been found in *Arabidopsis*. It has been suggested that it serves as an initiator or terminator of xylan synthesis (Peña et al. 2007). Based on xylan structure, it is likely that a few glycosyltransferases are required for its synthesis: initiation, elongation of the axis backbone, and sidechain addition (Rennie and Scheller 2014). Xylan is synthesized in the Golgi apparatus and secreted to the apoplast. Two members of glycosyltransferase family 43, *IRX9* and *IRX14* (Brown et al. 2007), and one member of GT47, *IRX10*, all coding xylosyltransferases, are required for the synthesis of the xylan backbone (Ratke et al. 2018). IRX10 protein interacts with the membrane-bound IRX9 and IRX14 proteins forming a membrane-bound complex that participates in xylan synthesis. Moreover, IRX7, IRX8, and PARVUS but not IRX9 and IRX14 are necessary for the presence of the specific reducing end sequence (Brown et al. 2007). IRX8 and PARVUS belong to GT family 8 that may catalyze the formation of α-glycosidic linkages suggesting that it is responsible for the addition of α-linked GalA residue to the xylan reducing end oligosaccharide sequence (Peña et al. 2007). GUX1 and GUX2, both members of GT48 family, have been identified as glucuronosyltransferases. GUX1 is the main contributor to the xylan substitution, while GUX2 contributes a minor proportion of GlcA decoration. The action pattern of both proteins is different leading to the formation of two different domains. The decorations of xylans might affect their interactions with other wall components such as cellulose or lignin. About two out of three glucuronosyl residues in *Arabidopsis* are 4-*O*-methylated (Bromley et al. 2013).

Arabinosyl side chains are abundant in secondary walls of grasses and conifers but absent in dicot plants. The xylosyl residues of the xylan backbone may be substituted with α-arabinosyl in the C-2 and C-3 positions. GT61 family members, from rice and wheat, have been identified as xylan arabinosyltransferases (XATs) responsible for α-(1,3) substitution (Anders et al. 2012). Moreover, another GT61 family member, xylosyl arabinosyl xylan transferase (XAX1), can form β-Xyl*p*-(1,2)-α-Ara*f*-(1,3) side chains. The dwarf phenotype of *xax1* mutant suggests the importance of the β-1,2-xylosyl substitution (Chiniquy et al. 2012). Recently, Zhong et al. (2018a) reported a xylan xylosyltransferase, a GT61 family member (XYXT1) in rice able to transfer xylose to the xylan backbone at the *O*-2 position.

Many cell wall polysaccharides may be acetylated to a different extent depending on species, tissues, and development state. In secondary walls, xylans are the main acetylated polysaccharide (Gille and Pauly 2012). Xylans are widely acetylated (40–60%) at the *O*-2 and *O*-3 positions of xylosyl residues in the polymer backbone (Scheller and Ulvskov 2010). Xylan acetylation is critical for secondary cell wall formation since a reduction in its level causes a decrease of its cellulose content and a dramatic effect on its structure (Yuan et al. 2016). Three different groups of proteins, RWA (reduced wall acetylation proteins), AXY9 (altered xyloglucan9), and several

TBL (trichome birefringence-like) proteins of the DUF231 family, are involved in xylan acetylation. RWAs are a family of proteins probably involved in the transport into the Golgi apparatus of acetyl-CoA, the donor of acetyl group for polysaccharide acetylation (Pawar et al. 2017; Rennie and Scheller 2014). In *rwa* mutants acetylation is decreased not only on xylan but also on multiple wall polysaccharides with growth phenotypes that range from mild to severe (Manabe et al. 2011). AXY9 mutation causes reduction in acetylation on both xyloglucan and xylan suggesting that AXY9 protein is part of the acetylation pathway acting downstream of the RWA proteins and upstream of polysaccharide acetyltransferases (Schultink et al. 2015). The TBL gene family is plant specific, and its function is related with cell walls (Bischoff et al. 2010). It contains a large number of genes, 46 members in *Arabidopsis*. The previous suggestion that TBL proteins might be involved in maintaining polysaccharide esterification has been confirmed at least for some of them that are acetyltransferase. So, TBL29 is an acetyltransferase specific for xylan, and it is not active against other wall polysaccharides (Xiong et al. 2013). Xylan acetylation in *2-O* and *3-O* positions was lower in *tbl29* mutant than in wild type, but the degree of acetylation of other wall polysaccharides was not affected (Urbanowicz et al. 2014; Yuan et al. 2013). Furthermore, TBL32 and TBL33 also are important for xylan acetylation in secondary walls of *Arabidopsis* (Yuan et al. 2016).

A relation between acetylation and glucuronic acid substitution has been found (Lee et al. 2014). Both substitutes may compete for the *O*-2 position of xylosyl residues. The *esk1 Arabidopsis* mutation specifically reduces xylan acetyltransferase activity and xylan acetylation at both *O*-2 and *O*-3 positions. In addition, *esk1* mutation increases the glucuronic acid substitution of xylan (Yuan et al. 2013). On the other hand, an increase in the acetylation of xylan has been found in the *gux/1/2/3* mutant which showed a complete loss of glucuronic acid substitution (Lee et al. 2014).

Xylan acetylation at regular intervals favored a stable interaction with cellulose leading to a cellulose-xylan network (Busse-Wicher et al. 2014). The nature of this interaction remains unclear. Simmons et al. (2016) showed that xylan has a threefold screw in solution, while it flattens into a twofold screw ribbon in presence of cellulose to bind to microfibrils. In the cellulose-deficient *irx3* mutant, xylan conformation reverts to the threefold screw conformation. Molecular dynamic simulations support that regular glycosyl substitutions favor the stabilization of twofold screw conformation of xylan increasing its affinity for cellulose microfibrils (Martínez-Abad et al. 2017).

Most xylan glucuronic acid side chains are methylated, about a 60% in the case of *Arabidopsis* (Zhong et al. 2005). Methyl groups are transferred from S-adenosylmethionine to glucuronosyl residues by GXM1, GXM2, and GXM3/GXMT1 (Lee et al. 2012; Urbanowicz et al. 2012), glucuronosyl methyl transferases of DUF579 family. A 75% reduction in methylation in the mutant gxm3/gxmt1 changed the lignin monomer composition, and it increased the glucuronoxylan released during thermal treatment (Urbanowicz et al. 2012). GXM1/2/3 are methyltransferases catalyzing 4-*O*-methylation of glucuronosyl residue side chains on xylan (Lee et al. 2012). The nucleotide sugar donor substrates for the biosynthesis

of xylan are UDP-Xyl, UDP-GlcA, and UDP-Ara*f*. They are predominantly synthesized in the cytosol from UDP-Glc. Thus, they need to be transported into the Golgi apparatus by nucleotide sugar transporters (NSTs). The cytosolic pool of UDP-Xyl has been proposed to be used directly for xylosylation reactions in the Golgi. On the other hand, UDP-Xyl synthesized into Golgi seems be used to produce UDP-Ara*p* (Zhao et al. 2018).

3.2 Mannans

Galactoglucomannan is the main hemicellulose in secondary walls of gymnosperm softwood, accounting for 20% of the walls compared to 5% in angiosperm hardwood (Stephen 1983; Willför et al. 2008). It is a linear polysaccharide constituted by (1–4)-linked β-D-glucose and β-D-mannose residues in a ratio of 1:1–4 (Capek et al. 2000; Lundqvist et al. 2002; Teleman et al. 2003). It also may include small amounts of α-D-galactose residues at *O*-2, *O*-3, and *O*-6 of some mannosyl and glucosyl residues. Moreover, acetyl groups may be located at all three free positions of mannosyl residues (Stephen 1983).

The glucomannan backbone is synthesized by enzymes of the cellulose synthase-like family (CSLA) (Fig. 4). Dhugga et al. (2004) first identified a cDNA encoding a mannan synthase that makes the β.1,4 mannan backbone of galactomannan in guar seed endosperm walls. Moreover, three *Arabidopsis* genes, *CSLA2, CSLA7*, and *CSLA9*, encode mannan synthase, and the same protein is able to use GDP-mannose or GDP-glucose as donor to synthesize a large homo- or heteropolymer. A similar mannan synthase activity for all plant *CSLA* genes has been postulated (Liepman et al. 2005).

In fenugreek seeds, the addition of α-galactosyl residues onto the C-6 position of mannose in the mannan backbone is catalyzed by a membrane-bound galactosyl transferase (MAGT) of family GT34 (Edwards et al. 1999; Yu et al. 2018). Two orthologs of fenugreek galactosyltransferase, named PtGT34A and PrGT34a, have been found in *Pinus taeda* and *P. radiata*.

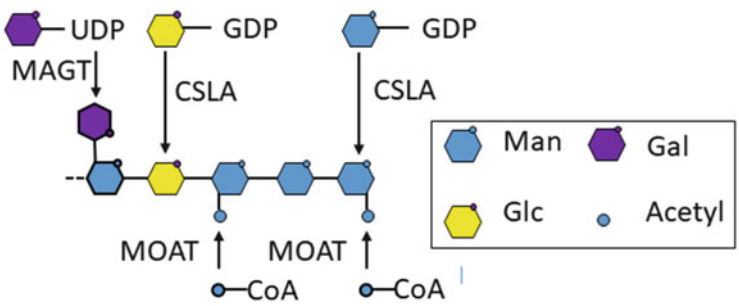

Fig. 4 Proteins involved in mannan biosynthesis. *MAGT* membrane-bound galactosyltransferase from GT34 family, *CSLA* mannan synthases, *MOAT* mannan acetyltransferase

In *Arabidopsis* and fenugreek, mannan synthesis-related proteins (MSR) have been shown to be necessary for mannan synthesis. However, their biochemical function remains unknown. They contain a DUF246 domain, which is plant specific. Several putative functions have been postulated: producing the primers necessary to initiate the mannan synthesis, synthesis of oligosaccharides linked to mannan synthases, or acting as interacting proteins to promote folding, stability, or activity of the complex (Wang et al. 2013a).

Recently, Zhong et al. (2018b) identified four DUF231 proteins in *Arabidopsis* (MOAT1–4) that specifically catalyze the acetylation of mannans. The abolition of acetylation by simultaneous RNA interference of the four MOAT genes in *Arabidopsis* did not show apparent changes in plant growth and development. The lack of phenotype may have been caused by the low content of glucomannan content in *Arabidopsis* walls. It would be necessary to study the abolition of mannan acetylation in gymnosperms with a high content of mannans in their walls.

Studies carried out with *Acetobacter xylinum* have shown strong affinity of glucomannan for cellulose (Iwata et al. 1998) and a reduction of crystallinity (Whitney et al. 1998). Glucomannan shows a twofold screw axis relatively flat and able to interact with cellulose microfibrils (Moreira and Filho 2008).

4 Lignin

The appearance of lignified secondary cell walls is the most important evolutionary event that allowed plants to conquer the terrestrial environment. Lignin is a very complex polyphenolic biopolymer that strengthens and waterproofs the secondary cell wall of specialized types of cells. It plays a key role in the adaptation of plants to terrestrial life. Although it is mainly deposited in cells of water-conducting and supportive tissues (Bonawitz and Chapple 2010), it also has an important role in other cell types, such as those of the seed coating, siliques (Barros et al. 2015), or anthers, giving it a key role in dehiscence and fertility (Borah and Kurana 2018). It also provides a defense against biotic and abiotic stresses.

Lignins are derived mainly from the monolignols *p*-coumaryl, coniferyl, and sinapyl alcohols, hydroxycinnamyl alcohol monomers which differ in their degree of methoxylation. When they are incorporated into the polymer, these monolignols form *p*-hydroxyphenyl (H), guaiacyl (G), and syringyl (S) units, respectively (Fig. 5). Lignin composition and the ratio of G, S, and H units vary not only among taxa but also among cell types, being influenced by the state of development and environmental factors and showing an unanticipated plasticity to accommodate changes in monomer composition (Boerjan et al. 2003; Sederoff et al. 1999). Despite these differences, gymnosperm (softwood) lignin is mostly composed of G units with low levels of H units, whereas the bulk of lignin in dicots angiosperms (hardwood) consists of G and S units with only traces of H units. In grasses, lignin also incorporates G and S units, but it has more H units than in dicots. In addition, C-lignin, a homopolymer of caffeyl alcohol, has been identified in the seed coats of

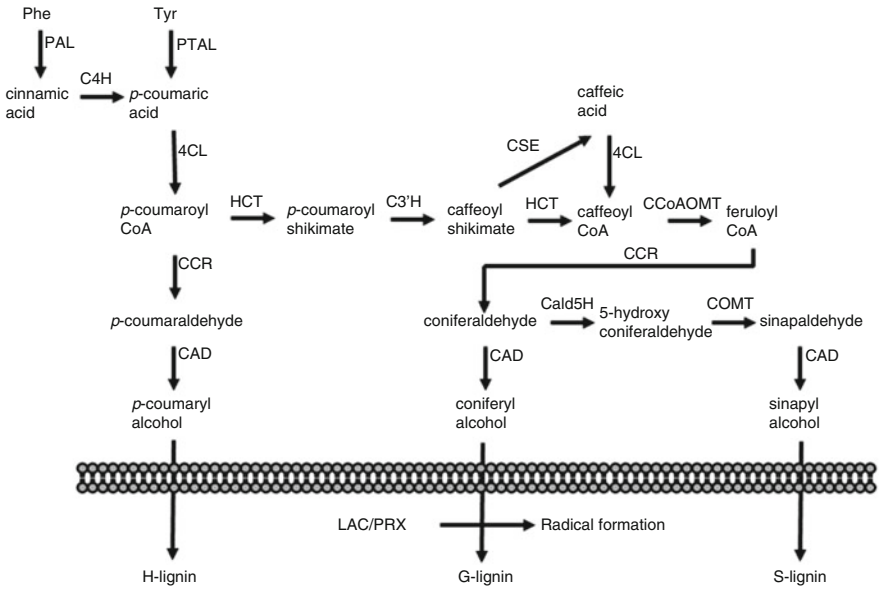

Fig. 5 Lignin biosynthesis pathway. *PAL* phenylalanine ammonia lyase, *PTAL* phenylalanine/tyrosine ammonia lyase, *C4H* cinnamate 4-hydroxylase, *4CL* hydroxycinnamate CoA ligase, *HCT* 4-hydroxycinnamoyl CoA: shikimate hydroxycinnamoyl transferase, *C3′H* *p*-coumaroyl shikimate 3′-hydroxylase, *CCoAOMT* caffeoyl-CoA 3-*O*-methyltransferase, *CCR* cinnamoyl CoA reductase, *CAld5H* coniferaldehyde 5-hydroxylase, *COMT* caffeic acid *O*-methyltransferase, *CAD* cinnamyl alcohol dehydrogenase, *CSE* caffeoyl shikimate esterase, *LAC* laccase, *PRX* peroxidase

monocot and dicot plants (Chen et al. 2012, 2013; Tobimatsu et al. 2013). Other phenylpropanoid components and monolignol conjugates are also incorporated into lignin (Karlen et al. 2017, 2018; Lan et al. 2016) adding complexity and structural diversity.

Instead of being exported to the apoplast, monolignols can also be stored as glycosylated conjugates in the vacuole (Dima et al. 2015; Le Roy et al. 2016). The presence of large amounts of coniferin, β-glucoside of coniferyl alcohol, during lignification in gymnosperms supports their participation in the process of lignification.

4.1 Biosynthesis

Lignin biosynthesis is a very complex process that can be divided into three stages: biosynthesis of lignin monomers in the cytosol, transport to the cell wall, and incorporation of monomers into the lignin polymer within the cell wall (Liu et al. 2018). The synthesis of lignin monomers (monolignols) starts with the general phenylpropanoid pathway. The amino acid phenylalanine derived from the

shikimate pathway in plastids is used as initial substrate. Phenylalanine is shuttled to the cytosol where it is deaminated by the phenylalanine ammonia-lyase (PAL) to produce cinnamic acid. *Arabidopsis* has four *PAL* genes, *PAL1* through *PAL4* (Raes et al. 2003). Monocots can also use tyrosine as a precursor (Higuchi et al. 1967). In a recent work, Barros et al. (2016) identified in *Brachypodium distachyon* a bifunctional ammonia-lyase (PTAL) and showed that L-Tyr is preferentially incorporated into the S units of lignin compared with L-Phe. Their results suggest that grass PTALs may explain the different compositions of grass cell walls. The next enzymatic steps leading to the synthesis of lignins involve successive hydroxylation reactions at one, two, or three positions of the aromatic ring, followed by methylation of one or two of these hydroxyl groups and reduction of the side-chain carboxyl to an alcohol group (Boerjan et al. 2003; Bonawitz and Chapple 2010).

Cinnamic acid is hydroxylated by cinnamate 4-hydroxylase (C4H) at C4 to form *p*-coumaric acid, with subsequent conversion to the corresponding CoA thioester by 4-coumaric acid CoA ligase (4CL). Here, the monolignol biosynthesis pathway diverges, and one branch is directed toward the synthesis of H-lignin through reduction of *p*-coumaroyl-CoA to *p*-coumaryl alcohol by cinnamoyl CoA reductase (CCR) and cinnamyl alcohol dehydrogenase (CAD). A second branch leads toward the synthesis of G- and S-lignins. In this case, the 3-hydroxylation needs the previous action of the 4-hydroxycinnamoyl-CoA shikimate hydroxycinnamoyl transferase (HCT) to convert the *p*-coumaroyl CoA into *p*-coumaroyl shikimate, substrate of the *p*-coumaroyl shikimate 3′ hydroxylase (C3′H) (Schoch et al. 2001) to produce caffeoyl shikimate. Then the reversible HCT catalyzes the transfer of the caffeoyl moiety of caffeoyl shikimate to CoA to form caffeoyl CoA which is successively methylated by caffeoyl-CoA *O*-methyl transferase (CCoAOMT) and reduced by CCR and CAD to feruloyl CoA, coniferaldehyde, and coniferyl alcohol, respectively, to form G-lignin. In addition, coniferyl aldehyde can be 5-hydroxylated and methylated by coniferaldehyde 5-hydroxylase (CAld5H, or ferulate 5-hydroxylase, F5H) and caffeic acid *O*-methyltransferase (COMT) to form sinapaldehyde which is reduced by CAD to sinapyl alcohol (S units).

The characterization in *Arabidopsis* of the *cse-1* and *cse-2* mutants of the *CSE* gene suggests the participation of the caffeoyl shikimate esterase (CSE) in the synthesis of phenylpropanoids. The analysis of the lignin composition in both mutants reveals an increase in the proportion of H units supporting the hypothesis that CSE is active before the divergence of the route toward the synthesis of the G and S units. Moreover, recombinant CSE uses very efficiently caffeoyl shikimate as substrate to form caffeic acid. Because 4CL catalyzes also the formation of CoA esters of caffeic acid, the activity of both enzymes can bypass the reversible HCT reaction (Chen et al. 2014; Vanholme et al. 2013). CSE orthologs have been identified in other dicot species such as *Vitis vinifera*, *Fragaria vesca*, *Cucumis sativus*, *Solanum lycopersicum*, *Medicago truncatula*, *Prunus persica*, *Eucalyptus grandis*, and *Populus trichocarpa*. They have also been found in some grasses like *Panicum virgatum*, but not in others such as *Brachypodium distachyon* and *Zea mays* (Ha et al. 2016). This suggests that the role of this pathway in lignification may not be universal.

The organization of the sequential enzymes of a metabolic pathway into a metabolon allows the efficient channeling of intermediate products from one to next and the control of the metabolic flow. In *Arabidopsis*, the successive hydroxylations of the aromatic ring require the presence of three cytochrome P450 monooxygenases, C4H, C3′H, and CAld5H (previously called F5H), which are membrane proteins anchored to the cytoplasmic surface of the ER. Previous studies suggested that these enzymes could interact with soluble enzymes in the same pathway to form a metabolon for substrate channeling (Achnine et al. 2004; Bassard et al. 2012). Moreover, in *Populus trichocarpa*, two C4H and one C3′H isozymes were shown to interact to form heteromeric membrane protein complexes (Chen et al. 2011). However, it has been found recently that C4H, C3′H, and CAld5H although located in close proximity on the ER membrane in vivo do not interact directly to form a complex (Gou et al. 2018). Instead, two membrane steroid-binding proteins (MSBP1 and MSBP2) located in the ER interact C4H, C3′H, and CAld5H. This suggests that MSBPs act as scaffold proteins to organize and stabilize the P450 enzyme complex and therefore control lignin biosynthesis.

Recently, studies with poplar have demonstrated interactions between CAD and CCR, soluble enzymes that catalyze the last steps of the synthesis of monolignols (Yan et al. 2018a). In this work using *PtrCAD1* y *PtrCCR2* RNAi transgenic plants, it was observed that in the transgenics with one gene silenced, the activity of the other enzyme decreased, which could be caused by the loss of the interaction between both proteins. Taken together, these results provide evidence supporting the formation of PtrCCR2/PtrCAD protein complexes in monolignol biosynthesis.

4.2 Transport of Monolignols

After being synthetized in the cytosol, monolignols must be transported to the apoplast where they are oxidized by enzymes, such as laccases and/or peroxidases, prior to be polymerized. However, the mechanism(s) to export monolignols from the cytosol are still unknown. Three possible routes for monomer transport across the cell membranes have been suggested: active transport via ABC transporters, passive diffusion, or diffusion facilitated by channels in the membrane (Perkins et al. 2019). The role of ATP-binding cassette transporters has been demonstrated in isolated vesicles of plasma membrane and tonoplast of *Arabidopsis* leaves and poplar roots (Miao and Liu 2010). These authors also demonstrated the existence of a selective transport through the membranes. While the vesicles of the plasma membrane transported preferentially monolignols, those of tonoplast preferred monolignol 4-*O*-glucosides, which implies the presence of different transporters. Yeast expressing the *Arabidopsis AtABCG29* exhibited a *p*-coumaryl alcohol transport activity. Moreover, the lignin in *abcg29* mutants has a reduced content not only in H but also in G and S units (Alejandro et al. 2012). Recently, the co-expression of four ABC transporters (*AtABCG29*, *AtABCG11*, *AtABCG22*, and *AtABCG36*), together with the transcription factor *MYB58* in *Arabidopsis* differentiating cultured

cells, has been reported, suggesting their participation in the lignification process (Takeuchi et al. 2018). However, in 2013, results of Tsuyama and coworkers, using membrane vesicles from xylem tissues of several tree species (angiosperms and gymnosperms), suggest that an H^+/coniferin antiporter present in tonoplast and endomembrane is involved in the transport of the monolignol glucosides (Tsuyama et al. 2013). Although some works support that mono- and dilignols can diffuse through the membranes (Boija et al. 2007), passive diffusion does not seem to play a role in the transport of monolignols toward the cell wall. Despite all the advances, how monolignols are deposited in the cell wall to be polymerized remains to be stablished.

4.3 Lignin Polymerization

Once exported to the apoplast, the first step in lignin polymerization entails the monolignol oxidation by two types of cell wall-localized enzymes, O_2-dependent laccases and H_2O_2-dependent peroxidases, to form monolignol radicals (Berthet et al. 2011; Shigeto and Tsutsumi 2016). After activation, the monomers are polymerized via a combinatorial radical coupling process (Tobimatsu and Schuetz 2019). This process is conditioned by the availability of the different monomers and the local matrix environment (Ralph et al. 2004). This mechanism allows the incorporation of different monomers in different combinations which account for the variability in the composition of the different types of lignin.

Although it is generally accepted that, unlike the biosynthesis of lignans, lignin polymerization takes place without the participation of dirigent proteins (DIRs), some studies suggest that these proteins have a role in directing the formation of specific chemical bonds during monolignol polymerization in specific cells (Barros et al. 2015; Davin and Lewis 2000; Tobimatsu and Schuetz 2019). In *Arabidopsis* roots, the DIR enhanced suberin 1 (ESB1) is specifically localized to the Casparian strip (Hosmani et al. 2013). Furthermore, the Casparian strip in the loss of function mutant *esb1* exhibited strong defects in lignin. These results support the role of ESB1 in the Casparian strip formation. Recently, a bioinformatic analysis of the DIRs present in the pear genome suggested the participation of PbDIR4 in lignin metabolism in fruits (Cheng et al. 2018).

4.4 Laccases

Plant laccases are members of a large family of multicopper oxidases (17 genes in *Arabidopsis thaliana*, 49 in *Populus trichocarpa*, and 29 in *Brachypodium distachyon*) (Barros et al. 2015; Lu et al. 2013; Wang et al. 2015) with broad substrate specificities and diverse expression patterns. Their involvement in lignification has been unambiguously established in *Arabidopsis lac4 lac11 lac17* triple

mutants (Zhao et al. 2013). The lignin deposition is almost undetectable causing a severe dwarf phenotype in these mutants. Furthermore, the expression of sugarcane *SofLAC* gene under the control of *AtLAC17* promoter in *atlac17* mutant led to the restoration of WT levels of lignin, although with different S:G ratio. Considering the different compositions of lignins, this could mean that in grasses, these enzymes have evolved to accommodate the different substrates available in their cell walls (Cesarino et al. 2013).

On the other hand, the identification of mutants for *AtlAC15* has provided evidence of its role in seed lignification, as well as in the elongation of *Arabidopsis* roots (Liang et al. 2006). Recently, Yan and coworkers have identified in cotton the *GhLACc15* gene, homologous to *AtLAC15*. Its overexpression in *Arabidopsis* increases lignification, increasing lignin G units, the G:S ratio, and resistance to *Verticillium*. Moreover, the silencing of the gene in cotton increases its susceptibility to the fungus (Yan et al. 2018b). Also, in *Brachypodium distachyon*, it has been demonstrated that the *BdLAC5* gene is involved in the culm lignification (Wang et al. 2015), whereas in corn, the role of two laccases, ZmLAC3 and ZmLAC5, in the lignin biosynthesis and lodging resistance under N-luxury conditions has been described (Sun et al. 2018).

4.5 Peroxidases

In plants, peroxidases (class III peroxidases, Prxs) are encoded by a large multigenic family, 73 genes in *Arabidopsis thaliana*, 93 genes in *Populus trichocarpa*, and 138 genes in *Oryza sativa* (Shigeto and Tsutsumi 2016) involved in a wide array of process. Together with laccases, they can catalyze the formation of the monolignol radicals required for lignin polymerization. However, the available data suggest that peroxidase and laccases do not have the same function and they do not function in a redundant route for lignin polymerization, at least in the vascular tissue of *Arabidopsis* (Zhao et al. 2013). The role of specific peroxidases in lignin polymerization has been demonstrated (Shigeto and Tsutsumi 2016). In tobacco, for example, the antisense suppression of *NtPrx60* led to a significant reduction in the level of lignin and a decrease of S and G units (Blee et al. 2003). Similar results have been obtained with the antisense suppression of an anionic peroxidase (*PrxA3A*) in hybrid aspen (Li et al. 2003). However, in both cases, few differences were observed in the growth of transgenic plants with respect to wild types. In *Arabidopsis*, several peroxidases have been suggested to have a role in the oxidation of monolignols. Knockout mutants of *AtPrx72* showed a reduction in lignin content and thinner secondary walls in interfascicular fibers, accompanied by slower growth (Fernández-Pérez et al. 2015; Herrero et al. 2013). Moreover, the overexpression of AtPrx37 under the control of CaMV 35S promoter resulted in a dwarf phenotype, along with an increase in the amount of esterified phenolics in the walls, suggesting a role for AtPRX37 in lignin deposition (Pedreira et al. 2011). In addition, a role in the formation of the Casparian strip has been shown for AtPrx64, probably in

conjunction with other peroxidases (Lee et al. 2013). On the other hand, in the *lac4 lac11 lac17* triple mutant, despite the severe reduction in lignin content and growth, the Casparian strip is not affected suggesting that laccases and peroxidases do not have redundant functions in lignification (Zhao et al. 2013). However, the role of both enzyme types in this process remains unclear.

5 Transcriptional Regulation of Secondary Cell Wall Synthesis

The transcriptional regulation of secondary cell wall synthesis is quite well understood, compared to regulation primary cell wall synthesis and many other physiological processes. At the center of the current model, based mostly on studies in *Arabidopsis*, are transcription factors (TFs) that act as "master switches," as their expression is sufficient and, in many cases, necessary to activate the complete program of secondary cell wall synthesis and deposition (Nakano et al. 2015). In *Arabidopsis*, the first layer of master switches is formed by ten TFs from the NAC family named VND1 to VND7, NST1, NST2, and NST3/SND1. These genes control the expression of a second layer formed by two redundant MYB master switches, MYB46 and MYB83. Constitutive expression of any of these transcription factors can lead to ectopic secondary cell walls in mesophyll, cortex, or epidermal cells (Kubo et al. 2005; McCarthy et al. 2009; Mitsuda et al. 2005, 2007; Zhong et al. 2006, 2007b; Zhou et al. 2014). The hierarchical organization of this network has facilitated the study of the regulation of secondary cell wall synthesis and could also facilitate the manipulation of this process for crop improvement as it appears to be conserved in other groups of angiosperms (Rao and Dixon 2018; Zhang et al. 2018a) (Fig. 6).

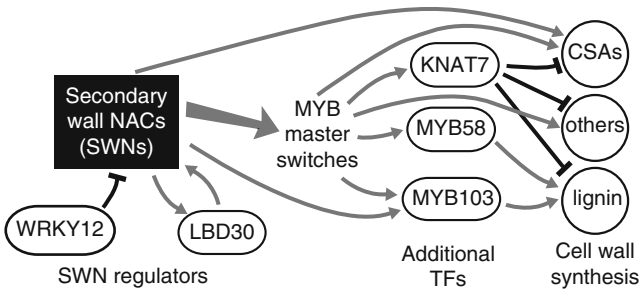

Fig. 6 Simplified regulatory network of secondary cell wall formation in *Arabidopsis*. Apart from the master switches, only a few selected transcription factors are included to show some examples of the connections that have been established

5.1 NAC Master Switches

The ten *Arabidopsis* NAC master switches, which have also been named secondary wall NACs (SWNs), form a distinct clade within the family (Nakano et al. 2015). Their closest homologs in *Arabidopsis* are a group of three TFs involved in the differentiation of root cap cells. Overexpression of any of them also causes ectopic secondary cell wall deposition, probably because they attach to similar binding sites as the SWNs (Bennett et al. 2010). Together, they form a larger clade that has been named VNS (for VND, NST/SND, and SMB related) and that includes a basal branch present in mosses and clubmosses (Nakano et al. 2015). In the moss *Physcomitrella patens*, these genes are involved in the differentiation of water-conducting hydroids, elongated cells that go through programmed cell death, as well as steroids, support cells with thickened walls (Xu et al. 2014a). Although both these cells lack lignified secondary cell walls, they are the functional precursors of the tracheary elements (TEs) and fibers of vascular plants and their developmental program seems to be the precursor for the transcriptional regulation of secondary cell wall deposition.

The function of SWNs as initiators of secondary cell wall deposition has been clearly stablished in several species other than *Arabidopsis*. In the gymnosperm *Pinus pinaster*, silencing of a secondary wall NAC resulted in the downregulation of a number of genes involved in secondary cell wall synthesis (Pascual et al. 2017). Constitutive overexpression of SWNs in poplar, tobacco, and the monocots *Brachypodium distachyon*, rice, or banana resulted in ectopic secondary cell walls, stablishing that NACs can also act as master switches in these species (Chai et al. 2015; Negi et al. 2015, 2016; Ohtani et al. 2011; Valdivia et al. 2013; Zhong et al. 2011b). Additionally, mutations, silencing, or dominant repression of SWNs from *Medicago truncatula*, rice, and maize resulted in defects in secondary cell walls (Xiao et al. 2018; Yoshida et al. 2013; Zhao et al. 2010). These results suggest that altering the expression of SWNs could be a useful tool to control secondary cell wall deposition in both gymnosperms and angiosperms. Overexpression of SWNs under promoters activated during secondary wall deposition has been shown to increase biomass in both *Arabidopsis* and poplar without deleterious effects on growth (Sakamoto et al. 2016; Yang et al. 2013). Similarly, dominant repression has been used to improve digestibility in tall fescue (Sato et al. 2018).

Secondary wall NACs in vascular plants can be divided in two main groups sometimes named VND and NST/SND like the respective *Arabidopsis* members. In *Arabidopsis*, VNDs are responsible for the deposition of secondary cell walls in tracheary elements. While VND6 and VND7 appear to have a larger effect, VND1 to VND5 are also able to activate many of the same targets (Kubo et al. 2005; Ohashi-Ito et al. 2010; Yamaguchi et al. 2011; Zhou et al. 2014). In contrast, NSTs are required for secondary cell wall deposition in fibers, the replum and endothecium of the siliques (NST1 and SND1/NST3), as well as the anther endothecium (NST1 and NST2), which is required for dehiscence (Mitsuda et al. 2005, 2007; Zhong et al. 2006, 2007a). This specialization of expression pattern does not seem to extend to

other species of angiosperms, as genes from both clades appear to be expressed indistinctly in fibers and tracheary elements of poplar, rice, maize, and switchgrass (Ohtani et al. 2011; Yoshida et al. 2013; Zhong et al. 2010a, 2011a, 2015). In poplar, while both clades are expressed indistinctly in secondary xylem, only VNDs appear to be expressed in primary xylem.

In *Arabidopsis*, the targets of both VND and NST/SND proteins show extensive overlap, as they all seem to recognize a similar binding site called SNBE, for secondary wall NAC binding element (Ohashi-Ito et al. 2010; Yamaguchi et al. 2011; Zhong et al. 2010c; Zhou et al. 2014). There is however a degree of specificity in the activation, as the induction of some targets varies strongly in response to different SWNs. There is less data from other species, and it is based mostly on transactivation assays, which show that the binding motif is conserved across species (Negi et al. 2017; Ohtani et al. 2011; Valdivia et al. 2013; Zhong et al. 2011a, b, 2015). It seems that in general VNDs are more effective than NSTs at activating genes involved in programmed cell death (Ohtani et al. 2011; Valdivia et al. 2013; Zhong et al. 2015). In some experiments, expression of *Arabidopsis* VNDs results in vessel-like discontinuous secondary cell walls, while NSTs induce uniformly thickened walls (Ohashi-Ito et al. 2010; Yamaguchi et al. 2011). However, *Arabidopsis* NSTs can also produce discontinuous walls when expressed in leaves, which suggests that additional factors are involved (Zhong et al. 2006). In other species, such as poplar or tobacco, overexpression of NSTs resulted in discontinuous cell walls, but these experiments were carried out in leaves (Ohtani et al. 2011; Valdivia et al. 2013). It is currently unclear what is the specific role of both clades in species other than *Arabidopsis*.

The activity or expression of SWNs has been shown to be modulated in *Arabidopsis* by interacting partners, by posttranslational modifications, and by a growing network of transcriptional repressors and activators (Endo et al. 2015; Hussey et al. 2011; Kawabe et al. 2018; Ohashi-Ito et al. 2018; Soyano et al. 2008; Sun et al. 2017; Taylor-Teeples et al. 2014; Wang et al. 2010; Wang and Dixon 2012; Yamaguchi et al. 2015; Yang et al. 2007, 2011). The regulatory network of SWNs appears to include positive feedback loops involving autoactivation and activation by other SWNs, as well as positive and negative feedback loops that include additional transcription factors like LBD15, LBD18, LBD30, SND2, MYB26, or MYB32. Among the upstream activators of SWNs, GATA12 and ANAC75 are both capable of inducing the deposition of ectopic secondary cell walls and could thus be considered additional master switches (Endo et al. 2015). Y1H experiments have identified E2Fc as a key upstream regulator of this network that acts as an inducer or repressor depending on concentration (Taylor-Teeples et al. 2014). The levels of different hormones also play an important role in the activation of these master switches, although the mechanisms are still unclear (Didi et al. 2015). Much less is known about SWN regulation in other species. Alternative splicing of an SWN resulting in a truncated form that acts as a dominant repressor seems to play a role in poplar (Zhao et al. 2014). Orthologs of the *Arabidopsis* SWN repressor WRKY12 appear to play a similar role in *Medicago*, *Populus*, maize, and switchgrass (Gallego-Giraldo et al. 2015; Wang et al. 2010; Yang et al. 2016). An

Arabidopsis TF called SND2 seems to participate in a positive feedback loop with SWNs, and orthologs in *Eucalyptus* and poplar could have a similar function (Hussey et al. 2011; Wang et al. 2013b). Finally, class III HD ZIP transcription factors which are involved in vascular patterning regulate the expression of SWNs in both *Arabidopsis* and *Populus* (Du et al. 2011; Endo et al. 2015).

5.2 MYB Master Switches

Among several hundreds of direct targets of SWNs identified in *Arabidopsis*, there are a large number of transcription factors, as well as some genes directly involved in cell wall synthesis such as cellulose synthases (Ohashi-Ito et al. 2010; Yamaguchi et al. 2011; Zhong et al. 2010c). Two of the direct targets are MYB46 and MYB83, two close homologs within the R2R3 MYB family which play a central role in the signaling network. Constitutive overexpression of either MYB83 or MYB46 results in ectopic secondary cell wall deposition in *Arabidopsis*, and the double mutant is unable to produce secondary walls (Ko et al. 2009; McCarthy et al. 2009; Zhong et al. 2007b). For this reason, these two genes have been considered a second layer of master switches under the direct control of secondary wall NACs. More than 100 genes were identified as direct targets of MYB46, which binds to a motif named SMRE for secondary wall MYB-responsive element (Kim et al. 2012, 2014; Zhong and Ye 2012). Among the direct targets are enzymes involved in the synthesis of lignin and hemicellulose, as well as the three cellulose synthases required for secondary cell wall deposition. It seems that activation by MYB master switches is strictly required for the correct expression of the cellulose synthases (Kim et al. 2013).

The central role of MYB master switches appear to be conserved in poplar where orthologs of MYB46 and MYB83 are capable of inducing deposition of ectopic secondary cell walls (Zhong et al. 2013). Orthologs from eucalyptus, pine, maize, rice, and switchgrass can bind SMRE motifs and complement *Arabidopsis* myb46 myb83 mutants, but their effect has not been demonstrated in the original species (Zhong et al. 2010b, 2011a, 2015). In addition, the orthologs from eucalyptus and pine have been shown to enhance secondary cell wall deposition in tobacco and, in the case of the pine ortholog, induce ectopic lignification (Goicoechea et al. 2005; Patzlaff et al. 2003). However, it is possible that the central role of the MYB46/83 clade is not be conserved in all other species, as suggested by the low levels of expression of their orthologs in cotton xylem (MacMillan et al. 2017). Direct activation of MYB46/83 orthologs by SWNs has been demonstrated in vitro for poplar, maize, and rice, but not for *Brachypodium* (Valdivia et al. 2013; Zhong et al. 2010a, 2011a).

5.3 Additional Transcription Factors

Among the closest homologs of MYB46 and MYB83 in *Arabidopsis*, there are a number of TFs that are also involved in the regulation of secondary cell wall deposition, such as MYB26, MYB61, MYB85, or MYB103 (Newman et al. 2004; Yang et al. 2007; Zhong et al. 2008). MYB26 can cause ectopic deposition of secondary cell walls through a feedback loop with SWNs and could thus be considered a MYB master switch, but its function in vivo seems limited to the endothecium. Both MYB61 and MYB85 cause ectopic lignification, while MYB103 overexpression only increases the thickness of secondary cell walls. In addition, mutants in MYB103 show a strong reduction in syringyl lignin (Öhman et al. 2012). Genes from this larger clade also appear to have similar roles in spruce, poplar, and *Brachypodium*, as shown by overexpression or dominant repression constructs (Bomal et al. 2008; Handakumbura et al. 2018; Hirano et al. 2013; Noda et al. 2015; Tian et al. 2013; Xu et al. 2017). However, in some cases, the role is not identical. The rice orthologs of both MYB61 and MYB103 appear to have a more critical role and to directly regulate cellulose synthesis (Hirano et al. 2013; Huang et al. 2015; Yang et al. 2014; Ye et al. 2015).

Additional *Arabidopsis* R2R3 MYBs that are more distantly related to AtMYB46 have also been linked to the regulation of secondary cell wall deposition. The close homologs MYB58 and MYB63, which are activated by the MYB master switches, appear to be specifically involved in the synthesis of lignin and are capable of inducing ectopic lignification (Zhou et al. 2009). This function appears to be conserved in their rice and sorghum orthologs (Hirano et al. 2013; Scully et al. 2015). However, the grass orthologs also seem to regulate xylan and cellulose synthesis, possibly due to the presence of binding motifs in the relevant promoters that are missing in *Arabidopsis*. An additional clade of *Arabidopsis* R2R3 MYBs with a possible role in secondary cell wall regulation is that formed by MYB4, MYB32, and other closely related TFs. These genes regulate the phenylpropanoid pathway and have been proposed to repress SWNs in a negative feedback loop, although their effect on secondary walls has not been clearly stablished (Fornalé et al. 2014; Jin et al. 2000; Wang et al. 2011). On the other hand, several maize orthologs strongly repress lignin synthesis in *Arabidopsis*, and in vitro studies and other experiments suggest that this repression also takes place in maize and other grasses (Sonbol et al. 2009; Vélez-Bermúdez et al. 2015). A similar mechanism appears to work in loquat and eucalyptus (Legay et al. 2010; Xu et al. 2014b). In addition, overexpression of a poplar ortholog of MYB4 resulted in repression of cellulose, xylan, and lignin biosynthesis genes (Yang et al. 2017). Another group of closely related genes activated by SWNs and with probable roles in promoting secondary cell wall deposition includes MYB52, MYB54, and MYB69 (Cassan-Wang et al. 2013b; Zhong et al. 2008). Recently, MYB15 has been shown to be necessary for defense-activated lignification (Chezem et al. 2017). Additionally, MYB75 seems to be a repressor of secondary cell wall formation (Bhargava et al. 2010).

Genes from other families also play important roles in the regulation of secondary cell wall deposition in *Arabidopsis*. Class I KNOX act as negative regulators of the biosynthesis of lignin and possibly other cell wall components in *Arabidopsis*, peach, poplar, maize tobacco, and switchgrass (Du et al. 2009; Mele et al. 2003; Townsley et al. 2013; Wuddineh et al. 2016). Some of the effects of this seems to be related to changes in gibberellin signaling. Another important component of the network is KNAT7, a class II KNOX gene that is a direct target of NAC and MYB master switches and represses secondary cell wall formation in fibers (Bhargava et al. 2010; Ko et al. 2009; Li et al. 2011, 2012b; Liu et al. 2014; Zhong et al. 2008). Its mechanism of action involves the formation of protein complexes with additional repressors such as OFP4, MYB75, and BLH6. The poplar ortholog of KNAT7 can rescue the *Arabidopsis* knat7 mutant, and this regulatory module of interacting repressors also appears to be conserved in rice (Li et al. 2012b; Schmitz et al. 2015). However, overexpression of one of the possible rice BLH6 orthologs resulted in ectopic secondary cell wall deposition, a result that is opposite to that observed in *Arabidopsis* (Hirano et al. 2013; Liu et al. 2014). It seems clear that in these lower levels of the regulatory network, there are important differences between species.

6 Conclusion

A plenty of studies on secondary cell wall biosynthesis have been published in the last two decades. Most genes involved in the biosynthesis of secondary wall components, including cellulose, xylan, glucomannan, and lignin, have been identified. Recently, Purushotham et al. (2016) demonstrated that reconstituted PptCESA8 is not only enough to synthesize in vitro glucan chains but also to bundle individual chains into cellulose microfibrils. However, the biochemical characterization of the proteins encoded by many of the discovered genes is generally lacking. Therefore, an effort will be necessary to reveal their role in the construction of secondary walls.

The complex network of transcription factors involved in the regulation of the synthesis and deposition of secondary cell walls is likely a reflection of the need to fine-tune cell wall composition in different cell types as well as to adjust this process to the availability of resources, to the demands of other metabolic pathways, or to biotic and abiotic stresses. Achieving a complete picture of the network of transcription factors will be necessary to understand the regulation of secondary wall formation in the context of the whole plant. This will greatly facilitate the modification of plant biomass, giving us the capacity to design walls fit for different purposes, such us better forage digestibility, higher saccharification, etc.

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Stress-Induced Microspore Embryogenesis in Crop Plants: Cell Totipotency Acquisition and Embryo Development

Pilar S. Testillano

Contents

Abstract Stress-induced microspore embryogenesis is an in vitro system in which the haploid microspore is reprogrammed by the application of stress treatments and, as a totipotent cell, enters into an embryogenesis pathway, producing double-haploid (DH) embryos and plants. DHs are important biotechnological tools in plant breeding programs, widely used by companies, as a source of new genetic variability, fixed in complete homozygous plants in only one generation step. However, their applications are limited in many crop and forest species due to low or null efficiency. Microspore embryogenesis is also a convenient system to analyze the cellular processes underlying cell reprogramming, totipotency acquisition, and embryogenesis. In the last years, investigation on stress-induced microspore embryogenesis has unveiled the involvement of several cellular processes like autophagy, cell death, epigenetic modifications, cell wall remodeling and auxin, whose role is not yet completely elucidated. In the present chapter, we review recent findings on the determinant factors that underlie stress-induced microspore embryogenesis induction and progression. These findings have provided new insights into the regulating mechanisms of microspore embryogenesis initiation and progression, opening up new intervention pathways through pharmacological treatments with small bioactive

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P. S. Testillano (✉)
Pollen Biotechnology of Crop Plants Group, Biological Research Center, CIB-CSIC, Madrid, Spain
e-mail: testillano@cib.csic.es

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compounds (autophagy, epigenetic, and auxin modulators), very promising to enhance in vitro embryo production yield for breeding programs of crop and forest species, especially for those recalcitrant.

Keywords AGPs, Autophagy, Auxin, *Brassica napus*, Cell death, Cell wall, DNA methylation, Epigenetics, Histone modifications, *Hordeum vulgare*, In vitro microspore culture, Pectins, *Quercus suber*, Stress response

1 Introduction

In higher plants, the haploid microspore, originated by meiosis in the anther, follows the gametophytic program to form the mature pollen that will be released for pollination. In vitro, upon the application of a stress treatment, the microspore can be deviated from its developmental program toward an embryogenesis pathway leading to embryo formation. After induction, a fraction of the microspore population of the culture, the responsive microspores, abandon their developmental program, are reprogrammed, and acquire totipotency and embryogenic competence (Bárány et al. 2005; Verdeil et al. 2007), while other microspores die (Fig. 1). This process, named stress-induced microspore embryogenesis, represents an important tool in plant breeding to obtain double-haploid plants, source of new genetic variability, fixed in complete homozygous plants in only one generation step (Maluszynski et al. 2003; Murovec and Bohanec 2012).

Stress-induced microspore embryogenesis is also a convenient model system to analyze the cellular processes underlying cell reprogramming, totipotency, and embryogenesis, as well as the switch to proliferation from plant differentiating cells (Testillano et al. 2005). The correct progression of the process depends on many factors which limit its efficiency, with several bottlenecks at defined developmental stages. This in vitro system is widely used by plant nursery and seed companies in their breeding programs, but it still presents low and even null efficiency in many crop and forest species. The occurrence of cell death and the low rates of reprogramming efficiency are major factors that highly reduce the process yield, at their initial stages. A better understanding of the processes involved in the induction will help to identify new targets and design new strategies to improve the efficiency of in vitro embryogenesis systems, even in recalcitrant species.

Most basic studies on microspore embryogenesis have been conducted in two model crop species, the monocot *Hordeum vulgare*, barley, and the dicot *Brassica napus*, rapeseed, species in which efficient and well-established in vitro systems of stress-induced microspore embryogenesis have been developed (Fig. 2), through isolated microspores cultures (Pechan and Keller 1988; Prem et al. 2012; Rodríguez-Serrano et al. 2012). The model plant *Arabidopsis thaliana* has not been used for the

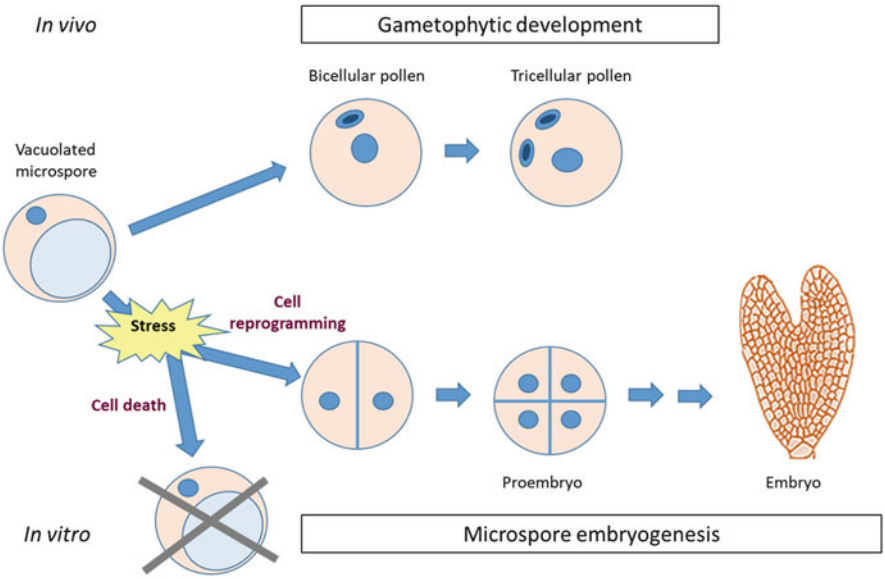

Fig. 1 Schematic representation of microspore embryogenesis. The process is initiated at the stage of vacuolated microspore and is induced by stress in vitro; responsive microspores are reprogrammed and divide to produce proembryos and later embryos. For comparison, it is represented the gametophytic developmental pathway that occurs in vivo, where the microspore divides asymmetrically to produce bicellular pollen and later tricellular pollen

study of microspore embryogenesis due to its recalcitrancy to the induction. In many other species, including crops and trees, the process is induced through anther cultures (Maluszynski et al. 2003). Moreover, production of double-haploid plants from anther cultures is especially useful for regeneration and breeding of fruit and forest trees, since the long regeneration time and strong inbreeding depression of these species make the traditional breeding methods impractical. In cork oak (*Quercus suber* L.), protocols for production of haploid embryos from anther cultures have been reported and later optimized (Bueno et al. 1997; Testillano et al. 2018a). Among fruit trees, *Citrus* is one of the best examples of successful regeneration of double haploids through anther cultures (Germaná 2009). Changes in various cell activities and modifications of the structural organization of subcellular compartments have been reported as accompanying the cell reprogramming process in some herbaceous and woody species (Bárány et al. 2005; Seguí-Simarro et al. 2006; Testillano et al. 2000, 2002; Solís et al. 2008). In the last years, several studies have reported the characterization of stress-induced microspore embryogenesis in vitro systems in forest and fruit trees, finding some common features in the developmental pattern with model species, as well as specific features of woody species (Rodríguez-Sanz et al. 2014a; Chiancone et al. 2015; Testillano et al. 2018a, b).

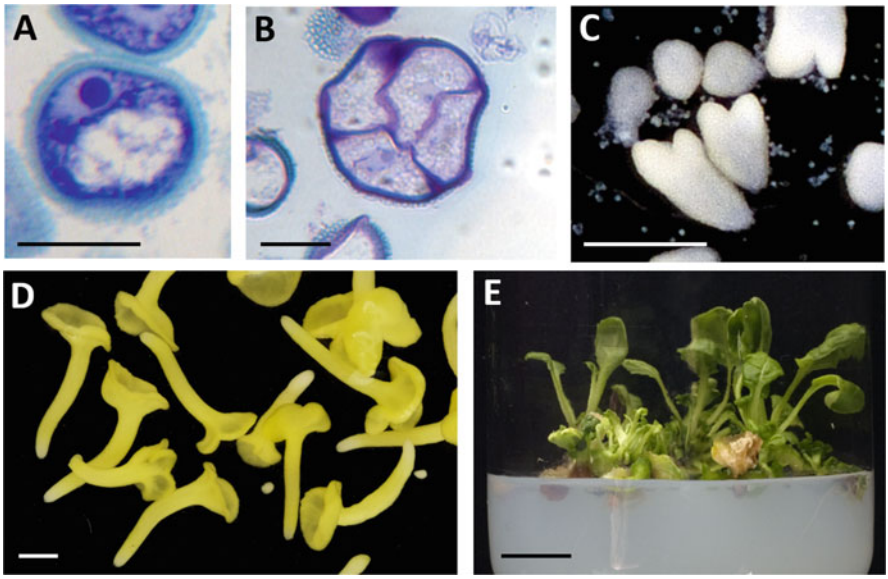

Fig. 2 Main stages of microspore embryogenesis in *Brassica napus*. (**a**) Vacuolated microspore at the beginning of culture. (**b**) Proembryo of a few cells, formed around 4 days after induction. (**c**) Globular, heart-shaped, and torpedo embryos. (**d**) Cotyledonary embryos formed in 30-day cultures. (**e**) In vitro plantlets regenerated from microspore-derived embryos. (**a**, **b**) Semithin sections stained by toluidine blue, bright field microscopy. (**c**, **d**) Details of in vitro cultures observed under stereomicroscope. Bars (**a**, **b**) 20 μm, (**c**, **d**) 1 mm, (**e**) 10 mm

Advances in the knowledge of the regulating mechanisms of microspore embryogenesis induction have been hampered by the difficulty of dissecting the early stages of the process by biochemical and molecular techniques, as well as by the limitation in applying genetic approaches to the embryogenesis-responsive genotypes. In isolated microspore cultures, after induction, a high number of nonresponsive microspores coexist with a relatively low number of responsive microspores and early proembryos that cannot be separated in culture samples at this early stage. In this sense, cellular approaches using in situ molecular identification techniques have shown important advantages and a high potential to analyze the process, by localizing key molecules and differential gene expression in embryogenic structures from the very initial stage of 2–3 cells, structures that can be distinguished from non-embryogenic ones by advanced imaging microscopy technologies (Testillano and Risueño 2009; Rodríguez-Sanz et al. 2015; Testillano and Rodríguez 2012).

In the last years, investigation on the regulation of stress-induced microspore embryogenesis has provided evidence of the involvement of several cellular processes whose role is not yet completely elucidated. In the present chapter, we review recent findings on the determinant factors that underlie stress-induced microspore embryogenesis induction and progression, with special attention to the role of autophagy, cell death, chromatin modifications, auxin, and cell wall remodeling.

Recent reports, using modern cell biology approaches, have proposed new strategies to improve microspore embryogenesis efficiency by pharmacological treatments with small molecules that are modulators of the involved processes (Solís et al. 2015; Berenguer et al. 2017a; Bárány et al. 2018; Pérez-Pérez et al. 2018a), opening up a completely new intervention pathway to increase the in vitro production of embryos and double-haploid plants in crop species, especially those recalcitrant, for breeding programs.

2 Stress-Induced Autophagy and Programmed Cell Death

In microspore embryogenesis systems, after the application of stress treatment to induce reprogramming, many cells die, limiting the efficiency of the process. Autophagy is a universal degradation pathway in all eukaryotes, including plants, that recycles cell materials upon stress conditions or during specific developmental processes. Together with a pro-survival role, autophagy has also been reported as cell death initiator and/or executioner (Masclaux-Daubresse et al. 2017; Hofius et al. 2017; Minina et al. 2013, 2014). Activation of autophagy involves induction of AuTophaGy-related *ATG* genes and activation of specific proteases (Avin-Wittenberg et al. 2018).

Several studies have shown that the inductive stress for microspore embryogenesis also lead to oxidative stress with production of reactive oxygen species (Zur et al. 2009; Rodríguez-Serrano et al. 2012). A recent report has demonstrated the activation of autophagy after the inductive stress of microspore embryogenesis in barley (Bárány et al. 2018). Autophagic structures, identified by specific ATG8 and ATG5 labelling and expression of *ATG6* and *ATG8a* genes, increased in microspore cultures, together with cell death levels, after stress (Fig. 3a). Autophagy inhibitors, like 3MA and concanamycin A, reduced cell death and increased microspore reprogramming efficiency, suggesting a role for autophagy in promoting cell death in this system. Among the numerous proteases that regulate plant cell homeostasis, many reports have provided evidence of the close connections between proteases and cell death processes (van der Hoorn and Jones 2004; van der Hoorn and Rivas 2018). In plants, Papain-like C1A Cys-Proteases, cathepsins, are the most abundant enzymes with proteolytic activity, with a role in plant senescence, PCD events, and proteolysis mediated by abiotic stress (Diaz-Mendoza et al. 2016; Velasco-Arroyo et al. 2016). Recent studies have shown the induction of expression of several cathepsin genes (*HvPap1*, *HvPap6*, and *HvPap12*), as well as proteolytic activity cathepsin B-, L- and H-like after the inductive stress and during the initial stages of microspore embryogenesis, in barley (Bárány et al. 2018). These proteases have been localized in small cytoplasmic spots of various sizes, probably corresponding to vesicles, lysosomal-like organelles, and small vacuoles of stress-treated microspores, a localization pattern that resembles that of autophagy structures. Moreover, when microspores were treated with E-64, which inhibits intracellular cysteine proteases, the levels of cell death decreased, suggesting the involvement of these proteases in cell death of microspore cultures of barley (Bárány et al. 2018).

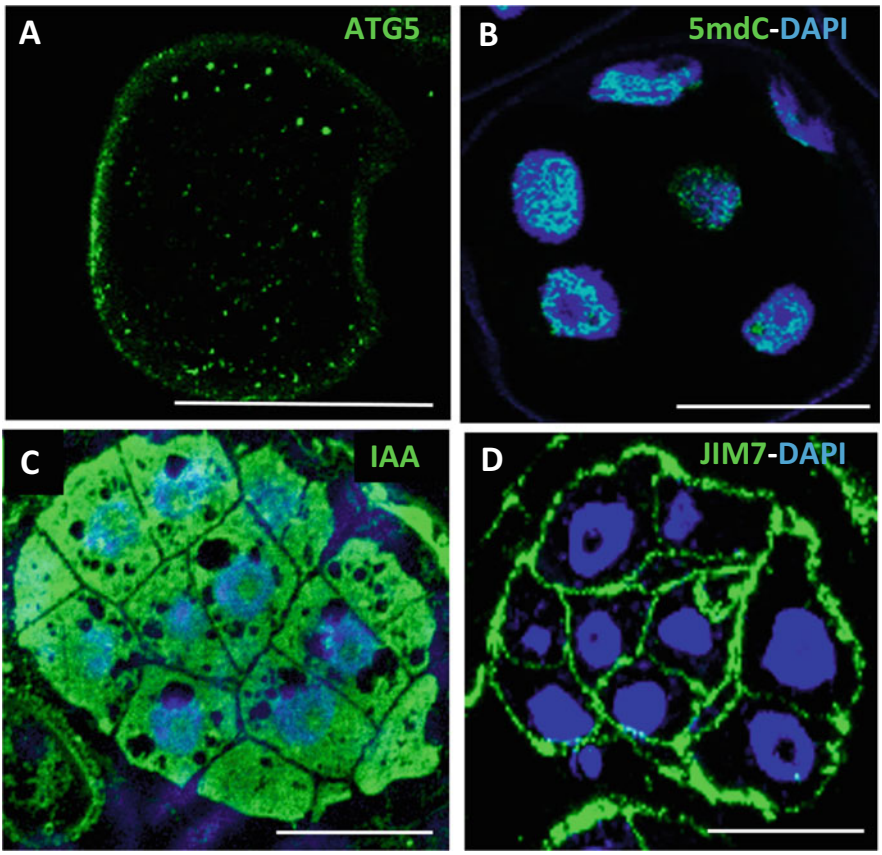

Fig. 3 Localization of key determinant molecules of microspore embryogenesis initiation. (**a**) ATG5 labelling showing numerous autophagosomes in cytoplasm of a vacuolate microspore after the inductive stress, in barley. (**b**) 5mdC labelling showing the distribution of methylated DNA over nuclei of an early proembryo of barley. (**c**) High accumulation of auxin (IAA) in cells of a proembryo of rapeseed. (**d**) Localization of esterified pectins by JIM7 antibody on cell walls of proembryo cells, in rapeseed. Confocal merged images of immunofluorescence (green signal) and DAPI staining (blue) of nuclei. Bars = 20 μm

Findings have revealed a role for autophagy in stress-induced cell death during microspore embryogenesis, with the participation of cathepsins, not only in barley but also in rapeseed (Berenguer et al. 2017b; Pérez-Pérez et al. 2018a), opening up new possibilities to enhance microspore embryogenesis efficiency with autophagy and/or protease modulators.

3 Epigenetic Reprogramming and Chromatin Modifications

Epigenetic modifications, mainly DNA methylation and histone methylation and acetylation, are key factors contributing to the functional status of chromatin that regulate gene expression during cell proliferation and differentiation, in both animals and plants (Kouzarides 2007). The mechanisms underlying cell reprogramming are thought to involve genome-wide changes in chromatin structure and gene expression. Defined changes in nuclear domains have been reported to accompany microspore embryogenesis induction (Testillano et al. 2000, 2005; Seguí-Simarro et al. 2006, 2011). Recent studies have indicated an epigenetic reprogramming during plant in vitro morphogenic processes (Miguel and Marum 2011) and during microspore embryogenesis (Solís et al. 2012; El-Tantawy et al. 2014).

This epigenetic reprogramming involves a global DNA methylation decrease with the activation of cell proliferation in early proembryos (Fig. 3b), as revealed by 5mdC immunofluorescence and quantification of global DNA methylation, and a subsequent DNA methylation increase with embryo differentiation, with similar profiles in several species like *Brassica napus* (Testillano et al. 2013; Solís et al. 2012), *Hordeum vulgare* (El-Tantawy et al. 2014), *Quercus suber* (Rodríguez-Sanz et al. 2014a), and *Quercus alba* (Corredoira et al. 2017). Moreover, in *B. napus*, the changes in the level and distribution of global DNA methylation during microspore embryogenesis induction and progression highly correlate with the expression patterns of the DNA methyltransferase *MET1*, suggesting the involvement of this enzyme in the process (Solís et al. 2012). Treatments with the DNA demethylating agent 5-Azacytidine (AzaC) on microspore in vitro cultures increased embryogenesis induction while they impaired subsequent progression, indicating that DNA hypomethylation favors microspore reprogramming, totipotency acquisition, and embryogenesis initiation, while embryo differentiation requires de novo DNA methylation (Solís et al. 2015).

Histone methylation and acetylation play an essential role in diverse biological processes ranging from transcriptional regulation to heterochromatin formation. Depending on which lysine residues are methylated and the degree of methylation, histone methylation is associated with both transcriptionally silenced and active chromatin domains. In general, H3K9 and H3K27 methylation is associated with silenced regions (Liu et al. 2010). Recently, it has been proved that microspore reprogramming and initiation of embryogenesis are associated with low levels of H3K9 methylation, correlating with gene expression profiles of the *BnHKMT SUVR4-like* histone methyl transferase and the *BnLSD1-like* histone demethylase. Moreover, pioneer analyses have reported the effects of the small molecule BIX-01294 for the first time in plants, revealing that it inhibited H3K9 methylation and promoted microspore reprogramming and embryogenesis initiation, in rapeseed and barley (Berenguer et al. 2017b).

Acetylation of histone lysines is characteristic of actively transcribed genes (Xu et al. 2005; Earley et al. 2007). N-terminal lysine residues of histones H3 and

H4 are acetylated by different histone acetyltransferases (HATs), enzymes which contribute to many plant development and adaptation processes, in combined action with histone deacetylases (HDACs) (Xu et al. 2005; Liu et al. 2012). Several reports have provided evidence to histone acetylation as modulator of cell totipotent state and cell reprogramming, during microspore embryogenesis (Li et al. 2014; Rodríguez-Sanz et al. 2014b). In *B. napus*, the expression profile of *BnHAT* correlates with the distribution pattern of acetylated histones, which show high levels of H3Ac and H4Ac in the vacuolated microspore, a totipotent cell with capacity of reprogramming and reentry into the cell cycle upon induction (Rodríguez-Sanz et al. 2014b).

4 The Role of Endogenous Auxin

Despite the work performed in recent decades, the effects of plant hormones in embryogenesis initiation and embryo development in established in vitro cultures are not well understood. In contrast to many somatic embryogenesis systems which are induced by exogenous hormone treatments, microspore embryogenesis is mostly induced by stress, which can trigger the response of endogenous hormones to determine new developmental cell fates. Auxins, and particularly indole-acetic acid (IAA), are major plant growth regulators involved in many plant developmental processes (Moreno-Risueño et al. 2010; Petrasek and Friml 2009), including embryogenesis where IAA biosynthesis increases throughout embryo development until early maturation. Many aspects of auxin action depend on its local biosynthesis and differential distribution within plant tissues, mainly regulated by its directional transport between cells (Petrasek and Friml 2009). During embryogenesis, redistribution of the efflux carrier proteins (PINs) lead to local increases of endogenous levels of auxins. It has been reported, in somatic embryogenesis, that local auxin accumulations are required to acquire embryogenic competence in specific domains of the explants (Elhiti and Stasolla 2011).

Studies in microspore embryogenesis of *B. napus* and *Q. suber* have shown that auxin accumulations were present in embryo cells from the very early stages (Fig. 3c) (Rodríguez-Sanz et al. 2015; Prem et al. 2012). Moreover, de novo auxin biosynthesis and upregulation of *BnTAA1* and *BnPIN1* (auxin biosynthesis and efflux transport genes) have been reported after microspore embryogenesis induction, as well as the blocking of the process with the inhibitors of auxin action and transport, PCIB and NPA (Rodríguez-Sanz et al. 2015). In contrast, during microsporogenesis, auxin levels and *TAA1* and *PIN1*-like gene expression are high in microspores at initial stages of development, while they progressively decreased during gametogenesis, in pollen grains (Pérez-Pérez et al. 2017). These findings indicate that auxin dynamics change with microspore reprogramming and auxin biosynthesis, activity, and transport are required for microspore embryogenesis initiation.

5 Cell Wall Remodeling: Pectin Methyl Esterases and AGPs Involvement

Modifications in cell wall components and pectin residues have been reported as being crucial for initiating cell responses in relation to cell fate and development. Pectins are major components of the primary plant cell walls; they are secreted into the cell wall in a highly methylesterified form and can be de-esterified in muro by pectin methylesterases or PMEs (Pelloux et al. 2007). The methylesterification of pectins changes significantly during plant growth and development (Willats et al. 2001, 2006). Changes in the methylesterification status of pectins have been related to the cell wall remodeling that occurs during diverse plant developmental processes. Recent reports have indicated that changes in cell wall mechanics controlled by the esterification/de-esterification status of pectins, mediated by PMEs and pectin methyl esterase inhibitors (PMEIs), underlie organogenesis initiation, early embryo growth, and embryogenesis progression. Nevertheless, the functional meaning of pectin-related cell wall remodeling in different cell types and processes still remains unclear.

Several studies have shown that the change of developmental program of the microspore involves changes in pectin esterification patterns which are associated with proliferation and differentiation events, in *Capsicum annuum*, *B. napus*, and *Q. suber* (Bárány et al. 2010; Rodríguez-Sanz et al. 2014a; Solís et al. 2016). In microspore embryogenesis, highly esterified pectins are characteristic features of early proembryo cell walls (Fig. 3d). In rapeseed, these changes in pectin esterification correlate with expression of *BnPME1*, indicating pectin-related modifications in the cell wall during microspore embryogenesis and suggesting a role of pectin esterification and cell wall configuration in microspore totipotency, embryogenesis induction, and progression (Solís et al. 2016). In other somatic embryogenesis systems, induced from leaves and immature zygotic embryos, embryogenic cell masses and early embryo cells showed high levels of esterified pectins, while in advanced embryos, differentiating cells exhibited walls rich in de-esterified pectins (Corredoira et al. 2017; Pérez-Pérez et al. 2018b). The findings indicate a role for PME and PMEI activities in the cell wall remodeling associated with microspore embryogenesis initiation and progression.

Arabinogalactan proteins (AGPs) are highly glycosylated hydroxyproline-rich proteins that are present in cell walls, plasma membranes, and extracellular secretions (Seifert and Roberts 2007). Increasing evidence has linked AGPs to many processes involved in plant growth and development, including somatic embryogenesis (Thompson and Knox 1998; Chapman et al. 2000; van Hengel et al. 2001). On the other hand, exogenous AGPs are known to affect somatic embryogenesis in different ways (Portillo et al. 2012), namely, as stimulating factors for embryogenesis induction (Yuan et al. 2012). However, less is known on the possible function of endogenous AGPs in plant cell reprogramming, totipotency, and embryogenesis induction.

The presence of secreted endogenous AGPs in maize microspore and zygote cultures has been reported to be a stimulating factor for embryo development (Borderies et al. 2004). Studies in *B. napus* microspore embryogenesis have provided evidence of the specific association of AGPs with the newly formed walls of 2–4 cell stage embryos, as well as the upregulation of *BnAGP Sta39-4* gene after embryogenesis induction (El-Tantawy et al. 2013). The addition of Yariv reagent, which specifically blocks AGPs, to microspore embryogenesis cultures, disturbed microspore embryogenesis initiation in a concentration-dependent manner (Tang et al. 2006). Taken together, these findings suggest the involvement of AGPs in the first embryogenic divisions of the microspore. Further work will be needed to shed light on the precise mechanisms of AGP action in this process.

6 Concluding Remarks

Investigation on stress-induced microspore embryogenesis of the last few years has unveiled key cellular processes involved in the induction and the control of plant cell reprogramming, totipotency, and embryogenic competence acquisition. Two major factors that highly affect the efficiency of stress-induced microspore embryogenesis are the occurrence of cell death and the low level of cell reprogramming. Early cell death events in microspore embryogenesis cultures are mainly due to the inductive stress, with participation of autophagy and several proteases. Cell reprogramming and totipotency acquisition of responsive microspores are regulated by epigenetic and hormonal mechanisms.

Several key factors have been identified as embryogenic markers, since they are differentially induced in embryogenic cells and their inhibition impairs embryogenesis initiation; they are hypomethylation of DNA, H3K9 demethylation, endogenous auxin accumulation, high esterification of pectins, and induction of AGPs expression. All these processes may be interconnected in the regulation of cell reprogramming and embryogenesis initiation. Increasing evidence has revealed the involvement of phytohormones, including auxin, in signaling processes of chromatin remodeling by epigenetic mechanisms to activate specific gene expression programs of development. Auxin has been reported to control cell wall remodeling during organogenesis initiation, by a reduction of cell wall stiffness that requires demethylesterification of pectins (Braybrook and Peaucelle 2013). Further investigations will be required to analyze the possible role of auxin and other hormones in pectin-related cell wall remodeling and epigenetic reprogramming during stress-induced microspore embryogenesis.

The recent reports on pharmacological treatments with autophagy and epigenetic modulators are very promising to enhance in vitro embryo production yield. Taken together, these findings are paving the way of new biotechnological strategies that will increase the efficiency of in vitro microspore embryogenesis systems by reducing cell death levels and enhancing cell reprogramming, totipotency, and embryogenesis initiation for breeding and conservation programs, even in recalcitrant crops.

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Potential of Microalgae Biomass for the Sustainable Production of Bio-commodities

Giorgio Perin and Tomas Morosinotto

Contents

Abstract Human activities are causing major negative environmental impacts, and the development of sustainable processes for production of commodities is a major urgency. Plant biomass represents a valuable alternative to produce energy and materials, but exploiting present crops for commodities production would however require massive resources (i.e. land, water and nutrients), raising serious sustainability concerns. In addition to efforts to improve plant, land and resource use efficiency, it is thus fundamental to look for alternative sources of biomass to complement crops. Microalgae are unicellular photosynthetic organisms that show a huge, yet untapped, potential in this context.

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G. Perin
Department of Biology, University of Padova, Padova, Italy

Department of Life Sciences, Imperial College London, London, UK

T. Morosinotto (✉)
Department of Biology, University of Padova, Padova, Italy
e-mail: tomas.morosinotto@unipd.it

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Microalgae metabolism is powered by photosynthesis and thus uses sunlight, a renewable energy source, and the exploitation of microalgae-based products has the potential to provide a beneficial environmental impact. These microorganisms have the ability to synthesize a wide spectrum of bioactive compounds, with many different potential applications (e.g. nutraceutics/pharmaceutics and biofuels). Several, still unresolved, challenges are however present such as the lack of cost-effective cultivation platforms and biomass-harvesting technologies. Moreover, the natural metabolic plasticity of microalgae is not optimized for a production at scale, and low biomass productivity and product yields affect competitiveness. Tuning microalgae metabolism to maximize productivity thus represents an unavoidable challenge to reach the theoretical potential of such organisms.

Keywords Bioactive compounds, Metabolic engineering, Microalgae, Photosynthetic metabolism, Sustainable economy

1 Microalgae Biomass Applications

1.1 Exploitation of Microalgae Biomass to Mitigate Environmental Consequences of Human Activities

Human activities are responsible for the massive release of greenhouse gases (GHG) and other pollutants in the environment (Moss et al. 2010). GHG accumulation in the atmosphere is causing serious environmental consequences, leading to global temperature increase, extreme weather events, oceans acidification and deoxygenation and is magnifying the pressures on food and water security, as well as on forests and other ecosystems functional biodiversity (Levy and Patz 2015; Allison and Bassett 2015). Available models indeed foresee a 30% net increase in anthropogenic emissions by the end of the century, if global economy is assumed to follow historical trends, with a consequent rise in the average global temperature up to 3°C (Walsh et al. 2015). According to the Paris agreement, net anthropogenic carbon emissions must be gradually reduced to zero in order to limit the temperature increase to less than 2°C and thus avoid the worst-case scenario (Schellnhuber et al. 2016; Rockström et al. 2017).

GHG emissions are not only connected with fuel combustion and energy production but are also due to other human activities. As example, agricultural productivity needs to increase to follow the growing world population, but intensive practices can have negative consequences such as eutrophication (United Nations 2017; Ewel et al. 2018). A short-term remodelling of human activities towards sustainability is thus a strong urgency. Replacing part of industrial processes currently supporting the global economy with more sustainable alternatives would

enable to preserve opportunities of economic development without constraining human welfare in the long term.

In such context, plant biomass represents a promising alternative to fulfill the demand of commodities. The photosynthetic metabolism requires only sustainable energy/carbon sources (i.e. sunlight and atmospheric CO_2) to fuel production of biomass, which can ultimately be converted into biofuels or valuable materials. However, scaling up crop cultivation comes at the expense of the natural environment, rising several concerns. Crop-derived biomass needs arable land/freshwater and nutrients, and redirecting part of the agricultural practice to fuel the production of other commodities besides food is thus expected to increase the pressure on land/resource use (i.e. increased fertilization and consequent nutrient run-off into environment causing eutrophication), with negative effects that could outweigh the benefits (Khanna et al. 2017). In fact, in order to replace the biomass diverted into the production of alternative bio-commodities, farmers worldwide would need to increase arable land thus converting present forests and grasslands to new croplands (Fargione et al. 2008; Whitaker et al. 2018). Since soils and plant biomass are the two largest biologically active stores of terrestrial carbon (Schlesinger and Bernhardt 2013), such practice would release a huge amount of CO_2 in the atmosphere, as a result of burning and consequent microbial decomposition of the biomass. In such a scenario, thus, the increase of arable land would cause an increase in GHG emissions, leading to a massive carbon debt only repairable in a long term (Searchinger et al. 2008).

It is thus necessary to find alternatives for the sustainable production of bio-commodities, beyond the simple expansion of area dedicated to crop cultivation. Production of plant-biomass feedstocks from marginal lands might be helpful in preserving the advantages of a photosynthesis-driven economy while minimizing the negative impacts. As example, the cultivation of perennial grasses and short-rotation trees would not require new arable land, thus providing a positive impact on GHG emissions also by reducing fertilizers and water usage. However, implementing standard crops with perennial grass cultivation on a global scale is not a trivial task (Robertson et al. 2017). In this context an emerging promising alternative is to use other biomass feedstocks such as microalgae.

Microalgae, as plants, support their metabolisms through photosynthesis, and they could thus represent an additional source of biomass to complement crops. Microalgae also present several other potentially interesting advantages. Their whole biomass is photosynthetically active with a potentially higher photon-to-biomass conversion efficiency (PBCE) with respect to plants (Melis 2009; Ooms et al. 2016). A larger PBCE would allow minimizing the pressure on environmental resources (i.e. land/water) to generate the biomass. Moreover, microalgae cultivation does not require arable land and the use of marine species would also reduce the pressure on the demand of freshwater (Usher et al. 2014). As a consequence, most of the resources needed to sustain microalgae cultivation do not compete with agriculture, not substantially perturbing crop productivity and food/feed production chain. Another consequence of a larger PBCE is that microalgae photosynthesis is highly effective in carbon sequestration, leading to the ability to withstand high CO_2

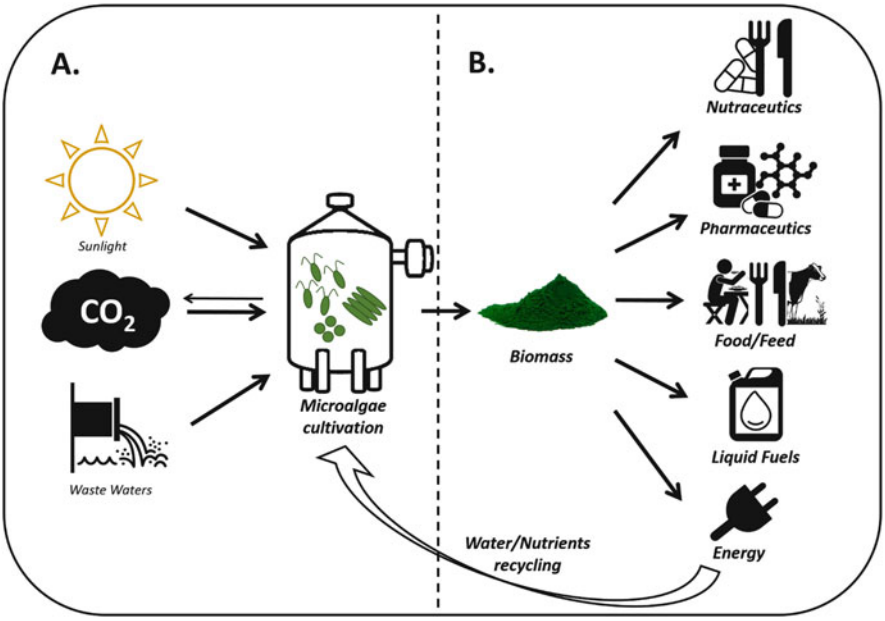

Fig. 1 Microalgae biomass-based economy. (**a**) Microalgae metabolism is powered by renewable energy/carbon sources (i.e. sunlight and CO_2) and can be supported by nutrients from civil, agricultural and industrial wastewaters, further improving environmental sustainability. CO_2 used for the cultivation can originate from an industrial plant, sequestering the emissions. (**b**) Biomass is feedstock for production of several molecules, finding application in various markets. Low-value compounds can be converted to liquid biofuels to fulfill part of the global energy demand, while high-value bioactive compounds find wide applications in both nutraceutical and pharmaceutical markets as food or feed additives. Biomass serves also for the production of energy to support water/nutrient recycling to the cultivation phase, potentially allowing energetic sustainability

concentrations, up to 90% (Salih 2011) and enabling their cultivation in connection to industrial sites, to mitigate overall anthropogenic GHG emissions.

Fertilizer input to sustain microalgae cultivation is instead expected to be substantial, given most species consist of ≈7% of nitrogen (N) and ≈1% of phosphorus (P) (Slade and Bauen 2013). Fertilizers currently come from finite resources/environmentally unsustainable processes (Haber-Bosch process and phosphate rocks for N and P, respectively), and large-scale microalgae cultivation would increase the demand for such feedstocks. However, current research efforts aim to tackle such issue exploiting the natural ability of some species to harvest nutrients from civil and industrial wastewaters, where they have been found to be particularly efficient in removing nitrates and phosphates (Ramos Tercero et al. 2014). Such feature leaves also room to implement microalgae as valuable tools to curb nutrient run-off into the environment as a consequence of intensive agricultural and industrial practices (Santos and Pires 2018) (Fig. 1a). It is thus possible to design highly sustainable processes where biomass production is combined with wastewater treatment and or water/nutrient recycling (Fig. 1). The high potential in sustainability has indeed been shown by economic

models where microalgae-derived biomass exploited to satisfy at least 40% of global energy/feed demands would result in massive environmental benefits and a drop in fossil fuel exploitation (Walsh et al. 2015).

A second main characteristic of algae biomass beyond high sustainability is the large spectrum of its potential applications. Microalgae are a highly diverse group of organisms, including species adapted to far different ecological niches, which shaped also their metabolic versatility. Some microalgae species can naturally accumulate high concentration of energy dense storage molecules, i.e. starch and triacylglycerols (Nascimento et al. 2013; Simionato et al. 2013b; Fazeli Danesh et al. 2018), that can be used as liquid biofuel precursors. As a consequence, microalgae could contribute to satisfy part of the primary energy demand on a global scale to sustain current anthropogenic activities. The global primary energy demand is currently fulfilled by fuels of fossil origin, which are finite and major contributors to the accumulation of greenhouse gases in the atmosphere. The constant growth in global population has increased the pressure on fossil fuel feedstocks, also increasing the environmental consequences to dramatic levels. Diversifying the feedstocks for energy production, including more sustainable alternatives, might allow fulfilling an increasing energy demand while also lowering the environmental impact. Microalgae are renewable feedstocks for energy production and have the potential to decrease the dependence from fossil fuels, with an expected twofold advantage: (1) lower GHG emissions and (2) develop a more stable energy market based on feedstocks less susceptible to price fluctuations (Milano et al. 2016).

Different algae species can also synthesize a wide spectrum of bioactive compounds (Appeltans et al. 2012; Maeda et al. 2018), potentially exploitable for the production of several commodities, such as food/feed, dyes, cosmetics and drugs in nutraceutical and pharmaceutical markets (Vanthoor-Koopmans et al. 2013; Gimpel et al. 2015; Bilal et al. 2017; Koutra et al. 2018; Renuka et al. 2018) (Fig. 1b). The synthesis of many bioactive compounds currently commercialized by biopharmaceutical and chemical industries relies on environmentally unsustainable chemical reactions (i.e. toxic catalysers) and thus the development of alternative processes based on naturally occurring enzymes would provide a further advantage in the long term (Ullrich et al. 2015).

All these potential advantages clearly open several challenges for their realization. As example, the generation of microalgae biomass to fulfill the demand of different commodities at scale would clearly require the development of a completely sustainable and economically competitive production chain. As example, the fraction of the biomass not converted into the desired product should not be discarded as waste but be exploited, for example, to generate energy via anaerobic digestion (Gonzalez-Fernandez et al. 2015). Logistics of the process will need to be highly integrated to maximize the advantages. As example, if cultivation occurs in marginal lands far from biorefineries where products are extracted, this could have a negative impact on the carbon balance (Robertson et al. 2017). The development of an integrated and optimized production chain enabling both a positive energy and carbon footprint is thus a seminal objective in order to develop a sustainable microalgae biomass-based economy (De Bhowmick et al. 2019).

1.2 Microalgae as Natural Source of Nutrients and Bioactive Compounds

Despite their potential as platforms for developing environmental-friendly industrial processes is now widely recognized, microalgae are presently on the market only as food/feed additives and for high-added value molecules production in cosmetics (Vanthoor-Koopmans et al. 2013; Gimpel et al. 2015; Sathasivam et al. 2017). There are known examples of microalgae that have been used as food and nutritional supplements for hundreds of years (Gantar and Svirčev 2008) and currently represent one valuable alternative for the sustainable production of protein feedstocks and to the fast-growing demand of consumers for "healthy food" (Kim et al. 2019). Among them, the green microalga *Chlorella vulgaris* Beijerinck and the cyanobacterium *Arthrospira platensis* Gomont, also known as *Spirulina platensis* (Gomont) Geitler, currently represent the most commercialized species, given their high nutrient/vitamin/protein and active polysaccharide content which enables them to provide valuable human diet/feed supplement, finding applications in nutraceutics (Kang et al. 2013; Choi et al. 2013; Yaakob et al. 2014) (Fig. 1b and Table 1). Microalgae biomass has been widely reported to have positive effects on human health, reducing cancer and cardiovascular diseases (Raposo and de Morais 2015; Luo et al. 2015; Wang et al. 2017) by increasing natural killer cell titers and anti-inflammatory response (Kwak et al. 2012).

Among the bioactive molecules microalgae naturally synthesize, the largest market share is currently covered by carotenoids, which had a value of 1.24 billion USD in 2016, and it is projected to reach 1.51 billion by 2021 (Markets and Markets 2016). Such molecules belong to a class of more than 600 natural organic pigments, which play different biological roles (Guedes et al. 2011; Raposo et al. 2015), representing the most abundant natural pigments in nature (Torregrosa-Crespo et al. 2018). Among them, β-carotene is the most abundant antioxidant of microalgae origin currently commercialized (Raposo et al. 2015). Its synthetic analogue consists only of the all-*trans* isomer, which was reported to be responsible for negative effects on plasma membrane cholesterol levels in mice studies (Harari et al. 2008). β-carotene purified from *Dunaliella salina* (Dunal) Teodoresco is instead highly enriched in the 9-*cis* isomer, with no negative effects on health reported so far. A second example is astaxanthin from the green microalgae *Haematococcus pluvialis* J. Von Flotow, 1844, that has antioxidant and anti-inflammatory activity and also finds application as natural dye for salmon and shrimp pigmentation (Zhang and Wang 2015) (Table 1). Fucoxanthin is the third most commercialized carotenoid for its antioxidant activity, recently proven to prevent epidermal hyperplasia and erythema when administered in creams on mice skin (Rodríguez-Luna et al. 2018). Moreover, microalgae naturally synthesize other pigments with strong bioactive properties, such as chlorophylls, reported to increase liver functionality and cell repair (García et al. 2017).

It is worth noting that the pool of bioactive pigments of microalgae origin we currently have access to is still limited to only the species so far isolated (Guiry 2012;

Table 1 Present main applications and biological properties for different microalgae genera

Application/product	Microalgae genera	Biological properties
Food	*Chlorella*, *Arthrospira*, *Tetraselmis*, *Nostoc*	Nutrient/vitamin supplement
Feed	*Chlorella*, *Arthrospira*, *Tetraselmis*, *Isochrysis*, *Phaeodactylum*, *Nannochloropsis*, *Thalassiosira*	Nutrient/vitamin supplement
Amino acids		
Mycosporine-like	*Aphanizomenon*, *Chlorella*, *Scenedesmus*	Sunscreen
Vitamins and carotenoids		
Vitamin B12	*Chlorella*, *Arthrospira*	Decrease fatigue, protect skin and against heart diseases
Tocopherol	*Arthrospira*	Free radical scavenger
β-Carotene	*Dunaliella*, *Haematococcus*, *Synechococcus*, *Nannochloropsis*	Food colourant, antioxidant, anti-inflammatory, antidiabetic, antitumour, with benefits for cognitive functions
Astaxanthin	*Dunaliella*, *Haematococcus*, *Chlorella*	Food colourant, antioxidant, anti-inflammatory, antitumour, cardiovascular and neurodegenerative diseases
Fucoxanthin	*Phaeodactylum*	Antiobesity, reduce cardiovascular risk factors, hepato-protective, skin-protective, anti-inflammatory and antioxidant effect
Zeaxanthin, Violaxanthin, Antheraxanthin, Canthaxanthin	*Dunaliella*, *Chlorella*, *Synechococcus*, *Nannochloropsis*, *Scenedesmus*, *Haematococcus*	Reduce cardiovascular risk factors, maintaining visual function, food additives, free radical scavengers
Proteins		
Glycoproteins	*Chlorella*	Anticancer, anti-inflammatory and anti-photoaging
Phycobiliproteins		
Phycocyanin, Allophycocyanin	*Arthrospira*	Dye for food and cosmetics, antioxidant
Phycoerythrin	*Porphyridium*	Fluorescent agent for diagnostic screenings
Chlorophyll		
Chlorophyll a	*Aphanizomenon*	Radical detoxifier and skin/liver repair
Alkenes		
Alkadienes, trienes	*Botryococcus*	Biofuels
Lipids		
PUFAs		
EPA	*Phaeodactylum*, *Nannochloropsis*, *Schizochytrium*	Nutritional supplement, aquaculture feed

(continued)

Table 1 (continued)

Application/product	Microalgae genera	Biological properties
DHA	*Schizochytrium*, *Isochrysis*, *Nannochloropsis*, *Pavlova*	Nutritional supplement, aquaculture feed
Linoleic/γ-linoleic acid	*Arthrospira*, *Dunaliella*, *Rhodomonas*, *Tetraselmis*	Protective against cardiovascular diseases
Acylglycerols		
DAGs	*Arthrospira*, *Dunaliella*	Biofuels
TAGs	*Nannochloropsis*	Biofuels
Polysaccharides		
Polysaccharides	*Chlorella*, *Nostoc*, *Arthrospira*	Thickeners and gelling agents, lubricants and antiviral and immune-stimulatory effect
Sulfonated polysaccharides	*Nostoc*, *Arthrospira*	Anticancer, antiviral and antioxidant
Sterols		
Brassicasterol, Sitosterol and Stigmasterol	*Dunaliella*	Antidiabetic, anticancer, anti-inflammatory, antioxidant activity
Phenolic and volatile compounds		
Phenolic compounds	*Chlorella*, *Nostoc*, *Chlamydomonas*	Antimicrobial and antioxidant activity
Neophytadiene, Phytol	*Dunaliella*, *Arthrospira*	Antimicrobial and antioxidant activity
Penta-/Heptadecane	*Synechocystis*, *Arthrospira*	Antimicrobial and antioxidant activity

Data here reported have been merged from de Morais et al. (2015), García et al. (2017), Sathasivam et al. (2017), Torregrosa-Crespo et al. (2018) and Kim et al. (2019)

Appeltans et al. 2012). Efforts to identify novel microalgae species will give the chance to isolate new promising candidates for carotenogenesis, possibly bearing broader pigments set to expand further the applications on human health, even if more effective screening methods for pigment accumulation are required (Aburai et al. 2018).

Besides pigments, microalgae biomass is an established sustainable feedstock also for fatty acids. Several marine species are highly enriched in omega-3 and omega-6 long-chain polyunsaturated fatty acids (PUFA), such as docosahexaenoic acid (DHA, C22:6; e.g. from *Isochrysis* sp. strain T-iso, *Pavlova lutheri* Butcher, 1952, several species of the *Nannochloropsis* genus), eicosapentaenoic acid (EPA, C20:5; e.g. *Nannochloropsis gaditana* L.M.Lubián, *Phaeodactylum tricornutum* Bohlin) and alpha-linolenic acid [e.g. *Rhodomonas salina* (Wislouch) D.R.A.Hill & Wetherbee, *Tetraselmis suecica* (Kylin) Butcher] (Kagan et al. 2014; Tsai et al. 2016) (Table 1), finding potential application as feed in aquaculture (Gimpel et al. 2015; García et al. 2017; Byreddy et al. 2018) and also as food supplement since such molecules have been reported to be beneficial for cardiovascular and inflammatory disorders. PUFAs are currently mainly derived from fish oils, whose

production is however rising several concerns for the long-term sustainability. Given the high content of oils in microalgae biomass, they might thus represent a valuable alternative to curb intensive fishing, which is strongly depleting current ocean fish stocks and marine biodiversity (Ryckebosch et al. 2014).

Polysaccharides (EPS) represent another important class of molecules naturally synthesized by some microalgae species, used in the food industry as thickeners and gelling additives (Liu et al. 2016), besides showing several positive effects on human health (Olasehinde et al. 2017) (Table 1). Other bioactive compounds derived from microalgae biomass are triterpenes called sterols (Luo et al. 2015), able to reduce low-density lipoprotein (LDL) abundance, promoting anti-inflammatory and positive cardiovascular effects (Olasehinde et al. 2017) (Table 1). Also complex molecules, such as glyco- and pigment-binding proteins (i.e. phycobiliproteins), are highly abundant in microalgae (Sonani et al. 2016). Among them, phycocyanin and phycoerythrin are currently the main molecules from *Spirulina platensis* to be commercialized for many different purposes, including natural dyes and nutraceutics with anti-inflammatory, neuroprotective and anticancer properties (Jiang et al. 2017) (Table 1).

Such wide pool of bioactive compounds is naturally synthesized by microalgae, thus enabling a direct administration of the biomass to complement diet. However, to reach a considerable effect, biomass needs to be administered in sufficient quantities, with possible negative consequences on supplemented food texture and flavour (Isleten Hosoglu 2018). A significant drawback to be considered also comes from the high nucleic acid content of some species, which are converted in uric acid with potential adverse health effects (Gantar and Svirčev 2008). In this context, the direct administration of cyanobacteria biomass rises even more concerns. Some species indeed synthesize hepato-/neurotoxins under certain environmental conditions, requiring a better comprehension of the cell physiology before commercialization (Shimizu 2003; Chu 2012). To prevent other biomass components to counteract the positive effect of the bioactive compounds listed in Table 1, the latter can also be purified from the biomass, enabling a more targeted effect on human health but also increasing costs (Kim et al. 2015).

Besides the above described applications, microalgae sustainability advantages have raised a strong interest as potential feedstocks for biofuel production to fulfill part of the global primary energy demand. In fact, several microalgae species are able to accumulate up to 60% dry weight in fatty acids (Simionato et al. 2013b; Jia et al. 2015) as triacylglycerols that are easily convertible in biodiesel through transesterification (Medipally et al. 2015; Park et al. 2015). The oleaginous trait is present in several microalgae, with *Nannochloropsis* species being the preferable hosts since showing an enrichment in the copy number of genes involved in key steps of lipid biosynthesis (Wang et al. 2014; Zienkiewicz et al. 2017).

1.3 Present Major Limitations of Algae Biotechnology

The great potential of microalgae opens the way for their integration in the current global economy as feedstock for different bio-commodities. However, despite microalgae biomass represents an eco-friendly and energetically sustainable solution to current industrial processes, the theoretical potential is still largely untapped, because implementing microalgae cultivation on a global scale is not commercially feasible yet (Carneiro et al. 2017). Critical issues still to be addressed include the development of efficient and cost-effective cultivation platforms and biomass-harvesting technologies (Acién Fernández et al. 2012; Lam et al. 2018). Moreover, except for fatty acids and other energy-rich storage compounds, the majority of bioactive compounds achieve unsatisfactory final yields (i.e. <1 up to 5% of biomass dry weight, depending on the cultivation conditions and the species) (Markou and Nerantzis 2013), therefore limiting the full realization of their potential.

In order to increase the production of microalgae-derived bio-commodities to allow a better integration in the global economy, intensive research efforts are thus still needed to overcome intrinsic limitations of the current state of the art and concretize such technology into an industrial reality. Such task is not trivial, and research efforts are indeed twofold: channelling more cellular resources towards bioactive molecules accumulation should in fact be complemented with more effective cultivation/harvesting technologies, tailored to large-scale production.

2 Algae Metabolic Engineering to Increase Yield in Bio-commodities

2.1 Challenges in Microalgae Metabolic Engineering

Pushing bioactive compound accumulation in microalgae relies on the ability to manipulate their metabolism, by overexpressing endogenous enzymes or introducing heterologous proteins triggering non-native reactions (Rasala and Mayfield 2015). However, regulatory networks are expected to be species-specific, limiting the development of universal strategies. The development of effective genome-editing approaches based on molecular mechanisms compatible with multiple hosts is currently opening the possibility to apply metabolic engineering to different species. Integrating genetic engineering with the native metabolism of the host is however not a trivial process, and research efforts still need to address multiple constraints. Rewiring the central metabolism might lead to unpredictable secondary effects on cell homeostasis due to the potential accumulation of toxic intermediates.

The impact of overexpression of enzymes depends on the molecular complexity of the target, which the native maturation steps of the host might not be able to support (Spadiut et al. 2014). Prokaryotic microalgae (i.e. cyanobacteria) are not able to efficiently perform post-translational modifications (Spadiut et al. 2014)

and are thus likely not suitable hosts for the expression of complex enzymes. The control of post-translational modifications (e.g. disulphide bonds formation and glycosylation) is indeed highly strategic because they are often seminal to support the biological activity of complex enzymes, but if hyperactive they might lead to negative consequences on human health. For example, the yeasts *Saccharomyces cerevisiae* Hansen, 1883, and *Pichia pastoris* (Guillermond) Pfaff tend to hyper-glycosylate recombinant proteins, possibly leading to immunogenic effects. Eukaryotic microalgae might thus represent promising solutions to achieve the right balance in this context, because they carry protein maturation processes similar to plants (Ullrich et al. 2015), enabling the correct folding also of complex enzymes and allowing post-translational modifications. Unlike plants, they are however unicellular hosts, allowing an easier transformation process (Böer et al. 2007) and saving time to isolate stable transformants (Peters and Stoger 2011).

Among microalgae, *Chlamydomonas reinhardtii* Dangeard, 1899, currently presents the most advanced molecular toolkit for genetic manipulation, and all three genomes (nuclear, chloroplast and mitochondrial) have been fully sequenced and are transformable (Remacle et al. 2006; Specht et al. 2010). However, metabolic engineering in such host has so far lagged behind expectations. Gene integration in the nuclear genome of eukaryotic microalgae mainly occurs by non-homologous end joining (NHEJ) (Gumpel et al. 1994), leaving room for positional effects curbing the transcription efficiency of genes of interest (GOI) and eventually leading to silencing (Scranton et al. 2015), particularly frequent for long coding sequences. On the contrary, plastid transformation is not affected by such constraints, enabling the accumulation of target enzymes up to 10% of total soluble proteins (Young and Purton 2015). However plastids, due to their prokaryotic origin, do not carry proteins maturation pathways, limiting the potential of such strategy (Scaife et al. 2015). Research efforts have thus been dedicated to counteract the above-mentioned constraints in *C. reinhardtii*, and promising strategies have been indeed identified to establish more reliable molecular toolkits in such host (Gimpel et al. 2015). As an example, merging the coding sequence of the enzyme to overexpress with the one of the selection marker, through the foot-and-mouth disease virus 2A (FMDV 2A) self-cleavage peptide, led to a 100-fold increase in the accumulation of the target protein (Rasala et al. 2012). A second strategy involves the insertion of introns in the coding sequence of the GOI, showing positive effect on gene expression (Dong et al. 2017). Introns likely play a seminal role in controlling gene expression through enhancers and mRNA processing activity (Baier et al. 2018). Moreover, in *C. reinhardtii* native gene expression deals with highly frequent introns also longer than exons. Therefore, mimicking the native gene structure might represent a promising strategy to push non-native genes overexpression in such host, as recently demonstrated (Baier et al. 2018). A similar approach could also be effective in other microalgae species, with a larger industrial production potential than *Chlamydomonas*. The introduction of introns within the GOI coding sequence increases the level of complexity of such strategy, but it is also expected to minimize the chances of lateral transgene transfer during the cultivation of transgenic microalgae, preventing the GOI to be expressed by prokaryotic organisms (Lauersen 2018).

Increasing subcellular translocation efficiency of the protein of interest is another important target to increase the efficacy of metabolic engineering. In this context, versatile and expandable vector toolkits, enabling an easy replacement of targeting peptides according to operational needs, have been developed, expanding the number of subcellular compartments currently targetable [i.e. ER, chloroplast, nucleus, mitochondria and intracellular microbodies (Lauersen et al. 2013, 2015)].

Most genome-editing tools have been applied to green algae but are rapidly expanding to other species, such as diatoms and *Eustigmatophyceae* (e.g. *Phaeodactylum* and *Nannochloropsis*), which show a larger industrial potential and in some cases also have a lower genetic complexity (Radakovits et al. 2012; Vieler et al. 2012) [e.g. lower intron frequency (Corteggiani Carpinelli et al. 2014)], theoretically enabling easier strategies for metabolic engineering. As an example, recently homologous recombination and CRISPR-based methods have been successfully developed in such species, enabling a more targeted genome editing (Wang et al. 2016; Dolch et al. 2017; Poliner et al. 2018a, b), but still important work is needed to develop efficient tools in multiple species to allow multigene expression.

2.2 *Improving the Content of Bioactive Compounds*

The synthesis of bioactive compounds in microalgae (Table 1) is often dependent on the exposition to environmental stressful stimuli, from nutrient limitation [nitrogen (N) and phosphorous (P)] and high salinity to saturating light (Chen et al. 2011, 2015; Simionato et al. 2011; Yin-Hu et al. 2012; Menon et al. 2013; Alcántara et al. 2015; Jerez et al. 2015; Alboresi et al. 2016). Such environmental conditions, however, negatively affect growth (Sun et al. 2013; Simionato et al. 2013b), as reported for N limitation which triggers an overall remodelling of cell central metabolism as a consequence of a reduced proteins and nucleic acid biosynthesis. This approach thus requires a two-stage process where first biomass accumulation is maximized, followed by a nutrient limitation phase to induce the accumulation of the desired molecules. This strategy, while very effective at lab scale, increases costs and complexity at the large scale, finally limiting productivity achievable at scale (Rawat et al. 2013).

In order to develop a microalgae-biomass-based economy scalable to fulfill bio-commodities needs on a global scale, bioactive molecule synthesis and biomass accumulation should operate simultaneously. Coupling these two aspects of microalgae cell physiology is however not a trivial process, requiring a deeper understanding of the molecular factors controlling the metabolic channelling of resources. Strategies to push for the accumulation of a specific molecule can act at multiple levels of a metabolic pathway, for instance, increasing its biosynthesis and downregulating the catabolism. Major efforts have however been so far dedicated to the former, with carotenogenesis and lipid biosynthesis being the major targets.

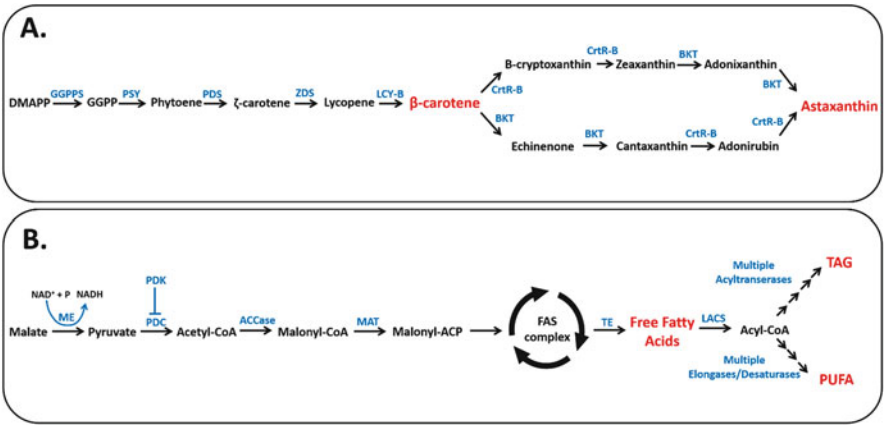

Fig. 2 Simplified biosynthetic pathways for carotenoids (**a**) and lipids (**b**) in microalgae. (**a**) Carotenogenesis. Abbreviations: Metabolites, *DMAPP* dimethylallyl pyrophosphate, *GGPP* geranylgeranyl pyrophosphate; enzymes, *BKT* β-carotene ketolase, *CrtR-B* β-carotene hydroxylase, *LYC-B* lycopene β-cyclase, *PDS* phytoene desaturase, *PSY* phytoene synthase, *ZDS* ζ-carotene desaturase. (**b**) Lipid biosynthesis. Abbreviations: Metabolites, *PUFA* polyunsaturated fatty acids, *TAG* triacylglycerols; enzymes, *ACCase* acetyl-CoA carboxylase, *ACP* acyl-carrier protein, *CoA* coenzyme A, *FAS* fatty acid synthase, *LACS* long-chain acyl-CoA synthetase, *MAT* malonyl-CoA/ACP transacylase, *ME* malic enzyme, *PDC* pyruvate dehydrogenase, *PDK* pyruvate carboxylase kinase, *TE* fatty acyl-ACP thioesterase. Major targets to increase microalgae commercial potential are indicated in red. Scheme adapted from Gimpel et al. (2015)

Carotenogenesis takes place in the plastid and is highly integrated with the central metabolism, making efforts for its modification highly challenging. A reasonable strategy to increase carotenoid accumulation is to push the abundance of their precursors (i.e. isoprenoids). Such group of compounds include many molecules of economic interest, representing good targets for metabolic engineering (Lauersen 2018), which even in the case of no success in increasing the accumulation of carotenoids might thus still enhance the commercial value of the microalgal host. Moreover, isoprenoid metabolic pathways are modulatory, making them easy targets to manipulate in multiple hosts. As an example, reliable non-native di-/tri-/sesquiterpenoid production has been recently achieved in *P. tricornutum* and *C. reinhardtii* (Lauersen et al. 2016, 2018; Wichmann et al. 2018; D'Adamo et al. 2018), increasing their commercialization potential.

Among isoprenoids, geranylgeranyl pyrophosphate (GGPP) is the precursor of carotenoid synthesis and likely a limiting metabolite for carotenogenesis. Overexpression of geranylgeranyl pyrophosphate synthase (GGPPS) in *Chlamydomonas* however failed in triggering a remodelling in isoprenoid profile (Fukusaki et al. 2003). Also attempts to increase keto-carotenoid production by overexpressing β-carotene ketolases (BKT) from *Dunaliella salina* failed to achieve the expected outcomes (León et al. 2007), highlighting the presence of a more complex regulation or additional rate-limiting steps (Fig. 2a).

Phytoene synthase (PSY) and phytoene desaturase (PDS) catalyse the first and second step in carotenogenesis, respectively, and have been identified as important targets to push β-carotene and keto-carotenoids (e.g. astaxanthin) synthesis in several species such as *H. pluvialis*, *D. salina*, *Chlorella zofingiensis Dönz 1934* and *C. reinhardtii* (Couso et al. 2011; Cordero et al. 2011; Liu et al. 2013, 2014) (Fig. 2a). However, the biotechnological optimization of carotenoid synthesis is more complex than expected because precursors (i.e. isoprenoids) are shared among different classes of pigments (e.g. carotenoids and chlorophylls) and prioritizing their channelling towards one or the other is not a trivial process, risking to unbalance carotenoid and chlorophyll abundance with detrimental consequences on cell physiology. Merging GGPPS with PSY was recently demonstrated to push the metabolic flux of GGPP towards carotenogenesis in *Arabidopsis thaliana* (L.) Heynh. (Camagna et al. 2018), leaving room for implementing such technology also in microalgae. In the future, identifying novel candidates (Sugiyama et al. 2017; Lao et al. 2018) and engineering simultaneously multiple enzymatic steps in the pathway might strengthen the metabolic flux redirection towards carotenoid synthesis with no secondary effects on cell physiology; the development of effective targeted genome-editing approaches is currently opening such possibility (Nymark et al. 2016; Banerjee et al. 2018).

Another major target for microalgae metabolic engineering is the increase in lipid accumulation. In the past years, different enzymatic steps of lipid biosynthesis pathway of several species have been targeted to enhance fatty acid accumulation or modify their composition profile, showing different degrees of success (Sun et al. 2018). Acetyl-CoA carboxylase (ACCase) catalyses the first step in fatty acid (FA) biosynthesis, converting acetyl-CoA into malonyl-CoA. However, a higher protein accumulation did not result in an increased lipid content in *Cyclotella cryptica* Reimann, Lewin & Guillard (Dunahay et al. 1996), while a positive effect was instead observed in *P. tricornutum* upon chloroplast transformation, highlighting possible other species-specific limiting steps in the biosynthesis pathway (Fig. 2b). Pyruvate dehydrogenase (PDC) catalyses the conversion of pyruvate into acetyl-CoA; therefore it contributes to control the availability of the substrate for FA biosynthesis. Pyruvate carboxylase kinase (PDK) controls PDC activity through phosphorylation, and its downregulation was shown to significatively increase FA content in *P. tricornutum* (Ma et al. 2014) (Fig. 2b). FA acyl-carrier protein thioesterases (TE) are another important target to remodel lipid biosynthesis, and their overexpression was shown to increase total FA content in *P. tricornutum* (Gong et al. 2011). Alternatively, the overexpression of Acyl-CoA: diacylglycerol acyltransferase (DGAT) in the same organism was shown to increase neutral lipid accumulation (Niu et al. 2013), pushing also the EPA content of the cell.

FA biosynthesis requires ATP and NADH to operate. Indirect strategies pushing energy/reducing power availability might thus be valuable alternatives to complement direct approaches in remodelling FA biosynthesis. As an example, malic enzyme (ME) converts malate into pyruvate, generating NADH. Its overexpression was shown to increase total lipid accumulation in *P. tricornutum* under nutrient-replete

conditions (Xue et al. 2015), likely as a consequence of the larger availability of reducing power (Fig. 2b).

Strategies affecting lipid biosynthesis might be also complemented with others rewiring their catabolism (Kong et al. 2018). Lipases and TEs involved in acyl-CoA hydrolysis are valuable target to knock-down in this context and in *P. tricornutum* were proven to increase total lipids (Trentacoste et al. 2013) and TAGs accumulation (Hao et al. 2018). One alternative to "classic" approaches targeting single enzymes is to identify master regulators of FA metabolism whose modification is instead expected to provide a more global remodelling of their accumulation. In such context, transcription factors (TFs) play a critical role and are indeed viable targets for lipid biosynthesis and biotechnological optimization (Hu et al. 2014; Banerjee et al. 2018). Overexpression of different TFs in *Nannochloropsis salina* D.J.Hibberd was shown to have a strong impact on biomass accumulation as well as on lipid biosynthesis (Kang et al. 2015, 2017; Kwon et al. 2018).

Another possibility is to target the lipid composition rather than the overall content. As example, the insertion of two higher plants TE in *P. tricornutum* resulted in a larger incorporation of short-chain FA in triacylglycerols (TAGs) (Radakovits et al. 2011). Polyunsaturated fatty acid (PUFA) market share is increasing over time, pushing for the development of reliable engineering strategies to increase their content in microalgae hosts. Desaturases and elongases are here expected to play a major role, driving the research efforts so far (Dolch et al. 2017) (Fig. 2b). The former have been shown to increase PUFA accumulation in *C. reinhardtii*, *P. tricornutum* and *Nannochloropsis oceanica* Suda & Miyashita, highlighting the feasibility of such practice (Zäuner et al. 2012; Peng et al. 2014; Kaye et al. 2015).

The strategies listed above prove the feasibility of genetic engineering to increase/remodel FA content in different microalgae species. Such approaches are however unlikely to be universal because of the huge metabolic diversity of microalgae species, likely to regulate FA metabolism in a species-specific manner. This feature has contributed to lag the achievements in such field behind expectation in the past years, mainly because of the monetary and time costs to elucidate metabolic network regulation and apply genetic engineering, tuned to different species.

It is also worth noting that a global remodelling of lipid metabolism to fulfill multiple bio-commodities productions is challenged by the natural competition for cellular resources between different lipid classes and by the difficulty in simultaneously modifying enzymes belonging to different subcellular compartments. Therefore, the metabolic strategies listed above ought to be tuned not only to the target strain biology but also to the eventual application of the biomass (i.e. human nutrition rather than feed, etc.). A deeper comprehension of both biosynthesis and catabolism of lipids as well as of the metabolic processes involved in length and saturation rate modification is seminal to such purpose, and filling this gap is of extreme importance because the nutritional value of the biomass depends on them (Medipally et al. 2015; Park et al. 2015).

Moreover, pushing bioactive molecule accumulation in microalgae biomass is just the first step to approach a broader impact on economy of microalgae-derived products. Accumulated molecules in some cases must be extracted from the biomass

to have a more targeted impact on human health. However, conventional extraction methods have several limitations (i.e. low efficiency and yield and high costs) and need further improvement to be effective but safe for humans and environmentally sustainable (Sosa-Hernández et al. 2018).

3 Strategies to Improve Microalgae Biomass Productivity

3.1 Challenges in Large-Scale Microalgae Cultivation

Microalgae are photosynthetic organisms; therefore their physiology is highly influenced by light availability, which has also been identified as a major parameter affecting growth (Ruiz et al. 2016). Microalgae are generally cultivated in open or closed platforms, namely, ponds or photobioreactors (PBR), respectively, which guarantee a controlled cultivation environment (De Vree et al. 2015). Such systems are exposed to excess irradiance to avoid energy input limitations, and microalgae are here cultivated at high densities to push productivity. In general, conditions experienced during industrial cultivation are different from the natural algae ecological niches, where cells are diluted and often experience nutrient/light limitations which contributed to the evolution of specific traits, often not advantageous in an intensive cultivation (Fig. 3).

As example, the general light limitation triggered the evolution of high pigment content per cell, to maximize the chances to prevail on other photosynthetic competitors for the available energy (Kirk 1994). Chlorophyll (Chl) is the main pigment responsible for light harvesting, which consequently is converted into excited forms, i.e. singlet chlorophyll ($^1Chl^*$), to ultimate fuel photochemical reactions, leading to the synthesis of ATP and NADPH. Pigments are bound to proteins called light-harvesting complexes (LHC), which accomplish the biological function of harvesting light energy and transfer it to the reaction centres of the two photosystems (PS) (Büchel 2015). An expanded light-harvesting apparatus is seminal in nature where cells are diluted, but in an intensive cultivation environment where cell concentration is high, it is detrimental for the overall population growth. A high efficiency in harvesting light, in fact, causes an inhomogeneous light distribution in the mass culture, with cells exposed to the light source capturing the majority of the incoming light, while inner layers instead experience energy limitation (Simionato et al. 2013a). Therefore, overall microalgae cultivation in PBR is strongly light-limited, compromising the achievable biomass productivity.

A second consequence can be found at the single-cell level. Microalgae generally can efficiently exploit light energy only until other factors (e.g. CO_2 availability) become limiting. If irradiance is higher, absorbed energy exceeds cells' photochemical capacity, causing an excess of $^1Chl^*$ that eventually leads to intersystem crossing and formation of Chl triplets ($^3Chl^*$). The latter can react with oxygen and generate singlet oxygen ($^1O_2$), which is a highly reactive molecule that oxidizes pigments, proteins and lipids, leading to damage and photoinhibition. To counteract the latter

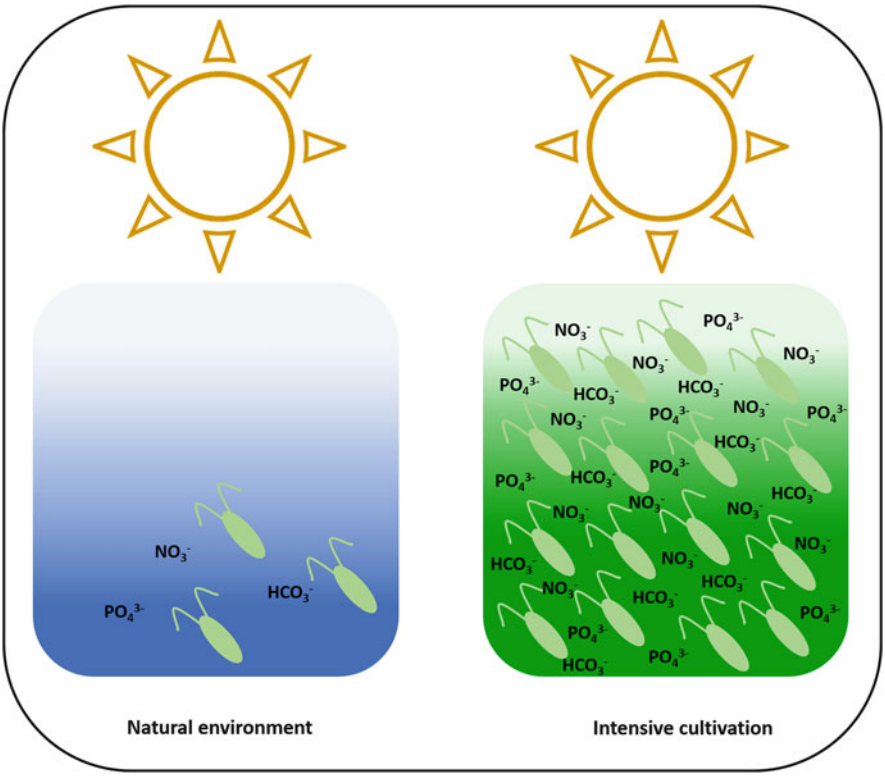

Fig. 3 Schematic representation of environmental differences microalgae face when switching from natural to intensive cultivation. In nature microalgae are generally diluted and often experience nutrients (here, i.e. NO_3^- and PO_4^{3-}), HCO_3^- and light limitation. During intensive cultivation, cells experience high cell concentrations and excess nutrients and HCO_3^- availability

scenario, microalgae evolved photoprotection mechanisms [i.e. non-photochemical quenching (NPQ) (Melis 2009)] which are molecular valves to allow a safe de-excitation of excess $^1Chl^*$ as heat. This means that in cells exposed to intense illumination, such as those at the PBR surface, a large fraction of the harvested energy [estimated up to 80% (Melis 2009)] is dissipated to avoid possible photodamage and oxidative stress, thus strongly decreasing their PBCE. At the same time, cells in inner layers do not receive enough light to run photochemical reaction. Light energy is here often below the compensation point, sometimes just enough to fulfill cell maintenance demands with therefore negative consequences on CO_2 fixation, which operates in suboptimal conditions, limiting biomass accumulation.

The situation is further complicated by the fact that cells in PBR also experience a changing light environment, as a consequence of daily and seasonal variations in the light supply and of the active mixing of the culture which suddenly switches cells from inner light-limited layers to fully exposed ones and vice versa. Microalgae naturally evolved mechanisms to cope with changing light environments to

maximize the chances of survival. Among them, microalgae can regulate pigment and photosynthetic component abundance according to their needs, from fast-occurring modifications of pigments content (i.e. between seconds and minutes) to slow-occurring ones (i.e. between minutes to hours), requiring de novo protein synthesis (i.e. antenna-size regulation) (Meneghesso et al. 2016). As an example, full NPQ activation in microalgae requires zeaxanthin (Nilkens et al. 2010; Murchie and Niyogi 2011), a pigment showing a direct activity in scavenging reactive oxygen species (ROS) (Kuczynska et al. 2015), but also essential to trigger conformational changes in specific classes of antenna proteins [light-harvesting complexes stress related (LHCSR and LHCX according to the species) (Büchel 2015)] to allow photoprotection. Such pigment is synthesized from violaxanthin by violaxanthin de-epoxidase (VDE), upon saturating light conditions, and converted back into the latter by zeaxanthin epoxidase (ZE) when cells return to limiting light, to close the so-called xanthophyll cycle. While the first reactions take seconds to occur, the latter can take several minutes (Goss and Jakob 2010). Despite microalgae are equipped with the required molecular mechanism to keep photosynthesis homeostasis in a changing light environment, kinetics of such mechanisms are incompatible with mixing in PBR, which generally operates in a millisecond-to-second timescale (Molina et al. 2001; Carvalho et al. 2011). As a consequence, cells exposed to the PBR surface do not have enough time to relax photoprotection before being exposed to light-limiting conditions in the inner layers, while cells in the latter do not have sufficient time to activate photoprotection mechanisms to counteract saturating irradiances of most exposed regions. Both cell populations therefore spend energy to inefficiently counteract light changes (Eberhard et al. 2008), again subtracting it to photochemical reactions and leading to a further reduction in light-use efficiency, ultimately leading to unsatisfactory PBCE values.

3.2 Metabolic Engineering to Increase Microalgae Light-Use Efficiency

Pushing microalgae PBCE is not a trivial objective, and strategies to optimize the entire cultivation process have been pursued in the past few years. In order to domesticate the photosynthetic metabolism to the artificial cultivation conditions, several issues need to be addressed (see Sect. 3.1) (Perin et al. 2014). The major strategies explored in the past years to pursue this objective were the reduction of the pigment content of the cell/reduction of the size of LHC (i.e. antenna proteins) and remodelling of photoprotection [for a detailed review on the topic, please refer to Perin et al. (2018)] (Fig. 4).

The first two strategies respond to the need for increasing available light for cells populating inner layers of the mass culture and pushing single-cell light-use efficiency in most exposed layers, enabling to channel more light energy into photochemistry, pushing CO_2 and biomass accumulation (Simionato et al. 2013a) (Fig. 4a).

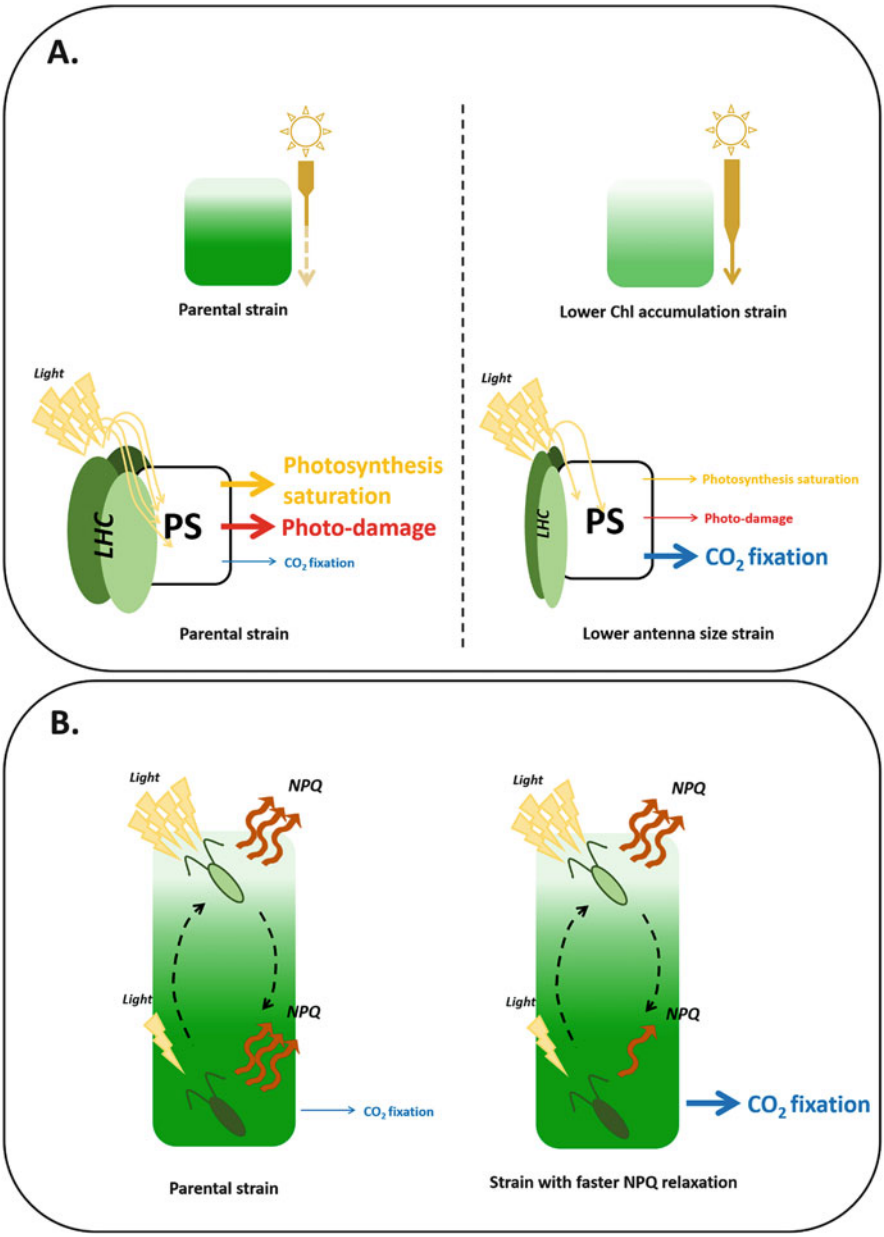

Fig. 4 Strategies to increase microalgae light-use efficiency in intensive cultivation. Schematic representation of the major strategies postulated to increase microalgae light-use efficiency in PBR. (**a**) Lower Chl accumulation and antenna size should increase the light penetration in the mass culture, increasing the amount of light exploitable by cells populating inner culture layers and push the saturation limit of photosynthesis, enabling more exposed cells to channel more light towards

A lower pigment content is in fact expected to reduce the amount of harvestable light by the first layers of a PBR, therefore shifting the saturating limit of photosynthesis towards higher irradiances but also increasing light availability in underneath layers (Oey et al. 2013) (Fig. 4a). Cells in such environment would thus receive an amount of energy exceeding just the one needed for cell maintenance, therefore enabling its channelling towards photochemical reactions, increasing CO_2 fixation and biomass productivity (Simionato et al. 2013a). It has indeed been estimated that only a fraction of the Chl molecules of a cell are seminal for the correct assembly of PS reaction centres [i.e. ≈15 and ≈30% for PSII and PSI, respectively, in *C. reinhardtii* (Melis 1991)], while the others are theoretically dispensable since not necessary for electrons transport reactions. Such strategy has been widely pursued in the past years mainly by applying forward genetic approaches in different microalgae species, with overall positive results in increasing light penetration in a mass culture (Bonente et al. 2011; Oey et al. 2013; Perin et al. 2015). However, pushing light availability in inner culture layers is not always expected to improve biomass productivity, and in fact, if the reduction of Chl molecules affects indispensable photochemistry reactions, the negative consequences on photosynthesis might compensate the advantages of a greater light availability.

The reduction in the size of LHC therefore represents a valuable complementary strategy to alter specific targets (Fig. 4a). Several strategies have been exploited for such purpose in the past, directly targeting LHC encoding genes (Polle et al. 2003; Bonente et al. 2011; Cazzaniga et al. 2014; Perin et al. 2015). Also indirect approaches proved their effectiveness in reducing LHC size, by targeting co-/post-translational molecular factors (Beckmann et al. 2009; Wobbe et al. 2009; Kirst and Melis 2014; Sharon-Gojman et al. 2017; Jeong et al. 2018).

Overall, several strains generated following those strategies indeed showed an improved light-use efficiency and the ability to saturate photosynthesis at higher irradiances with respect to the parental strains (Bonente et al. 2011; Cazzaniga et al. 2014; Perin et al. 2015), validating the above-described approaches to effectively improve the photosynthetic metabolism. However, LHC proteins are not only involved in light harvesting but play a complex biological role, with some classes also involved in regulating photoprotection (see Sect. 3.1) (Horton and Ruban 2005). A reduction in such proteins could therefore trigger unexpected secondary effects, by lowering cell ability to withstand intense illumination and increasing susceptibility to saturating irradiances. Moreover, LHC proteins are bound to both PS, and if LHC reduction is not balanced among them, it might lead to further negative consequences. As an example, the reduction of LHC classes only bound to PSII might result in a reduction in overall light-use efficiency because light energy absorbed by PSI could not be utilized by the linear electron transport, if a consistent

Fig. 4 (continued) photochemical reactions, enhancing CO_2 fixation and biomass accumulation. (**b**) Photoprotection remodelling is expected to provide a valuable contribution to reduce the amount of energy lost. In particular speeding up NPQ relaxation would increase the amount of energy that more exposed cells channel towards CO_2 fixation, once reaching inner culture layers. Abbreviation: *PS* photosystem

adjustment in PSII/PSI ratio would not be introduced (Polle et al. 2000). The above-discussed possible drawbacks of LHC reduction might however be counterbalanced by more targeted approaches, in which only LHC involved in light harvesting will be affected, also potentially balancing their reduction among the two PS. The development of CRISPR-based genome-editing approaches is indeed opening such possibility, by targeting also microalgae species with a larger industrial potential (Banerjee et al. 2018; Verruto et al. 2018).

Metabolic engineering of the photosynthetic metabolism is thus more likely to be successful when balancing light-harvesting and photoprotection efficiencies (Perin et al. 2018). The latter is a seminal feature of microalgae cells to curb photoinhibition and consequent cell damage (Sect. 3.1), and modifications in light-harvesting efficiency should never compromise it. In fact, cells in most exposed PBR layers still need to be able to withstand intense illumination and avoid photodamage, and an overall photoprotection reduction is expected to have serious negative consequences which would compensate for a lower energy loss (Perin et al. 2017a). Rather than modulating NPQ intensity, a preferable strategy could be to remodel photoprotection activation/relaxation kinetics, to enable photosynthesis to respond faster to sudden changes in the light environment (Fig. 4b). As discussed in Sect. 3.1, photoregulation kinetics generally lag behind the timescales of the environmental changes in light intensities experienced in PBR, and therefore a faster response might contribute to respond more promptly to them, increasing light-use efficiency while preserving photoprotection ability. NPQ activation in microalgae is controlled by the xanthophyll cycle; therefore speeding up its kinetics might have an influence on the timescale of NPQ activation/relaxation. Modulating xanthophyll cycle kinetics was indeed recently demonstrated to speed up NPQ relaxation in tobacco plants, pushing biomass accumulation in the field (Kromdijk et al. 2016).

Not only light harvesting and photoprotection contribute to control light-use efficiency, also electron transport reactions in fact play a major role, with alternative electron transport involved in regulating photosynthesis in a changing light environment (Cardol et al. 2011). Strengthening such pathways in microalgae species that lost some of such components during evolution might therefore represent a promising complementary strategy to those listed above. As an example, flavodiiron (Flv) proteins avoid over-reduction in the electron transport chain in fluctuating light conditions, redirecting electrons from NADPH to oxygen and preventing PSI to be photodamaged (Rochaix 2014). Their introduction in *Arabidopsis* and tobacco plants was indeed proven to speed up the recovery of photochemistry in fluctuating light conditions (Yamamoto et al. 2016; Gómez et al. 2018). Such approaches have not been attempted in microalgae so far, but the elucidation of the regulatory network of photoprotection in different microalgae species (Goss and Lepetit 2015) together with the development of more effective genome-editing approaches might open such possibility.

Overall, metabolic engineering of photosynthesis was proven to be feasible in different microalgae species, leading to significant improvements in PBCE and

biomass productivity in some cases. However, such results were observed at the lab scale, and mutant performances in PBR need still to be assessed. The cultivation environment was indeed demonstrated to have a huge impact on genetically engineered microalgae, highlighting how operational conditions may enhance or reduce the performances of strains isolated in the lab (Perin et al. 2017b). Identifying the right genetic traits to target in order to push PBCE in PBR is clearly not a trivial process, since algae performances respond to many environmental parameters. Not only the dynamic environment, but also parameters related to the PBR design and operation such as light path, culture density, mixing rate, temperature and pH might have an influence on photosynthesis, as they control light, CO_2 and nutrients availability. It is therefore impossible to extrapolate the impact of a genetic modification in the PBR environment from simple lab-scale evaluations. The elucidation of the impact that different cultivation parameters have on microalgae physiology is therefore seminal to speed up engineering strategies and to direct them towards the biological targets expected to have the greatest impact on PBCE. It should be underlined, however, given the complexity of the final cultivation environment, it is likely that there will be no superior genetically modified strain applicable in all circumstances, but instead the genetic modification ought to be tailored to the local environment and PBR characteristics.

4 Conclusions

The massive environmental impact of current anthropogenic activities calls for the development of alternative sustainable processes to fulfill the growing demand of commodities. Microalgae are able to exploit clean energy/carbon sources to synthesize a wide spectrum of bioactive compounds and biofuel precursors and thus are highly promising in this context. Their metabolic versatility can also enable the production of diverse commodities for multiple markets (i.e. from livestock feed to human healthcare and fuels).

Such potential is however still not realized, and several technological barriers are present, as demonstrated by the relatively small number of operating industrial plants. The cost of microalgae biomass production is still too high and several efforts are being indeed dedicated to the development of cost-effective cultivation platforms and biomass-harvesting technologies. In addition to operational parameters, microalgae metabolism will also need to be optimized to intensive production to achieve maximal productivity. Genetic approaches to improve microalgae metabolism have already demonstrated, at least at the lab scale, their potential in channelling more cellular resources to the synthesis of compounds of interest.

Biomass productivity is the second target requiring optimization to achieve efficient solar-driven production of commodities, and microalgae light-use efficiency is the key limiting parameter which currently curbs the maximum achievable production. Several strategies have been proven effective to tune the photosynthetic metabolism and select beneficial traits to push growth during intensive cultivation.

Improved strains have however been so far mainly tested at small scale, and the influence of conditions experienced during large-scale intensive cultivation must be taken in full account for the development of a robust process. Both aspects should be seriously taken into consideration in the future to move from the proof-of-concept stage of current achievements in microalgae biotechnologies to a concrete industrial application.

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Crassulacean Acid Metabolism and Its Role in Plant Acclimatization to Abiotic Stresses and Defence Against Pathogens

Marta Libik-Konieczny, Elżbieta Kuźniak, Ewa Surówka, Ireneusz Ślesak, Michał Nosek, and Zbigniew Miszalski

Contents

Abstract The type of plant photosynthetic metabolism (C_3, C_4 or CAM) seems to affect plant sensitivity to environmental stresses. CAM is a metabolic strategy allowing plants to maintain photosynthesis under stress conditions. Evolutionary developed adaptations of CAM plants to climate changes, especially CO_2 and water availability, may also affect their resistance to biotic stresses. Experimental data

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M. Libik-Konieczny, E. Surówka, and I. Ślesak
Franciszek Górski Institute of Plant Physiology, Polish Academy of Sciences, Kraków, Poland

E. Kuźniak
Department of Plant Physiology and Biochemistry, Faculty of Biology and Environmental Protection, University of Lodz, Łódź, Poland

M. Nosek
Institute of Biology, Pedagogical University, Kraków, Poland

Z. Miszalski (✉)
Franciszek Górski Institute of Plant Physiology, Polish Academy of Sciences, Kraków, Poland

Malopolska Centre of Biotechnology, Jagiellonian University, Kraków, Poland
e-mail: miszalski@ifr-pan.edu.pl

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obtained with the *Mesembryanthemum crystallinum*/*Botrytis cinerea* (C_3-CAM intermediate plant/necrotroph) pathosystem indicated that the CAM mode of photosynthesis favoured the expression of resistance when compared with C_3 plants. We suggested that microenvironmental conditions encountered by the pathogen in the plant tissue were one of the reasons why the fungus penetration of the CAM leaves was less effective than of the C_3 ones. CAM-related postinoculation conditions encountered by the fungi in the plant tissue could be crucial for disease development. Differences between the reaction of C_3 and CAM plants to the pathogen resulted most likely from photorespiratory activity, accompanied by changes in the reactive oxygen species (ROS)-redox signalling and salicylic acid (SA)-mediated local and systemic response.

Keywords Antioxidant system, Crassulacean acid metabolism, *Mesembryanthemum crystallinum*, Oxidative stress, Pathogen, Photosynthesis, Resistance

Abbreviations

AA	Ascorbic acid
ABA	Abscisic acid
ALT	Alanine aminotransferase
APX	Ascorbate peroxidase
AST	Aspartate aminotransferase
CAM	Crassulacean acid metabolism
cAPX	Cytosolic ascorbate peroxidase
CAT	Catalase
CBB	Calvin-Benson-Bassham cycle
CCM	CO_2-concentrating mechanism
CuZnSOD	Copper/zinc superoxide dismutase
DAMPs	Damage-associated molecular patterns
DHAR	Dehydroascorbate reductase
ETI	Effector-triggered immunity
Fd-GOGAT	Ferredoxin-dependent glutamate synthase
GPX	Guaiacol peroxidase
GR	Glutathione reductase
GS	Glutamine synthetase
GSH	Reduced glutathione
GSSG	Oxidized glutathione
HR	Hypersensitive response
JA	Jasmonic acid
MAMPs	Microbe-associated molecular patterns
MDHAR	Monodehydroascorbate reductase
MnSOD	Manganese superoxide dismutase
NAD-GDH	Glutamate dehydrogenase

NAD(P)-MDH	NAD(P)-malate dehydrogenase
NAD(P)-ME	NAD(P)-malic enzymes
NLR	Nucleotide-binding leucine-rich repeat receptor
NPR1	Non-expressor of pathogenesis-related protein 1
NO	Nitric oxide
NR	Nitrate reductase
OAA	Oxaloacetic acid
PAMPs	Pathogen-associated molecular patterns
PEP	Phospho*enol*pyruvate
PEPC	Phospho*enol*pyruvate carboxylase
PCD	Programmed cell death
PGA	Phosphoglyceric acid
PR	Pathogenesis-related protein
PRRs	Pattern recognition receptors
PTI	PAMP-triggered immunity
ROS	Reactive oxygen species
RubisCO	Ribulose-1,5-bisphosphate carboxylase/oxygenase
SA	Salicylic acid
SAR	Systemic acquired resistance
SOD	Superoxide dismutase
WUE	Water use efficiency

1 Introduction

In the coming decades, the change in the global redistribution of plant species and plant communities according to experts' forecasts seems inevitable. Such forecasts are based on (1) data of the Intergovernmental Panel on Climate Change (2007) showing that the global land surface temperature has been warming by 0.27°C per decade since 1979 (Kelly and Goulden 2008) and (2) predictions indicating the increase in the global average surface temperature by 1.5–4.5°C and CO_2 concentration up to 770 ppm (present concentration around 400 ppm) in 2100 (Kumar et al. 2017). The significant changes of these parameters not only affect environment (e.g. global spatio-temporal patterns of water and nutrient availability) and occurrence of plants but also their metabolism as well as the interactions with organisms, including symbionts and pathogens.

Since plants, divided into three major photosynthetic types – C_3, C_4 and crassulacean acid metabolism (CAM) – based on differences in their mechanism of CO_2 assimilation, are evolutionary adapted to unique ecological niches, changes in environmental factors can affect each group of plants in a different manner. For instance, the increasing CO_2 level will intensify photosynthesis mainly in C_3 plants not equipped with the CO_2-concentrating mechanism (CCM) contrary to C_4 and CAM plants. However, simultaneous increases in temperature and CO_2 level can be

unfavourable for C_3 plants. On the other hand, such changes may favour C_4 and CAM plants, known as well adapted to stress condition as they have high light saturation points, higher carboxylation efficiency, lower CO_2 compensation point and higher water use efficiency (WUE) in comparison with C_3 plants (Lüttge 2010). It has been suggested that temperature increases may extend the geographic range of pathogens leading to the establishment of new plant-pathogen interactions. In general, the climate changes can have positive, neutral or negative effects on these interactions, since both plants and their potential pathogens, which function as two coevolving groups, are locked in continual antagonism involving alternating cycles of selection to increase resistance or virulence, respectively (Wróblewski et al. 2009).

Better adaptation of C_4 and CAM plants to a hotter and drier climate (Lüttge 2010; Yang et al. 2015) has recently triggered increase in the exploration of CAM plants in scientific research and in many branches of industry, including food and biomass production (Yang et al. 2015). Therefore, future studies on plant-pathogen interactions in the context of the role of host photosynthetic metabolic type in the induction of disease defence mechanisms could be of special importance. It might help to fully understand the multidimensional nature of plant-pathogen interactions and to produce disease-resistant crop plants that are resilient to climate change.

2 Crassulacean Acid Metabolism (CAM) as a Specific Mode of CO_2 Assimilation

The majority of plants growing in the temperate zone are of the C_3 type since they produce three-carbon phosphoglyceric acid (PGA) as the first stable product of CO_2 fixation in the Calvin-Benson-Bassham (CBB) cycle. In contrast, plants that belong to the CAM type have developed a photosynthetic metabolism with a four-carbon organic acid – oxaloacetic acid (OAA) – as the first product of CO_2 assimilation (for details see Fig. 1). This primary carboxylation (β-carboxylation) is carried out by phospho*enol*pyruvate carboxylase (PEPC) that catalyses fixation of HCO_3^- to phospho*enol*pyruvate (PEP), a three-carbon compound. OAA is most often converted into malate by NAD(P)-malate dehydrogenase (NAD(P)-MDH). In many CAM plants, citric acid co-accumulates with malate (Lüttge 1988; Cushman and Bohnert 1999).

CAM plants in the typical course of their metabolism assimilate CO_2 at night when the stomata are opened. Malate synthesized during the night in the cytosol is accumulated in the vacuole due to the activity of tonoplast ATPase and the inward-rectifying malate-anion channel (Fig. 1). This process leads to strong acidification of the vacuole. Malate stored in the vacuole is rectified during the day by diffusion or through the inward-rectifying malate-anion channel (Lüttge et al. 1995; Cushman and Borland 2002; Ratajczak et al. 2003; Hafke et al. 2003; Elter et al. 2007).

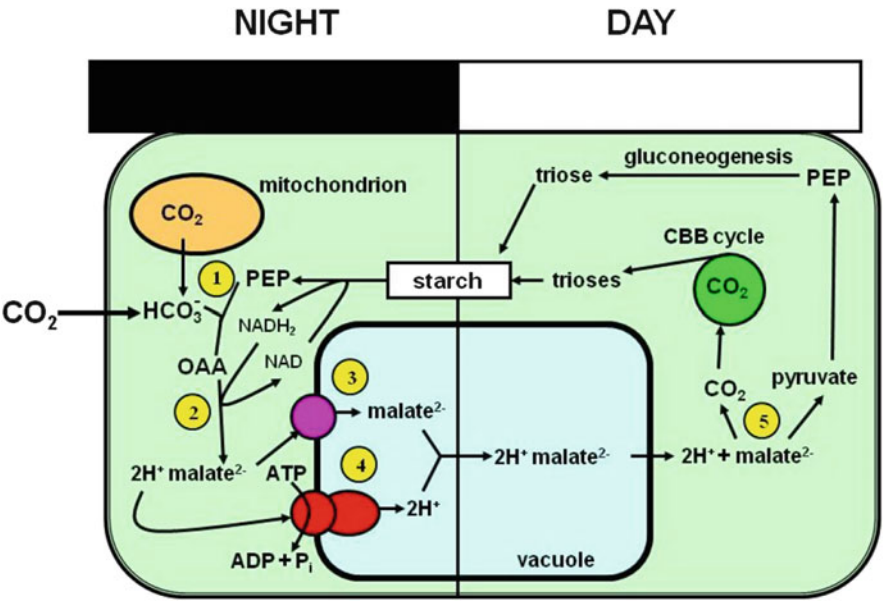

Fig. 1 Simplified scheme of basic processes of the CAM pathway as an example of ME-CAM-type *Mesembryanthemum crystallinum* L. (1) phospho*eno*lpyruvate carboxylase (PEPC); (2) NAD(P)-malate dehydrogenase (NAD(P)-MDH); (3) anion channel; (4) V-type H^+-ATPase; (5) NAD(P)-malic enzyme (NAD(P)-ME); *CBB cycle* Calvin-Benson-Bassham cycle, *OAA* oxaloacetic acid, *PEP* phospho*eno*lpyruvate (based on Ślesak et al. 2002, modified)

In the ME-CAM plants, malate is decarboxylated in the cytosol to CO_2 and pyruvate by NAD(P)-malic enzymes (NAD(P)-ME). In PEPCK-CAM plants, malate is transformed into OAA by MDH. Further, PEP carboxykinase (PEPCK) converts OAA to PEP and CO_2, which is then released and fixed in CBB cycle (Cushman and Bohnert 1999).

The CAM mode of CO_2 assimilation exhibits many similarities to the C_4 type, because the first product of CO_2 fixation (OAA) and many enzymes, as, for example, PEPC and NAD(P)-MEs, are present in both modes of CO_2 assimilation; however, in C_4-type plants, CO_2 is taken up during the day. Additionally, in C_4 photosynthesis CO_2 fixation and CBB cycle are spatially separated: carboxylation and decarboxylation reactions are carried out in mesophyll and bundle sheath cells (Kranz-type leaf anatomy), respectively (Edwards et al. 2004; Bräutigam et al. 2017). In general, CAM and C_4 photosynthesis processes are similar, as both CAM and C_4 plants have evolved a CO_2-concentrating mechanism (CCM) (Griffiths 1989; Lüttge 2002, 2004). In the process of CO_2 assimilation, CAM and C_4 plants use the same set of enzymes as C_3, and main differences between them are based on various spatio-temporal regulations and the occurrence of specific isoforms of the key enzymes (Cushman and Bohnert 1999; Bräutigam et al. 2017).

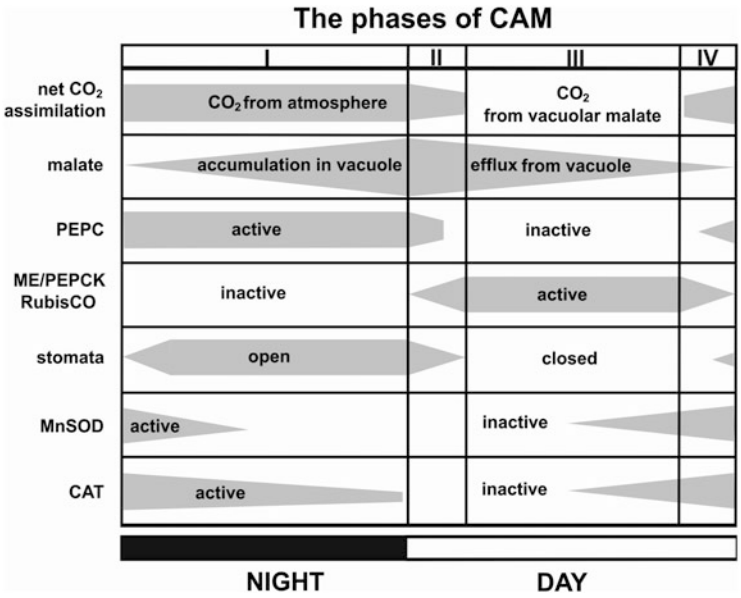

Fig. 2 The main phases of CAM are indicated over the course of 24 h photoperiod. Diurnal changes in some enzymes activities, metabolic fluxes, CO_2 uptake and stomata activity are indicated by shadings (based on Cushman and Bohnert 1999, modified)

2.1 CAM Phases

Four basic phases of CO_2 assimilation can be observed in plants with fully developed CAM (Fig. 2) (Osmond 1978; Cushman and Bohnert 1999; Osmond et al. 1999; Cushman and Borland 2002; Lüttge 2002). At night (phase I), when stomata are opened, the accumulation of malate in vacuoles occurs as the result of PEPC activity. At the beginning of the day (morning, phase II), stomata gradually close, and decarboxylation of malate begins to predominate in CO_2 metabolism. Liberated CO_2 is fixed by ribulose-1,5-bisphosphate carboxylase/oxygenase (RubisCO) (secondary carboxylation), and PEPC activity decreases. Later during the day (phase III), decarboxylation of malate prevails, followed by carboxylation via CBB cycle. The mechanism of CCM in phase III is counterbalanced by increased O_2 concentration, and this may support formation of reactive oxygen species (ROS) (Lüttge 2002) and induction of an oxidative stress (see Sect. 3), followed by the increase in the activities of antioxidant enzymes like manganese superoxide dismutase (MnSOD) (Niewiadomska et al. 2004) and catalase (CAT) (Niewiadomska et al. 1999) (Fig. 2). Photorespiration activity, a potential sink of O_2 consumption, is not eliminated in CAM plants because with CO_2 assimilation at high concentration behind closed stomata, photosynthetic oxygen evolution simultaneously also leads to O_2 concentrating of up to 40% (Lüttge 2010). Thus, photorespiration remains still active and participates in the amelioration of oxidative stress (Lüttge 2002; Miszalski et al.

2001; Niewiadomska and Borland 2007). In the C_3-CAM intermediate species *Mesembryanthemum crystallinum*, CAT activity was significantly reduced in the CAM state compared to the C_3 state. However, during the diurnal cycle, it increased in the middle of phase III and showed a peak in the phase IV (Niewiadomska et al. 1999). At the end of the day (late afternoon, phase IV), after depletion of CO_2 from the malate pool, CO_2 can be derived from respiration or from the atmosphere through opened stomata. At the same time, RubisCO activity decreases, and PEPC activity starts to increase (Cushman and Bohnert 1999; Osmond et al. 1999; Cushman and Borland 2002; Lüttge 2002).

2.2 CAM as an Adaptation to Abiotic Stresses

Taxonomical and phylogenetic studies suggest that CAM and C_4 plants evolved from C_3 plant ancestors (Moore 1983). It is considered that CAM evolved as a water-saving process due to the reduction of stomata transpiration during the day; however, it is also suggested to have occurred in response to the decreased atmospheric CO_2 level and drying Earth's climate that began during the Cretaceous (ca. 100 million years ago) and continued until the Miocene. Nowadays, many researchers seem to favour the latter hypothesis, suggesting that the decrease in CO_2 availability ("CO_2 availability stress") and not the lack of water was the primary cause of CAM evolution, leading to the development of CCM (Cushman and Bohnert 1999; Sage 2002; Kelley and Rundel 2003). The main determinants of functional CAM can be summarized as follows: (1) nocturnal malate accumulation in the vacuole, (2) decrease in stomata conductance during the day associated with decrease in net CO_2 uploading and assimilation of internal CO_2 released from vacuole and (3) increase in PEPC activity at night and in decarboxylating enzyme activities during the day (Fig. 2). CAM represents a plastic carbon fixation pathway in plants, and its intensity depends on plant development status and environmental conditions (see also below; Cushman and Bohnert 1999; Cushman and Borland 2002).

2.3 Plasticity of CAM

While CAM was described for the first time in *Crassulaceae*, it is not limited to this family but widespread in many other taxa (Silvera et al. 2010; see also below). The constantly expanding group of CAM species is not uniform. It includes plants where CAM induction is part of a genetic programme and remains under full developmental control, referred to as obligatory or constitutive CAM species. Among them we can find many representatives of *Crassulaceae* and *Cactaceae* used in early studies concerning metabolic control and functional significance of CAM (Osmond 1978, 2007; Winter and Smith 1996). Obligatory CAM species are expected to express CAM permanently in mature organs/tissues since this metabolism is initiated at the specific stage of

developmental programme, irrespective of environmental conditions. On the other hand, there are plants where CAM is an optional way of carbon assimilation, and its initiation and inhibition are largely under environmental control. These plants, called C_3-CAM intermediate or CAM facultative species, are characterized by high plasticity of photosynthesis, described as the ability to induce or, if already present, magnify CAM intensity under stress conditions, usually drought. One of the first reports confirming CAM inducibility in response to environmental factors was presented over 50 years ago for *Kalanchoë blossfeldiana* (Gregory et al. 1954), and the first report on CAM inducibility by salt stress in *M. crystallinum* (common ice plant) was presented by Winter and von Willert (1972). Similar plasticity of photosynthesis was reported for several species from families including *Bromeliaceae*, *Clusiaceae*, *Crassulaceae*, *Montiaceae*, *Piperaceae*, *Portulacaceae* and *Talinaceae* (reviewed by Winter and Holtum 2014). This author proposed also an interesting comparison describing the relationship between facultative and obligatory CAM plants. Obligatory and facultative CAM were suggested to represent endpoints of a continuum between CAM fully controlled by ontogeny and by environmental factors, respectively. Functional discrepancies between obligatory and facultative CAM plants were transparently illustrated in the study of Winter et al. (2008). It shows evidence supporting the hypothesis of CAM continuity but also gives insight on distinctive differences in understanding the endpoints of the mentioned metabolic continuum. The most interesting observation is the presence of facultative CAM components in constitutive CAM species. For example, C_3 metabolism is the predominant type of their photosynthesis during the initial stage of plant ontogenesis (Winter et al. 2008).

2.4 *C_3-CAM Intermediate* Mesembryanthemum crystallinum *as a Model System to Study Plant-Stress Factor Interactions*

In some facultative CAM plants, the plasticity is also extended, i.e. after a stressor withdrawal photosynthesis of these plants returns back to C_3-type metabolism. This phenomenon was observed in *M. crystallinum* (see below). This semi-halophyte remains one of the most comprehensively studied C_3-CAM intermediate species (317 papers published with its name in the title, Web of Science, 2018 http://thomsonreuters.com/thomson-reuters-web-of-science/). *M. crystallinum* is recognized as one of the best model plants for photosynthetic plasticity-oriented studies. First evidence of CAM reversibility and notion of environmental control of its induction was reported by Vernon et al. (1988). This study, however, did not gain expected interest, and during next few years the ice plant was often described as a halophyte with developmentally programmed C_3-CAM shift, accelerated by salinity and drought (Yen et al. 2001; Cushman and Bohnert 2002; Black and Osmond 2003; Dodd et al. 2003; Hurst et al. 2004; Niewiadomska et al. 2004). Recent studies focussed on the response of *M. crystallinum* to the removal of the mentioned

stressors, confirmed highly flexible photosynthesis, switching from CAM to C_3 when the stressor was absent (Winter and Holtum 2014; Nosek et al. 2018). Moreover, both C_3 and CAM plants exhibit tolerance to abiotic stresses, especially those connected to limited water availability. This is unequivocally related with the ice plant origin and extended genome plasticity. The ice plant was employed as a model in experiments regarding induction and modulation of PEPC (Cushman et al. 1989; Höfner et al. 1987; Ostrem et al. 1987); the role of plant hormones, i.e. cytokinins, abscisic acid and SA, during stress-induced C_3-CAM transition (Chu et al. 1990; Kuźniak et al. 2013; Schmitt and Piepenbrock 1992); shift-accompanied alterations of the antioxidant system; and the resulting modification of cell redox status (Miszalski et al. 1998; Niewiadomska et al. 1999; Ślesak et al. 2002, 2008; Niewiadomska and Miszalski 2008) or ROS per se (Surówka et al. 2016), as well as studies concentrated on the response to common pollutants, i.e. sulphur dioxide (Surówka et al. 2007) and ozone (Niewiadomska et al. 2002; Hurst et al. 2004). *M. crystallinum* was also used in experiments regarding plant-metal interaction. Increased tolerance towards high Ni and Cu doses and Zn hyperaccumulator features were confirmed (Thomas et al. 1998; Kholodova et al. 2005; Kholodova et al. 2011; Amari et al. 2014; Shevyakova et al. 2003). Our research involving soil-grown ice plant interaction with increased Cd^{2+} concentrations showed photosynthetic metabolism-dependent tolerance towards this heavy metal (Nosek et al. unpublished).

As shown above, *M. crystallinum* is widely used as a model for studies on plant tolerance to abiotic stress (Bohnert and Cushman 2000). Little is known, however, about its resistance to pathogens although it was reported to be successfully infected by *Pestalotia* sp. (Yen et al. 2001), *Botrytis cinerea*, *Pseudomonas syringae* (Libik-Konieczny et al. 2011) and *Pythium aphanidermatum* (You et al. 2015). These *M. crystallinum*-pathogen interactions provide a unique opportunity to study the co-regulation of primary metabolism, modifications of redox homeostasis and defence reactions to pathogens which still await elucidation (Rojas et al. 2014).

3 Reactive Oxygen Species (ROS) and Antioxidant System in CAM Plants

Changes in cellular redox homeostasis resulting from disturbance of metabolic processes are caused by a wide range of various abiotic and biotic stress factors (Das and Roychoudhury 2014; Czarnocka and Karpiński 2018). The common denominator of plant responses to stress factors is the phenomenon known as "oxidative stress" consisting of the overproduction of reactive oxygen species (ROS) and/or suppression of the antioxidant system activity (Gill and Tuteja 2010). The main sites of ROS production in plant cells are cell wall, apoplast, plasma membrane, mitochondrion, chloroplast, peroxisome, endoplasmic reticulum and cytosol (Ślesak et al. 2007; Szymańska et al. 2017; Surówka et al. 2019). ROS include mainly superoxide anion

radical ($O_2^{\bullet -}$), hydrogen peroxide (H_2O_2) and hydroxyl radical ($OH^{\bullet}$) (for review see Das and Roychoudhury 2014). Under physiological conditions, the antioxidant system keeps ROS at the level which enables proper functioning of a cell. They function as signalling molecules that mediate physiological processes, such as cell growth, differentiation and metabolism. On the other hand, stress-induced excess production of ROS damages DNA, proteins and lipids, thereby causing cell death (Cross et al. 1987).

The content of ROS in plant cells remains under tight control of the antioxidant system. This system is a network of enzymatic and non-enzymatic molecules able to maintain redox homeostasis that is a fundamental condition for plant cell functioning.

Efficiently working antioxidant machinery consists of enzymatic components like superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX), guaiacol peroxidase (GPX), glutathione reductase (GR), monodehydroascorbate reductase (MDHAR) and dehydroascorbate reductase (DHAR) as well as of non-enzymatic antioxidants like ascorbic acid (AA), reduced glutathione (GSH), α-tocopherol, carotenoids, flavonoids and osmolyte proline. These components act together to prevent oxidative stress induction and mitigate damages caused by ROS overproduction, and they are generating redox signals engaged in defence mechanisms against stress.

Two opposite hypotheses regarding ROS production in CAM plants were proposed (Sunagawa et al. 2010). CAM was suggested to increase oxidative stress during the light period due to the relatively high concentration of O_2 and the increase in electron transport behind closed stomata (Spalding et al. 1979, Lüttge 2010). However, CAM may also prevent the production of ROS during daytime due to CO_2 concentration resulting from the decarboxylation process, which might reduce overenergization of the photosynthetic machinery under water-limited conditions (Griffiths 1989). Fluctuation of intracellular CO_2 concentration and alterations of CO_2/O_2 ratio are natural consequences of CAM (see Sect. 2.1). Both of them affect the diurnal fluctuations of photorespiration. As a result, the rate of ROS generation during the light phase is modified, and the new oxidative status in the cell is established (Niewiadomska et al. 1999). It was revealed that CAM plants were especially equipped to deal with oxidative stress. Namely, studies on CAM intermediate *Sedum album* exhibited increased activities of ROS-scavenging enzymes like APX, SOD, GR and MDHAR during C_3 to CAM transition (Castillo 1996). Moreover, comparative studies performed on the wild-type and a CAM-deficient mutant of *M. crystallinum* showed that the key metabolic elements necessary for CAM might be part of an overall strategy for alleviating potential damage caused by increased oxidative load as a consequence of abiotic stresses (Borland et al. 2006; Cushman et al. 2008; Sunagawa et al. 2010). Early reports connecting CAM induction and antioxidant system response of the ice plant showed that during salt treatment, the activity of all superoxide dismutase forms was induced (Miszalski et al. 1998). It appears, however, that upregulation of mitochondrial MnSOD is a typical reaction to the induction of CAM (Miszalski et al. 1998; Broetto et al. 2002).

Salinity-induced CAM was found to be correlated with upregulated cytosolic ascorbate peroxidase (cAPX) gene expression and increased APX activity (Slesak et al. 2003; Nosek et al. 2015a, b, 2018). CuZnSOD gene upregulation and protein activity increase were also found during CAM induction with excess light (Hurst et al. 2004). On the other hand, H_2O_2-induced CAM was accompanied by increased α-tocopherol and proline concentrations in ice plant tissues (Surówka et al. 2016). Altogether, these results suggest that in facultative CAM species, stress-induced CAM is related to the substantial reorganization of the antioxidant system towards its enhanced capacity. It is important, however, to keep in mind that enhanced antioxidant capacity of CAM-performing tissues and the expected increase of tolerance towards abiotic and biotic stresses are rather the results of acclimation to a stressful episode than part of CAM photosynthesis. As ROS-mediated signalling is controlled by the balance between ROS generation and scavenging, these CAM-related regulations may influence the local and systemic defence response against biotic stress. Both salinity and pathogen infection can be signalled throughout the plant by cell-to-cell autopropagating vascular ROS waves (Jiang et al. 2012; Gilroy et al. 2014). As to the local response, ROS overproduction at the sites of infection mediating HR was reported to be a powerful resistance mechanism against obligatory biotrophic pathogens depriving them of a food source (Greenberg and Yao 2004). Some necrotrophs, such as *B. cinerea*, were shown to benefit from host cell death during HR (Govrin and Levine 2000). In *M. crystallinum* grown under greenhouse conditions, we found that the HR-like reaction, accompanied by H_2O_2 accumulation, was an effective defence mechanism against *B. cinerea* at the early step of its growth in the plant tissue (Kuźniak et al. 2010).

4 Plant-Pathogen Interactions: A General Overview

Biotic stress can be defined as the effect of interaction between a given plant and other living organisms. Plant infection with pathogens can be considered as a two-way interaction in which a plant recognizes and responds to a pathogen attack, while a pathogen affects plant metabolism creating an optimal environment for its own growth and development (Kushalappa et al. 2016; Méndez and Romero 2017).

In general, plant pathogens can be classified as biotrophic, necrotrophic and hemibiotrophic based on their lifestyle and interaction with a host (van Kan et al. 2014; Gill et al. 2015; Méndez and Romero 2017). Biotrophs supress the host immune system and maintain host viability to obtain nutrients from living host tissue. In contrast, necrotrophs kill host cells and obtain nutrients from dead host tissue. Most interactions between plants and pathogens are hemibiotrophic; there is an initial biotrophic phase followed by a necrotrophic one at later stages of infection (Chowdhury et al. 2017). Two main types of plant responses to pathogen attack are known: host resistance and nonhost resistance (van Kan et al. 2014; Gill et al. 2015; Kayum et al. 2016; Lee et al. 2017; Méndez and Romero 2017). Host resistance is generally controlled by single R (resistance) genes (gene-for-gene resistance), while

nonhost resistance is believed to be a multigene and longer-lasting trait. In addition to those plant-pathogen interactions, several other models have been recently described, including zig-zag model, invasion model and multicomponent model (Andolfo and Ercolano 2015; Méndez and Romero 2017; Silva et al. 2018).

Plant response to pathogen infection is based on innate immunity of each cell and on systemic signals generated at infection sites (Jwa and Hwang 2017; Peyraud et al. 2017). The first line of defence is triggered in plants by the perception of microbe- or pathogen-associated molecular patterns (MAMPs or PAMPs) via membrane-bound pattern recognition receptors (PRRs), leading to basal immunity, known as PAMP-triggered immunity (PTI). PTI provides protection against nonhost pathogens and limits disease caused by virulent pathogens. The second line of defence refers to plant cell-based surveillance system using intracellular nucleotide-binding leucine-rich repeat (NLR) receptors to recognize specific pathogen effectors. This leads to R gene-mediated effector-triggered immunity (ETI). ETI is highly specific and often accompanied by the hypersensitive response (HR) and systemic acquired resistance (SAR). An additional surveillance system consists of endogenous damage-associated molecular patterns (DAMPs) that are able to trigger immune responses. Moreover, defence responses include activation of multiple signalling pathways and transcription of defence-related genes that limit pathogen proliferation and/or disease symptom expression. In addition, ROS are produced, cell wall defence mechanisms are activated, and defence hormones, such SA, ethylene and jasmonic acid (JA), modify plant response (de León and Montesano 2013; Delaunois et al. 2014; Chowdhury et al. 2017; Jwa and Hwang 2017; Gill et al. 2015; Lee et al. 2017; Méndez and Romero 2017; Peyraud et al. 2017).

The interaction between plant and pathogen can be compatible (when a pathogen infects susceptible host plants causing disease symptoms) and incompatible (when a pathogen encounters a nonhost or a resistant host plant) (Doughari 2015; Kayum et al. 2016).

The ability of a pathogen to cause a disease on a plant depends on many internal elements like host and pathogen physiology and biochemistry as well as external determinants such as environmental conditions (Doughari 2015). Plant sensitivity to pathogen infection might be influenced by plant developmental stage (Develey-Rivière and Galiana 2007), infected organs/tissue (e.g. leaves, the vascular system), basal levels of antioxidants and their activity as well as the type of CO_2 fixation metabolism (Rojas et al. 2014; De Gara et al. 2003).

Most of the knowledge concerning plant-pathogen interactions is based on the data from C_3 plants (Peyraud et al. 2017; Velásquez et al. 2018). There are scarce data on the diseases of CAM and C_3-CAM plants (Table 1). Little is also known about the defence mechanisms and the differences in physiological response induced in C_3 and CAM plants (Table 2).

ROS play a major role in plant-pathogen interactions. ROS production can be either a hallmark of successful pathogen recognition and activation of plant defences or serve as an effective weapon used by the invading pathogen which facilitates disease development (Jwa and Hwang 2017). Preparedness of plants performing CAM to salt stress, including the complex relationship between ROS and antioxidant

Table 1 Examples of natural pathogens of CAM or C_3-CAM intermediate plants in their habitats or in the greenhouse conditions

Organism	Pathogen species	Plant species	Name of the disease and the symptoms	References
Viruses	*Ampelovirus* *Capsicum chlorosis virus*	*Ananas comosus*	Mealybug wilt disease Yellow spot	Joy and Sindhu (2012)
	Impatiens necrotic spot tospovirus *Kalanchoë mosaic potyvirus* *Tomato spotted wilt tospovirus* *Kalanchoë top-spotting badnavirus*	*Kalanchoë blossfeldiana*	Impatiens necrotic spot *Kalanchoë* mosaic Spotted wilt Top spotting	Wick (2017)
Bacteria	*Acetobacter aceti* *Gluconobacter oxydans* (*Acetobacter suboxydans*) *Erwinia herbicola* (*Enterobacter agglomerans*) *Pantoea ananatis* *Tatumella morbirosei* *Tatumella ptyseos* (*Pantoea citrea*)	*Ananas comosus*	Pink disease	Rohrbach and Pfeiffer (1976), Cha et al. (1997), Kado (2003), Marín-Cevada and Fuentes-Ramírez (2016)
	Rhodococcus fascians	*Kalanchoë blossfeldiana*	Bacterial fasciation	Wick (2017)
	Erwinia chrysanthemi *Erwinia carotovora*		Bacterial soft rot	Putnam and Miller (2007)
	Agrobacterium tumefaciens		Crown gall	
Fungi	*Choanephora cucurbitarum*	*Mesembryanthemum* spp.	*Choanephora* rot (blossom end rot or wet rot)	Data base of plant disease in Japan (https://www.gene.affrc.go.jp/databasesmicro_pl_diseases_detail_en.php?id=11326)
	Botrytis cinerea		*Botrytis* leaf spot	Kagiwada et al. (2010) Maeda et al. (2010)
	Phytophthora cinnamomi	*Ananas comosus*	The *Phytophthora* heart (top) rot disease	Joy and Sindhu (2012)
	Phytophthora nicotianae *Chalara paradoxa*		Base (butt) rot Fruitlet core rot (green eye) disease	
	Fusarium guttiforme *Penicillium funiculosum*		Fusariosis diseases White leaf spot	
	Thielaviopsis paradoxa		Butt rot disease	Nurnadirah et al. (2018)

(continued)

Table 1 (continued)

Organism	Pathogen species	Plant species	Name of the disease and the symptoms	References
	Botrytis cinerea Pers.:Fr *Cladosporium* sp. *Fusarium* sp. *Rhizoctonia solani Kühn*	*Kalanchoë blossfeldiana*	*Botrytis* blight *Cladosporium* leaf spot *Fusarium* stem rot *Rhizoctonia* root and crown rot	Wick (2017)
	Scytalidium sp., e.g. *S. lignicola*, *Scytalidium* sp., *Lasiodiplodia* spp., *Fusarium* spp.	*Opuntia ficus-indica*	Squamous rot	de Souza et al. (2017)
	Alternaria alternata	*Agave americana*	Disease symptoms: from round and golden brown spots to coloured longitudinal streaks	Gautam (2013)
Nematodes	*Meloidogyne javanica* (root-knot nematode) *Pratylenchus brachyurus* (root lesion nematode) *Rotylenchulus reniformis* (reniform nematode)	*Ananas comosus*	Nematode-associated diseases	Joy and Sindhu (2012)

processes, as it was mentioned above (see Sect. 3), can influence the plant-pathogen interaction. CAM species might be a valuable source of information concerning plant-pathogen interactions at the physiological and molecular levels.

Plants integrate diverse environmental stimuli via a network of interacting signalling pathways in which ROS, redox switches and phytohormones were suggested to form nodes of convergence at which the abiotic and biotic signalling crosstalk occurs (Sewelam et al. 2016; Chojak-Koźniewska et al. 2018). The signalling pathways induced by individual stress factors interact in antagonistic and synergistic ways, and this crosstalk determines the final whole-plant response. The overlap between signalling pathways, especially those mediated by ROS, production of which is accelerated by many stresses, was suggested to underpin cross-tolerance (Pastori and Foyer 2002). This phenomenon allows plants to tolerate multiple stresses after exposure to one specific stressor. For example, in C_3 *Arabidopsis* plants, pretreatment with ozone induced tolerance towards subsequent biotic stress caused by *Pseudomonas syringae* infection. Plants infected without pretreatment were severely damaged and died (Sharma et al. 1996). Similarly, drought enhanced tomato defence response against *B. cinerea* (Achuo et al. 2006). In other studies, however, abiotic stress was shown to suppress disease resistance in C_3 plants (Prasch and Sonnewald 2013). The salt-stressed cucumber plants were more prone to angular leaf spot disease caused by *Pseudomonas syringae* pv. *lachrymans* (Chojak-Koźniewska et al. 2017). In *M. crystallinum*, however, the salt stress-induced shift

Table 2 Selected metabolites/enzymes and the effect of their action after pathogen infection of CAM and C_3-CAM intermediate plants

Plant-pathogen interaction	Metabolites/ enzymes involved in plant defence	The nature of changes in metabolite/enzyme content/ activity involved in plant defence	Physiological response	References
M. crystallinum/ *Pseudomonas syringae*	Antioxidants	The increase in total SOD and CAT activities in C_3 plants and stable level in CAM plants	The enhanced antioxidant potential depended on the type of photosynthetic carbon metabolism	Libik-Konieczny et al. (2011)
		The decrease in GPX in C_3 plants and the increase of activity in CAM plants	The increase in the concentration of H_2O_2 in C_3 plants and a decrease in CAM plants	
M. crystallinum/ *Botrytis cinerea*	Antioxidants	The increase in total SOD and CAT activities in C_3 plants and stable level in CAM plants	The enhanced antioxidant potential depended on the type of photosynthetic carbon metabolism	Libik-Konieczny et al. (2011)
		The decrease in GPX in C_3 plants and the increase of activity in CAM plants	The increase in the concentration of H_2O_2 in C_3 plants and a decrease in CAM plants	
		The lower expression of CAT gene *catL* in C_3 midrib parts in comparison to mesophyll In CAM plants upregulation of *catL* expression in mesophyll	Establishment of leaf defence by spatial modification in the expression of genes involved in the modulation of ROS level	Nosek et al. (2015a, b)
		The increase of ascorbate peroxidase, class III peroxidases and glutathione transferase activities in NaCl-adapted callus	The modification of the infection pattern in the salt-adapted callus and the enhanced antioxidant potential related to metabolic adaptations to salinity	Kuźniak et al. (2011)
		The increase in tocopherol content in C_3 plants	Increased membrane protection against oxidative stress in C_3 plants	Gabara et al. (2012)
		Higher constitutive levels of AA content and APX activity in CAM plants The increase in GR activity, stronger in CAM plants	Higher ascorbate-related antioxidant potential in CAM plants than in C_3	Kuźniak et al. (2013)
		Higher constitutive glutathione level and glutathione redox ratio in CAM plants; local infection-induced glutathione redox ratio decrease, especially in C_3 plants	Glutathione-dependent redox signalling contributes to defence response in a photosynthetic metabolism-specific manner	Kuźniak et al. (2013)
		Local accumulation of S-thiolated proteins in infected C_3 and CAM plants, stronger in C_3 ones	The contribution of protein S-thiolation in local plant defence depends on photosynthetic metabolism	(Kuźniak et al. 2013)
M. crystallinum/ *Botrytis cinerea*	Organic acids	Differences in the direction of changes in malate content in C_3 and CAM plants. Weak diurnal oscillations of malate content in CAM plants	The involvement of the malate valve in plant-pathogen response and its dependence on the type of photosynthetic carbon metabolism	Libik-Konieczny et al. (2012), Kuźniak et al. (2010)
	Malic enzymes	Differences in regulation of NADP-ME activity in C_3 and CAM plants and different expression of NADP-ME gene	Differences between C_3 and CAM plants in the direction of changes in primary metabolism providing energy, reducing	Libik-Konieczny et al. (2012), Nosek et al. (2015a, b)

(continued)

Table 2 (continued)

Plant-pathogen interaction	Metabolites/ enzymes involved in plant defence	The nature of changes in metabolite/enzyme content/ activity involved in plant defence	Physiological response	References
		(*nadpme1*) in mesophyll and veins of C_3 and CAM plants	equivalents and carbon skeletons for defence responses to halt the pathogen growth	
	PEPC and nitrogen metabolism-related enzymes	Earlier increase in PEPC and Fd-GOGAT activities and later inhibition of NR activity in CAM than in C_3 plants PEPC and nitrogen metabolism-related enzymes involved in metabolic reprogramming in systemic leaves of CAM plants	CAM-specific, infection-induced alteration of carbon/nitrogen status in infected and systemic leaves promotes the expression of defence mechanisms	Gajewska et al. (2018)
	Phytohormones	Local and systemic accumulation of free SA in CAM and C_3 plants. The effect was stronger in CAM plants	SA-dependent signalling promotes defence response in CAM plants	Kuźniak et al. (2013)
	Callose, lignins	C_3 and CAM plants accumulated callose and lignin to similar levels after infection	The local immune response not related to callose and lignin deposition	Kuźniak et al. (2013)
Kalanchoë daigremontiana/ Agrobacterium tumefaciens	Antioxidants	The increase in CAT activity in tumour tissue	Changes in the tissue organization. The decrease of H_2O_2 content in tumour tissues and induction of defence mechanisms against pathogen	Tkalec et al. (2012)
		The increase in peroxidases class III activities and the induction of new isoenzymes		
		The increase in polyphenol oxidase activity in tumour tissue		

towards CAM metabolism appeared to improve defence response against *B. cinerea* allowing plants to respond more quickly to the pathogen (Kuźniak et al. 2010). We found that in *M. crystallinum*/*B. cinerea* and *M. crystallinum*/*Pseudomonas syringae* pathosystems, the activities of two antioxidant enzymes SOD and CAT were unchanged in CAM-performing plants, in which infection development was inhibited (Table 2) (Libik-Konieczny et al. 2011). In other CAM species, like *Kalanchoë daigremontiana*, no changes in CAT, peroxidase class III and polyphenol oxidase activities were described in the tissues next to the sites of infection with *Agrobacterium tumefaciens* (Tkalec et al. 2012). These data indicate that CAM metabolism might create a specific microenvironment with high antioxidant potential, sufficient to inhibit pathogen development.

Here, common biochemical and hormonal regulations related to ROS-redox homeostasis, interactions between carbon and nitrogen metabolism and SA signalling were argued to be important. Moreover, the supporting role of CAM-specific chloroplast-derived retrograde redox signals cannot be neglected (Nosek et al. 2015a, b).

5 The Role of Carbon and Nitrogen Metabolism in Plant's Defence Against Pathogens

Plant responses to pathogens depend on their metabolic status in many ways. First, successful pathogen colonization is based on nutrient acquisition from the plant tissue (Fatima and Senthil-Kumar 2015). Second, the resistance response requires an increased supply of carbon skeletons and nitrogen sources, energy and reducing equivalents derived from primary metabolic processes, mainly photosynthesis and nitrogen assimilation (Nunes-Nesi et al. 2010). In some plant-pathogen interactions, this increased demand for metabolites fuelling defence is accompanied by reduction in photosynthetic metabolism, followed by source-to-sink transition of the infected tissue (Bolton 2009; Wang et al. 2018). Third, some primary metabolites play a signalling role in plant defence against pathogens (Reignault et al. 2001; Morkunas and Ratajczak 2014). It was shown that sugars downregulate photosynthetic genes and induce pathogenesis-related ones (Bolouri Moghaddam and Van den Ende 2012).

Although intensively studied, the role of photosynthesis in plant defence against pathogens remains unclear and needs more studies. In numerous plant-pathogen interactions, light was shown to be required to increase plant resistance by regulating defence-related gene expression (Bechtold et al. 2005; Chandra-Shekara et al. 2006; Nagendran and Lee 2015). The dependence of defence activation on light was confirmed in the *M. crystallinum*/*B. cinerea* pathosystem; however, we found that the C_3 or CAM type of photosynthesis was not the primary factor determining the defence mechanisms (Kuźniak et al. 2010). We suggested that the postinoculation microenvironmental conditions encountered by the pathogen in the plant tissue were one of the reasons why the fungus penetration of the CAM leaves was less effective than of the C_3 ones (Fig. 3). Although the mechanisms by which *B. cinerea* hyphae penetrated the leaf tissues appeared to be similar in C_3 and CAM plants, and based on the physical pressure and enzyme secretion, the pathogen performed better in the C_3 plants as shown by the morphology and hyphae growth intensity (Gabara et al. 2012).

Unavailability of nutrients matching the pathogen requirements can limit the nonhost pathogen growth and was suggested an important determinant of nonhost resistance (Senthil-Kumar and Mysore 2013). The vascular tissue, especially phloem, serves as a nutrient niche for pathogens. We found that in *B. cinerea*-infected leaves of *M. crystallinum*, xylem vessels, which are poor in nutrients, were filled with numerous fungal hyphae with visible symptoms of destruction. Electron microscopy showed that the morphological signs of *B. cinerea* hyphae degeneration in xylem were similar in C_3 and CAM plants (Gabara et al. 2012). It cannot be excluded, however, that the fungal hyphae penetrating the xylem vessels failed to establish an effective nutritional relationship not because of the lower availability of nutrients but due to the exposition to H_2O_2 which accumulates in the leaf vasculature under stress (Ślesak et al. 2008; Nosek et al. 2015b). Interestingly, plant cells were shown to be less susceptible to H_2O_2 than some fungal pathogens (Lu and Higgins 1999).

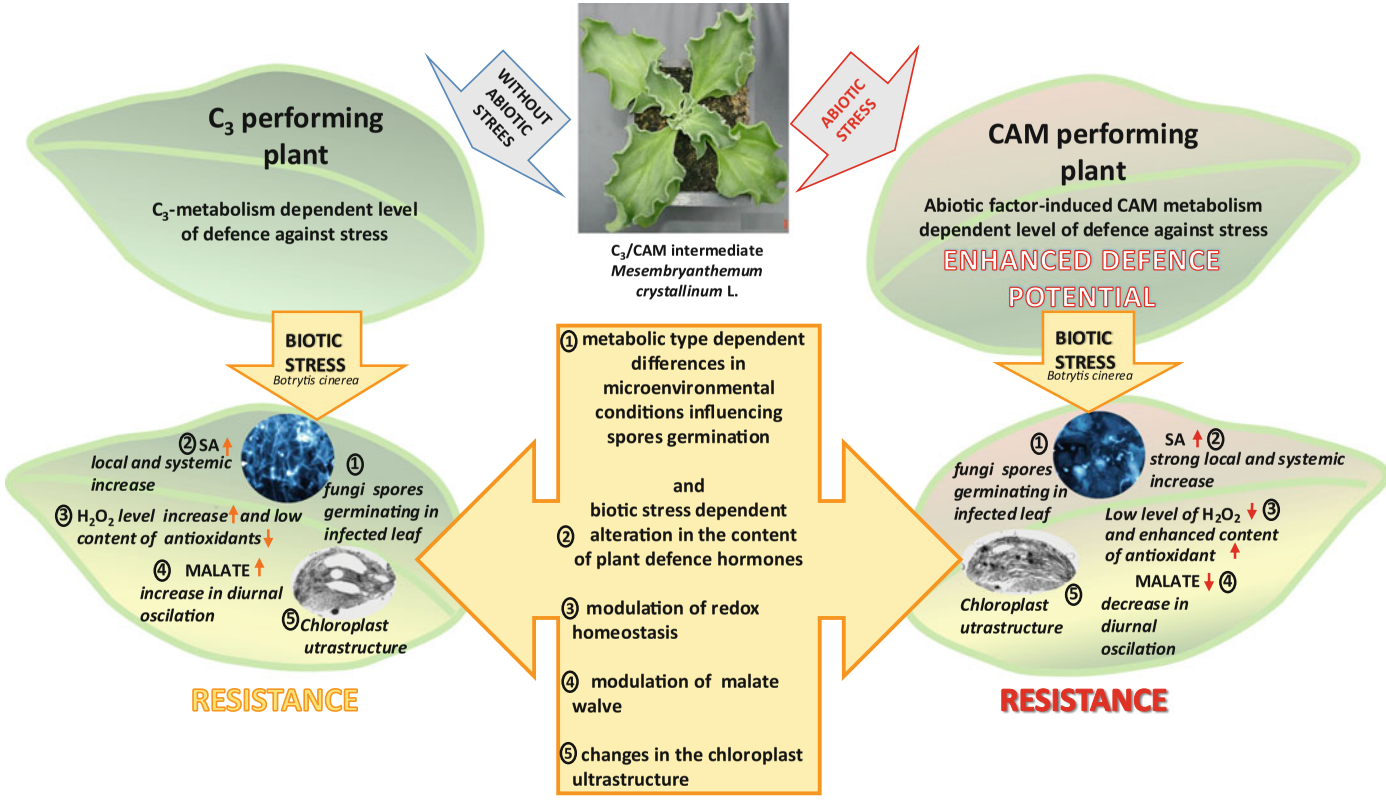

Fig. 3 Scheme of C_3-CAM intermediate *M. crystallinum* plant response to biotic factors, e.g. *Botrytis cinerea* in the relations to induced photosynthetic metabolism type. For details see Sect. 3 and Table 2

While addressing the role of leaf vasculature in pathogenesis, its photosynthetic activity should be also taken into consideration. In *M. crystallinum*, leaf veins are photosynthetically competent, and chloroplasts resemble those in bundle sheaths of C_4 plants. However, their role depends on the photosynthetic mode of carbon assimilation. In C_3-performing plants, they are suggested to be involved in the supply of CO_2 for carboxylation, whereas in CAM ones in providing malate and in H_2O_2 signalling (Libik et al. 2004; Kuźniak et al. 2016). This could be beneficial for the infected CAM-performing plants, as the malate-mediated mechanisms via malate shuttle and activity of NADP-malic enzyme (NADP-ME) can facilitate the allocation of carbon resources to defence reactions and redox signalling (Corpas and Barroso 2014; Heyno et al. 2014). Moreover, the malate valve might communicate the chloroplast redox state to other organelles integrating the cellular response to stress (Dietz et al. 2016).

The biochemical features of the microenvironmental niches were also suggested to affect *B. cinerea* pathogenesis in *M. crystallinum* at the inoculation sites. The resistance response was stronger in CAM plants, than in C_3 ones, because malic acid accumulating in their mesophyll cells (Table 2) is a poor carbon source for *B. cinerea* and inhibits its spore germination (Vercesi et al. 1997; Unger et al. 2005). Similarly, the salt stress-induced lower water potential and high contents of polyols in CAM leaves could create a less favourable microenvironment for pathogen growth and modulate the post-penetration defence (Kim et al. 2005; Elad and Evensen 1995).

An invading pathogen interferes with the source-sink balance in plants and creates a sink in the infected tissue withdrawing nutrients for its growth (Lemoine et al. 2013). We found that at the sites of *B. cinerea* inoculation, dead plant cells within necrotic spots were filled with starch grains, regardless of the type of photosynthesis. However, in the leaf areas surrounding the lesions, the source-sink relationship was differentially changed by the pathogen, depending on the photosynthetic metabolism. In C_3-performing plants, the number of starch grains in chloroplasts remained constant, whereas their surface decreased and glucose content increased. In CAM-performing plants, the decrease in the number of starch grains was accompanied by lowered glucose content (Fig. 3) (Gabara et al. 2012). As the induction of pathogenesis-related (PR) genes was found to be hexokinase-dependent (Rojas et al. 2014) and in CAM plants glucose-6-phosphate is preferentially released as the starch degradation product (Neuhaus and Schulte 1996), we suggested that these pathogen-induced metabolic changes could support the defence responses in CAM plants.

Regardless of the type of photosynthetic metabolism, a hypersensitive response (HR)-like reaction successfully restricting the pathogen growth was observed in the *B. cinerea*-infected leaves. Programmed cell death (PCD) events, however, were observed only in C_3 plants (Kuźniak et al. 2013). These data confirm the concept that necrotrophic pathogens, unlike biotrophs, trigger PCD in plants to induce disease by producing PCD-inducing pathogenicity factors (Govrin and Levine 2000; Govrin et al. 2006). Their secretion to the leaf tissue could also be determined by biochemical differences between the environmental niches at the inoculation sites in C_3 and CAM plants.

The SA signalling pathway is pivotal for plant defence against biotrophic pathogens, while its role in plant interactions with necrotrophs is less clear and appears to depend on the pathosystem (Rivas-San Vicente and Plasencia 2011; Verma et al. 2016). As SA is also important for salt and oxidative stress tolerance in plants (Jayakannan et al. 2015), the studies on facultative halophyte and C_3-CAM intermediate plant species can shed light on the mechanisms of SA action.

SA can promote the antioxidant defence in plants. SA interacts with glutathione in a regulatory loop in which the SA-mediated increase in glutathione content enhances the antioxidant potential and simultaneously activates the redox-dependent NPR1 (non-expressor of pathogenesis-related protein 1) defence signalling (Mou et al. 2003; Herrera-Vásquez et al. 2015). Correlation of glutathione with disease resistance has been reported for numerous plant-pathogen interactions, including *M. crystallinum*/*B. cinerea* (Kuźniak and Skłodowska 2005; Großkinsky et al. 2012; Kuźniak et al. 2013). Moreover, glutathione was shown to control the signalling role of SA in plant responses to stress (Foyer and Noctor 2009).

The HR-like response was also assumed to signal the stress acclimation response in tissues noninfected by the pathogen (Heath 2000). In *M. crystallinum*/*B. cinerea* interaction, SA, glutathione and protein S-glutathionylation were involved in these signalling processes in a photosynthetic metabolism-dependent manner (Kuźniak et al. 2013) (Fig. 3). We found infection-induced accumulation of free SA, at the expense of its glucoside (Table 2). This was observed both, locally, in tissues adjacent to the necrotic sites and, systemically, in the non-inoculated upper leaves supporting the signalling role of SA. These changes co-occurred with those in the glutathione pool, manifested by a general oxidative shift towards oxidized glutathione (GSSG) accumulation in the vicinity of the necrotic lesion and by reduced glutathione (GSH) content increase in systemic leaves tailored for C_3-performing plants. At the local level, changes in the glutathione pool were accompanied by those in protein S-glutathionylation via which glutathione can also regulate the functions of cells under stress (Zaffagnini et al. 2012). In CAM plants, the SA and glutathione-related redox signalling after infection could depend on the supply of glycine via the photorespiratory pathway as both the levels of cysteinylglycine, which can thiolate proteins and interfere with redox signalling, and of free glycine were lower than in C_3 ones (Table 2) (Kuźniak et al. 2013; Nakano et al. 2006). The SA signalling is functionally linked to the nitric oxide (NO) pathway, and these signalling molecules interact in plant defence responses activated after pathogen attack at the local and systemic levels (Klessig et al. 2000). However, the interference between the SA and NO pathways is not clear, as NO was shown both to act synergistically with SA in activating defence responses and to antagonize its action. NO initiates the biosynthesis of SA and promotes the SA-related HR. Simultaneously the NO-mediated S-nitrosylation of NPR1 protein promotes its oligomerization and suppresses the regulatory role of nuclear-located NPR1 monomers in SA-dependent gene expression (Mur et al. 2013). The mechanisms of NO generation in plants, via, e.g. nitrate reductase (NR), were suggested to be important arbiters in the interplay between these interacting signalling pathways, influencing the amplitude of immune responses (Gupta et al. 2011; Mur et al. 2013).

NR is regulated by sugars at the post-transcriptional level and by amino acids at the transcript level. Sugars such as glucose and glucose-6-phosphate activate it (Iglesias-Bartolome et al. 2004), while glutamine and glutamate inactivate it (Scheible et al. 1997). This relationship appeared to be important for CAM plant defence against *B. cinerea*, due to both the biochemical characteristics of pathogen-induced source-to-sink transition of the infected tissue and changes in nitrogen metabolism-related enzymes (Kuźniak et al. 2013; Gajewska et al. 2018).

The role of nitrogen metabolism in plant defence is widely accepted. Nitrogen supply and the form of nitrogen available to plants and pathogens affect disease development (Snoeijers et al. 2000; Thalineau et al. 2016; Wang et al. 2018). Similarly to the effects described for primary carbon metabolism, infection-induced changes in nitrogen metabolism were shown to promote either plant defence or disease development, depending on the plant-pathogen interaction (Liu et al. 2010; Seifi et al. 2013). Some pathogens modify plant nitrogen metabolism for their nutritional benefit (Divon and Fluhr 2007). This picture is further complicated by the fact that nitrogen acts as signal for CAM expression (Ota and Yamamoto 1991; Rodrigues et al. 2014). *M. crystallinum* performing CAM showed higher NR and glutamate dehydrogenase (NAD-GDH) activities but lower glutamine synthetase (GS) and alanine aminotransferase (ALT) activities in comparison to C_3 plants. Higher NR activity in CAM plants resulting from NaCl treatment could have an impact on NO signalling, according to the scenario described above. In cucumber, salt stress increased NO concentration, and NR activity was responsible for its biosynthesis (Reda et al. 2018). In *M. crystallinum*, *B. cinerea* infection inhibited NR activity in the inoculated leaves of C_3 and CAM plants. The activity of GS remained unchanged, and the local response of glutamate-yielding enzymes, i.e. ferredoxin-dependent glutamate synthase (Fd-GOGAT), aspartate aminotransferase (AST), ALT and NAD-GDH, was specifically related to the metabolic type of photosynthesis. In CAM plants, Fd-GOGAT activity increased earlier, and NR was inhibited later than in C_3 plants (Gajewska et al. 2018). Interestingly, in CAM plants the nitrogen-metabolizing enzymes can participate in transmission of the local infection signal throughout the plant as their activity changes in the healthy upper leaves mirrored those in the infected ones. This was not observed in C_3 plants (Gajewska et al. 2018).

CAM type of photosynthesis was beneficial for the expression of resistance to *B. cinerea*, while C_3 was not. This could be also related to the interactive effects of abscisic acid (ABA) and SA accumulating after infection. Both plant hormones are known to be involved in abiotic and biotic stress defence, and the final plant response depends rather on the ABA/SA equilibrium than the absolute hormone levels (Jiang et al. 2010). As abiotic stress-induced ABA is known to influence the outcome of plant-pathogen interactions (Xu et al. 2013; Osakabe et al. 2014), the biosynthesis of ABA induced by decreased water potential in CAM plants could interfere with the resistance responses, e.g. those mediated by SA.

6 Conclusions

It was shown that CAM-performing plants of *M. crystallinum* were more tolerant to some abiotic stresses than the C_3 ones. This was related to CAM photosynthesis and nitrogen assimilation and higher basal levels of enzymatic and non-enzymatic antioxidants during some phases of the diurnal cycle (Niewiadomska and Borland 2007). The increased abiotic stress tolerance of CAM plants was suggested to be specifically connected to the photorespiration fluctuation (especially during III and IV phases of CAM) accompanied by the depletion of the malate/citrate pool (Fig. 2) and by oxygen production (Lüttge 2002, 2010; Miszalski et al. 1998) that leads to ROS generation (Voss et al. 2013). One could expect that the antioxidant system keeping ROS under control can be also involved in controlling pathogen development, as it was documented that ROS compounds often play a central role in the coordination of key metabolic processes and defence response against pathogens. As the global climate change is expected to lead to the emergence of new stress combinations affecting crop production, studies on the biotic factor response of facultative CAM plants, previously exposed to abiotic stress which induced the C_3-CAM transition, can provide reliable information on how plants deal with the unfavourable environment and respond to multiple stressors. By understanding these regulatory mechanisms, one could predict the outcome of pathogen infection under environmental pressure caused by abiotic stresses.

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The Role of the Shikimate and the Phenylpropanoid Pathways in Root-Knot Nematode Infection

Noureddine Hamamouch and Essarioui Adil

Contents

Abstract Plant-parasitic nematodes are costly burdens to crop production because of their intricate relationship with the host plants, wide host range, and the level of postinfection damage. Limitations on the use of chemical pesticides have brought increasing attention in studies on alternative methods for nematode control. Among the strategies of nonchemical nematode management is the identification and implementation of host resistance. Plant resistance involves the production of morphological barriers to prevent pathogens from entry into host cells or may include the synthesis of certain biochemicals that interfere with the subsequent development of pathogens. Among plant biochemical responses to infection is the synthesis of

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N. Hamamouch (✉)
Laboratory of Biotechnology and Sustainable Development of Natural Resources, Polydisciplinary Faculty, University Sultan Moulay Slimane, Beni Mellal, Morocco

E. Adil
National Institute for Agricultural Research, Errachidia, Morocco

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important and diverse compounds from the shikimate and the phenylpropanoid pathways. Many of these compounds are bioactive, playing important roles in defense against biotic and abiotic tresses. This review gathers information from across a large body of studies focusing on the role of the shikimate and the phenylpropanoid pathways in plant-nematode interactions.

Keywords Parasitic nematodes, Host resistance, Shikimate pathway, Phenylpropanoid pathway

1 Introduction

Plant-parasitic nematodes (PPNs) are agricultural pests that cause significant crop loss, estimated at up to $US 125 billion globally per annum (McCarter 2009; Nicol et al. 2011). There are over 4,100 species of PPN described to date (Decraemer and Hunt 2006), and they are classified into three groups, Triplonchida, Dorylaimida, and Tylenchida, with the majority of agriculturally damaging nematodes belonging to the last order (Tytgat et al. 2000).

PPNs deploy different parasitic strategies; they are either migratory or sedentary and can be either endoparasitic (enter the host and migrate through host tissues causing extensive damage) or ectoparasitic (nematodes never enter the host but simply migrate through the soil, using roots as an ephemeral food source as they encounter them) (Wyss and Grundler 1992; Sijmons et al. 1994; Tytgat et al. 2000). The feeding process damages the root system and reduces the plant's ability to absorb water and nutrients. Typical nematode damage symptoms are a reduction in root biomass, a distortion of root structure, and/or enlargement of the root. Nematode damage of the plant's root system also provides an opportunity for other plant pathogens to invade the root and thus further damage the plant. The most economically important nematodes, which belong to the sedentary endoparasitic group, are the root-knot nematodes (RKN, *Meloidogyne* species) and cyst nematodes (*Globodera* and *Heterodera* species), followed by the migratory endoparasites, the root lesion nematodes (*Pratylenchus* species), and the burrowing nematodes (*Radopholus* species) (Bird and Kaloshian 2003; Jones et al. 2013).

PPNs have evolved a highly specialized feeding structure, termed stylet (a protrusible hollow mouth spear) (Fig. 1), used to penetrate cells to allow feeding on plant tissues (Kikuchi et al. 2017). Parasitic nematodes secrete an array of effectors into host cells through their stylet to initiate the formation of specialized feeding structures known as syncytia for cyst nematodes and giant cells for root-knot nematodes (Fig. 1) (Wyss and Grundler 1992; Golinowski et al. 1996; Mitchum et al. 2013). These plant feeding cells, which are characterized by dense cytoplasm, enlarged multiple nuclei, a fragmented central vacuole, and proliferation of organelles (Jones 1981; Golinowski et al. 1996), act as the permanent source of nutrients for further nematode development. These secretions are accompanied by an extensive alteration of gene expression of parasitized plant cells and roots including genes related to defense responses, cell wall modifications, and metabolic pathways

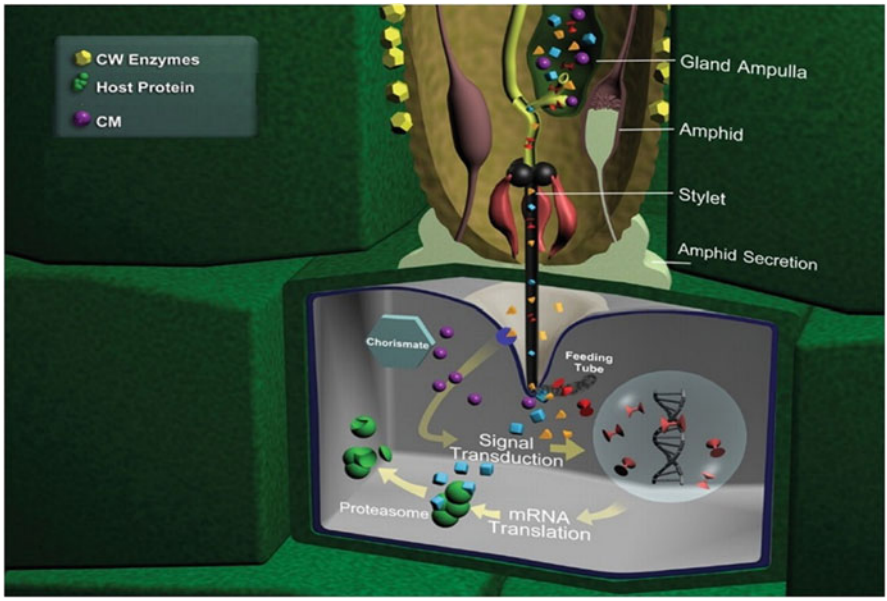

Fig. 1 A model of potential interactions of secreted nematode effectors into a host cell. Secretions are released into the host cells through the nematode's feeding spear (stylet). Cell wall (CW)-modifying proteins are secreted during migration of infective juveniles through host plant tissues. Other nematode secretions are involved in the formation of specialized feeding cells by the nematode, including effects on host cell metabolism by secreted CM. Modified from Davis et al. (2004)

(Puthoff et al. 2003; Jammes et al. 2005; Alkharouf et al. 2006; Ithal et al. 2007; Gheysen and Mitchum 2009; Szakasits et al. 2009; Barcala et al. 2010). Two of the plant metabolic pathways that are involved in plant interactions with parasitic nematodes are the shikimate and the phenylpropanoid pathways.

2 The Shikimate Pathway

The shikimate pathway (Fig. 2) connects primary metabolism to aromatic acids (phenylalanine, tyrosine, and tryptophan) biosynthesis. In a sequence of seven metabolic reactions, phosphoenolpyruvate and erythrose 4-phosphate are converted to chorismate. The enzyme chorismate mutase (CM) then coverts chorismate to prephenate, which is the first step of phenylalanine and tyrosine biosynthesis.

Although the shikimate pathway does not exist in animals, CM gene has been found in the root-knot nematode (Lambert et al. 1999; Doyle and Lambert 2003; Gao et al. 2003; Huang et al. 2005), potato cyst nematode (Jones et al. 2003), sugar beet cyst nematode (*Heterodera schachtii*) (Vanholme et al. 2009), and soybean cyst nematode (Bekal et al. 2003). Although these species of nematodes possess either a

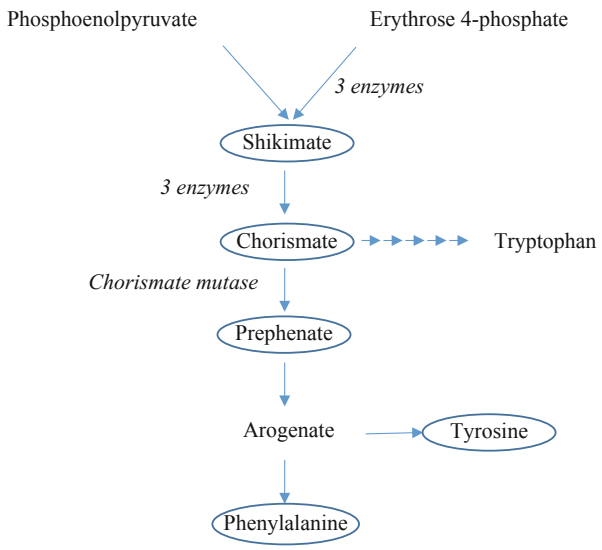

Fig. 2 A simplified shikimate pathway

single or two CM genes, they still lack the other genes of the shikimate and aromatic amino acid biosynthesis pathways (Lambert et al. 1999; Popeijus et al. 2000; Bekal et al. 2003). Because plants have the shikimate pathway and nematodes have a secreted form of CM, this enzyme is thought to interfere with the plant's endogenous shikimate pathway and alter the regulation of this pathway for the benefit of the parasite. The secreted nematode CM may increase the flow through the cytosolic branch of the shikimate pathway, thereby decreasing the biosynthesis of plastid-derived phenolics. Increased flux through the cytosolic branch of the shikimate pathway may be one strategy the nematode uses to decrease accumulation of plastid-derived phenolic compounds known to mediate plant defense responses, thereby suppressing plant defense.

Transgenic expression of the *Meloidogyne javanica* nematode MjCM1 gene in plant suppresses lateral rot formation and the development of the vascular system, which can be rescued by exogenous application of indoleacetic acid, suggesting that the expression of MjCM1 reduces auxin levels (Doyle and Lambert 2003). Since chorismate is also a precursor for the synthesis of the plant hormones auxin and salicylate, the expression of the MjCM1 in plant cells is thought to competitively reduce the fluxes toward (1) the synthesis of Trp and its downstream hormone auxin and (2) the synthesis of salicylate directly from chorismate.

3 The Phenylpropanoid Metabolism

The phenylalanine ammonia lyase (PAL) is a key enzyme in the phenylpropanoid pathway; it catalyzes the non-oxidative deamination of phenylalanine (one of the end products of the shikimate pathway) to trans-cinnamate and directs the carbon flow from the shikimate pathway to the various branches of the phenylpropanoid metabolism, including lignols (precursor to lignin and lignocellulose), flavonoids, isoflavonoids, anthocyanins, and stilbenes, coumarins, hydroxycinnamic acid conjugates, and lignans (Fig. 3) (D'Auria and Gershenzon 2005).

Expression of PAL gene upon nematode infection has been documented in different studies. Transcription of PAL gene increased in resistant, but not in susceptible, soybean cultivars after *Heterodera glycines* and *M. incognita* infections (Edens et al. 1995). In addition, induced resistance in tomato plants activates the expression of PAL gene when the plant is infected with *M. incognita* (Vasyukova et al. 2007) and with the potato cyst nematode (*Globodera rostochiensis*) (Uehara et al. 2010). Similarly, in maize-resistant lines, expression of PAL4 gene was highly induced upon infection with *M. incognita* compared to susceptible lines (Starr et al. 2014).

Moreover, activity of PAL enzyme after nematode infection has also been reported. PAL enzyme activity increased in resistant tomato roots infected with RKN (Breuske 1980), in resistant soybean cultivars after *H. glycines* and *M. incognita* infection (Edens et al. 1995), and in resistant potato plants infected with *H. rostochiensis* (Giebel 1973).

Recent studies using comparative microarray analysis of an incompatible and compatible response of soybean plants to *H. glycines* indicated no major change in expression of genes encoding PAL in the compatible interaction. However, at 3, 6, and 9 days after infection in the incompatible interaction, genes encoding PAL

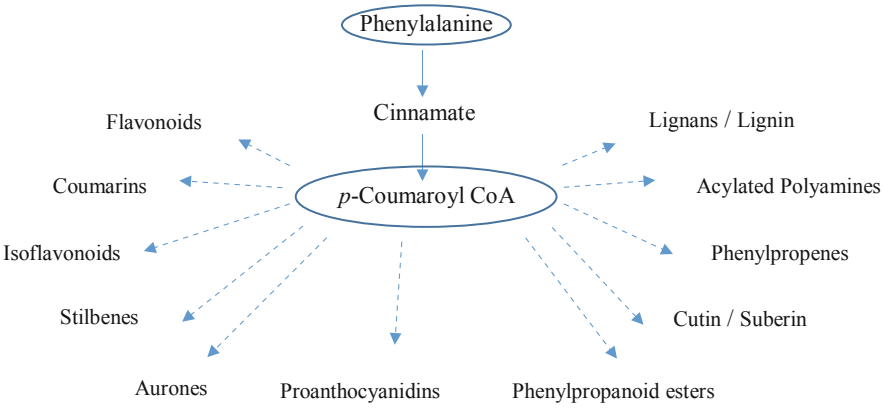

Fig. 3 A simplified phenylpropanoid biosynthesis pathway

increased in expression 20 to more than 40 times, indicating an increased metabolic flow into the pathway (Klink et al. 2007a).

The observed increased PAL gene expression and PAL enzyme activity upon nematode infection may induce the phenylpropanoid pathway for the timely synthesis of flavonoids, lignin, and phenolics to protect the resistant plants from nematode infection, or it may induce synthesis of salicylic acid as a signal transducer to induce defense mechanisms (Silverman et al. 1993; Vernooij et al. 1994). Though these results reflect whole plant response to nematode infection in terms of PAL gene expression or PAL enzyme activity, they do not necessarily reflect the expression level of the gene inside the nematode feeding structures. Transgenic plants transformed with promoter: GUS reporter gene offer the advantage of directly observing the gene expression level inside the nematode feeding structures. Transgenic tobacco and *Arabidopsis* plants expressing the PAL promoter: GUS indicated that PAL gene was strongly downregulated in both *H. schachtii* and *M. incognita* feeding structures (Goddijn et al. 1993). Absence of PAL activity could be a prerequisite for nematode feeding site development since phenylpropanoids play key roles in the protection against pathogens. The effect of PAL overexpression, inside the nematode feeding site, on nematode development remains to be examined.

4 Lignin

From phenylalanine, lignin biosynthesis proceeds via a series of side-chain modifications and ring hydroxylations and *O*-methylations leading to the production of hydroxycinnamyl alcohols, also known as monolignols. Monolignols are the building blocks of lignin, which confers structural support, vascular integrity, and resistance to plants against pathogens.

Lignin synthesis plays a role in plant-nematode interactions. It has been reported that resistance to migratory nematodes correlates with increased lignin deposition in the cell walls of resistant banana plants (Wuyts et al. 2006; Dhakshinamoorthy et al. 2014). In addition, susceptible tomato plants treated with benzothiadiazole (BTH), which acts as a priming agent in plant defense leading to a reduction in penetration and development of the root-knot nematode *M. incognita,* revealed accumulation of higher lignin levels at later infection stages compared to non-treated plants (Veronico et al. 2018).

The timing of its synthesis influences strongly plants' susceptibility to nematode infection. Expression of lignin synthesis-related genes was faster and greater in resistant tomato cv. Rossol roots after infection with RKN compared to susceptible tomato cv. Roma (Veronico et al. 2018; Wuyts et al. 2006).

In soybean plants, upregulation of genes involving lignin biosynthesis has also been shown in the nematode feeding structures. Global analysis of gene expression changes in soybean plants infected with nematode revealed that genes involved in lignin biosynthesis are upregulated in both giant cell and syncytium such as the gene encoding quercetin 3-*O*-methyltransferase (OMT) (Ithal et al. 2007) and caffeoyl-

coA-*O*-methyltransferase genes (CCAO-OMT12 and 10) (Ibrahim et al. 2011). In addition, soybean genes encoding a family of 21 extensin peroxidases that participate in lignin biosynthesis were also upregulated in *M. incognita*-formed giant cells (Ibrahim et al. 2011).

In *Arabidopsis*, it has been shown that root-knot nematodes activate expression of *Arabidopsis* caffeic acid *O*-methyltransferse 1 (COMT1) gene early in cells of the swelling gall, in giant cells, and in surrounding dividing cells (Quentin et al. 2009). COMT1 expression was maintained in the mature root gall until 21 days after inoculation, in both the giant cells and their neighboring cells. However, knockout of COMT1 did not have any observable effect on the mean number of galls established 3 weeks after inoculation (Quentin et al. 2009).

Induced expression of gene involved in lignin biosynthesis inside the nematode feeding sites is not surprising, as both giant cells and outer walls of syncytia induce thickening of the cell wall through callose or lignin deposition (Grundler et al. 1998) and peroxidase activity (Ithal et al. 2007) probably to withstand the rising pressure of feeding sites.

5 Flavonoids

Flavonoids constitute a large class of secondary carbon-based metabolites present in all land plants. More than 10,000 different types of flavonoids have been described from a variety of plant species. Flavonoids are induced in response to pathogen attack including fungi (Christensen et al. 1998), bacteria (Shirley 1996), and insects (Misra et al. 2010; Diaz Napal et al. 2010; Thoison et al. 2004).

Flavonoids are also induced in response to nematode infections. Specifically, flavonoids have been shown to be induced in response to infection with the root-knot nematodes (Hutangura et al. 1999) and also in or around developing syncytia of *H. schachtii* early during infection and around galls formed by *Xiphinema diversicaudatum* (Jones et al. 2007). However, mutant lines defective in various parts of the flavonoid biosynthetic pathway did not show a reduction in nematode development; on the contrary, these plants were more susceptible to nematode infection than the control plants suggesting that flavonoids are produced in plants as part of the defense response to nematode infection.

There are several flavonoid subgroups based on their structural properties, including the chalcones, flavones, flavonols, flavandiols, anthocyanins, condensed tannins, aurones, isoflavonoids, and pterocarpans (Winkel-Shirley 2001; Hassan and Mathesius 2012).

6 Flavonols

Flavonols, a subclass of flavonoids, are the most abundant flavonoids. They possess potent-free radical scavenging activity (Braca et al. 2003) and have insecticidal properties (Simmonds 2003). In fact, soybean (*Glycine max*) genotype PI 227687, which accumulates the flavonol rutin, has been used widely in breeding programs as a source of insect resistance (Hoffmann-Campo et al. 2006). Flavonols are also involved in modulating auxin transport and signaling (Bohm 1998; Harborne and Williams 2000; Kobayashi et al. 2004). Several studies support a role of auxin in nematode feeding site formation of both cyst and root-knot nematode (Gheysen and Mitchum 2011; Goverse et al. 2000; Hutangura et al. 1999; Karczmarek et al. 2004). Moreover, flavonols have been shown to have direct effect on chemotaxis, motility, and egg hatching of many nematode species (Wuyts et al. 2006). Kaempferol (flavonol) has been shown to inhibit egg hatching in *Radopholus similis* (Wuyts et al. 2006). The flavonols (kaempferol, quercetin, and myricetin) repelled and slowed *M. incognita* juveniles (Wuyts et al. 2006). Patuletin, patulitrin, quercetin, and rutin were shown to kill the juveniles of *H. zeae* at various concentrations and duration of exposure (Faizi et al. 2011).

Flavonol effect on PPRs is species-specific. Using similar concentrations of flavonols, kaempferol, quercetin, and myricetin repelled *M. incognita* and *R. similis* juveniles but not *Pratylenchus penetrans*, whereas the flavonols inhibited the motility of *M. incognita* juveniles, but not *R. similis* and *P. penetrans* juveniles (Wuyts et al. 2006).

The differences in flavonol effects in different nematode species are likely due to the differences in chemosensory receptors, flavonoid receptor binding affinities, cell signaling cascade, and solute permeability across the cuticle in different species.

In *Arabidopsis*, a gene encoding flavonol synthase1 (FLS1) enzyme is strongly downregulated in both syncytia and giant cells as indicated by histochemical analysis of GUS gene expression (Fig. 4), suggesting that downregulation of this gene is

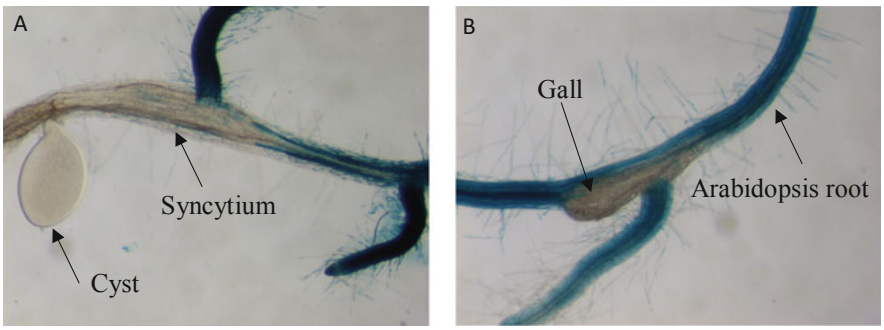

Fig. 4 Expression analysis of *Arabidopsis AtFLS*-1 gene during nematode infection. Histochemical localization of GUS activity directed by *AtFLS-1p*::GUS fusion in *H. schachtii*-induced syncytia (**a**) and in *M. incognita*-induced giant cells (**b**)

important in the formation and/or maintenance of the nematodes' feeding sites. Downregulation of flavonol synthase gene in the nematode feeding sites is surprising, as nematodes have been shown to induce reactive oxygen species formation in their feedings sites (Siddique et al. 2014; Melillo et al. 2006) and flavonols have very strong scavenging activity. It would be interesting to see if overexpression of FLS1 and fls1 mutant has any effect on nematode's development.

7 Isoflavonoids

Isoflavonoids also play a role in plant-nematode interaction. It has been reported that isoflavonoids are produced in infected roots of both *H. glycines*-resistant Hartwig and susceptible Essex soybean (Kennedy et al. 1999). A recent study by Chin et al. (2018) showed that isoflavonoids are elicited in high amounts in *Medicago truncatula* in response to *M. javanica* infection. Examination of transgenic *M. truncatula* plants which over- and under-produced isoflavonoids indicated that the early production or very high accumulation of isoflavonoids resulted in less severe infection. Specifically, the isoflavonoid, afromosin, and an isoflavonoid derivative, medicarpin, were effective in inhibiting nematode motility and in repelling nematodes in vitro, and the accumulation of the isoflavonoid formononetin and medicarpin in the roots of resistant white clover is believed to exhibit a defensive role on the stem nematode *Ditylenchus dipsaci* (Cook et al. 1995); the isoflavonoid glyceolin was found to accumulate close to the nematode's head in a resistant cultivar but not in susceptible plants (Huang and Barker 1991).

Expression of genes involved in isoflavonoid production has been shown to increase during nematode infection. Klink et al. (2007b) examined gene expression in roots of soybean cv Peking infected with incompatible and compatible populations of *H. glycines* and found that expression of the gene encoding chalcone synthase (CHS) was more than 40-fold in the incompatible interaction at 3 and 9 days postinfection. However, there was no change in the compatible interaction, while one gene encoding chalcone isomerase (CHI) was elevated 4-, 6-, and 17-fold in the incompatible interaction.

Global analysis of gene expression changes in soybean after infection with *H. glycines* also showed that the CHS and CHI are upregulated in nematode-infected roots (Ithal et al. 2007). However, when CHS gene expression was inhibited by RNAi in *Medicago trunculata*, root-knot nematodes were still able to form gall although the galls formed were smaller compared to control plants (Wasson et al. 2009).

8 Coumarins

Coumarins are ubiquitously found in higher plants where they originate from the phenylpropanoid pathway, and they are involved actively against a wide range of microbes (Brooker et al. 2008). Coumarins are induced in response to fungal and insect attack (Olson and Roseland 1991; Rahman 2000) and may be involved in defense against pathogenic fungi and insects (Brooker et al. 2007; Razavi et al. 2010; Sharma et al. 2006). In plant-nematode interaction, coumarins (8-geranyloxypsoralen, imperatorin, and herclenin) lethal to *B. xylophilus* have been identified in the roots of *Heracleum candicans* Wall (Wang et al. 2008). Coumarins (osthole, columbianadin, bergapten, and xanthotoxin) isolated from roots of *Angelica pubescens* (Duhuo) were very toxic against *B. xylophilus* with a mortality rate of 95.25% in 72 h at 1.0 mg/mL (Guo et al. 2018). Coumarins from *Ficus carica* leaves (psoralen and bergapten) also exhibited a strong effect on *B. xylophilus* with a mortality rate of 91% within 72 h at 1.0 mg/mL. Furanocoumarin (xanthotoxin, psoralen, bergapten, and oxypeucedanin) from parsley exhibits significant nematicidal activity against *M. incognita*, *M. hapla*, and *M. arenaria*. Addition of fresh parsley paste to soil reduced the number of *M. incognita* females and plant galls on tomato roots (Caponi et al. 2015).

9 Tannins

Tannins are a group of water-soluble polyphenolic compounds that have the ability to precipitate proteins and other molecules such as polysaccharides, lipids, as well as metal ions (Schofield et al. 2001; Jakobek 2015). They are found in higher plants and are grouped into two classes, termed condensed (syn. Proanthocyanidins) and hydrolyzed tannins. Tannins are toxic to a wide range of fungi, bacteria, and yeast (Scalbert 1991). Studies on the effect of tannins on plant-parasitic nematodes are few. Tannins from chestnut significantly reduced egg hatching of the root-knot nematode *M. javanica* (Maistrello et al. 2010; D'Errico et al. 2018). Tannins in the extract of *Fumaria parviflora* have been shown to have strong nematicidal effects on J2 and eggs of *M. incognita* (Nax et al. 2013). Low concentration of tannic acid (less than 40 mg/L) increased hatching of *H. glycines* eggs. However, higher concentrations inhibit egg hatching (Chen et al. 1997).

The behavioral response of nematode species to tannic acid is variable. Hewlett et al. (1997) found that tannic acid was attractive for *M. arenaria* and *M. incognita*, whereas it was repellent for *R. similis*, and no effects were observed on *H. glycines*. The behavioral response of *Meloidogyne* J2s to tannic acid indicates that tannic acid may be used by the nematode as a chemical signal to locate roots of host plants. Soil treatments with tannic acid were found to control *M. arenaria* on squash (Mian and Rodriguez-Kabana 1982a, b).

10 Conclusion

The phenylpropanoid-derived compounds play essential roles in plant defense against pathogens. They have been identified in all defense reactions, including constitutive phytoanticipins, inducible phytoalexins, signaling molecules, and many other metabolites that are yet to be identified. Several benefits may result from the identification and characterization of phenylpropanoid-derived compounds such as nematode repellants, hatching stimulants or inhibitors, and nematoxicants. These compounds can be developed for use as nematicides themselves, or they can serve as model compounds for the development of chemical synthesized derivatives with enhanced activity. In addition, transcript profiling of key genes of the phenylpropanoid pathway during nematode infection could identify potential gene targets for nematode control strategies. Genes whose expression augments in response to nematode infection can be used as targets for knockout purposes. Overexpression of genes whose expression is downregulated during nematode can also be used as alternative method in nematode control strategies.

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Functional Diversity of Photosynthesis, Plant-Species Diversity, and Habitat Diversity

Ulrich Lüttge

Contents

Abstract Photosynthesis is the basis of productivity. With that it is prerequisite of growth, which is the driver of competition between plants and therefore of fitness for occupying space. Modes of photosynthesis are C_3 , C_4, and C_2 photosynthesis and crassulacean acid metabolism (CAM), each with plasticity of modifications conveying flexibility and considerable functional diversity. At the level of macrohabitats, i.e., ecosystems and biomes, vegetation with dominant C_3 and C_4 photosynthesis, respectively, or prominent contribution of CAM, can be distinguished. At finer scaling levels, this becomes more difficult. At the community level, C_4 and CAM vegetation as well as C_3 vegetation, where CAM and C_4 species are conspicuously immersed, can be observed. At the microhabitat level species with different modes of photosynthesis often are found to occur sympatrically side by side even with very similar life-forms and appear to be equally successful in adapting or acclimatizing to environmental conditions. They get involved in interactions of competition and

U. Lüttge (✉)
Department of Biology, Technical University of Darmstadt, Darmstadt, Germany
e-mail: luettge@bio.tu-darmstadt.de

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facilitation. Diversity of modes of photosynthesis and their plasticity create various options, but at the level of microhabitats, ecophysiological performance with respect to photosynthesis often does not explain distinct distributions of plant species. Plasticity and diversity imply complexity. "Diversity creates diversity." Understanding biodiversity means understanding complexity. The diversity of arrivals of plants carrying a diversity of functional traits of photosynthesis at sites governed by chance and their establishment at dynamically variable conditions appears best leading us to an understanding of why so often we find that ecophysiological performance per se is insufficient for explaining local distribution of plants and habitat occupation.

1 Introduction

Fitness of plants is often considered in a basically traditional Darwinian sense of reproductive fitness. It was rejected that photosynthetic performance could be closely related to fitness: "Direct correlation between photosynthetic rates and fitness are rarely observed in natural populations; ..... it is impossible to say that variation in photosynthetic rate, per se, contributed directly to individual fitness" (Ackerly et al. 2000; see Lüttge 2007c). On the other hand, photosynthesis is the basis of productivity of individual plants. This is prerequisite of growth, and growth is the driver of competition between plants and with that their fitness (Cahill 2013; Souza and Lüttge 2015). Clonal propagation, i.e., just by growth, demonstrates the success of plants and hence fitness in the absence of sexual reproduction. Clonal vegetation may have enormous extensions. In the Qira Oasis of the Taklimakan Desert in China, a clone of the trees of *Populus euphratica* Oliv. has been identified covering an area of at least 4 ha (Brulheide et al. 2004). *Populus tremuloides* Michx. is found to form clones of 43 ha in the Wasatch Mountains in Utah, USA (Grant 1993), or even 81 ha elsewhere (Kemperman and Barnes 1976). This substantial growth and competition for space occupation cannot happen without photosynthetic productivity. It reveals vegetative fitness.

Sage and Stata (2015) have addressed diversity in a review of how "photosynthetic diversity meets biodiversity." Diversity means variety. We can consider variety at many levels, e.g., the floristic level of various individual species in a habitat, the level of various functions carried by species, and the level of various habitats. These will be covered in the present essay. Sage and Strata say that, ".... a failure to understand how photosynthetic diversity influences ecological and evolutionary processes handicaps our ability to understand patterns of biodiversity through time." In this vein, while functional diversity is an emergent outcome of integration and interaction of many different functional traits, we may focus here on functions of photosynthesis which by themselves again are displaying diversity.

The advanced technical development and miniaturization of equipment for measuring gas exchange of leaves and chlorophyll fluorescence has it allowed to move from physiological autecology of individual plants and species to functional

photosynthetic synecology at natural sites in the field (Lüttge and Scarano 2004, 2007; Lüttge 2005). We can ask the question here if functional diversity of photosynthesis relates to niche occupation. For obtaining a survey of the functional diversity of photosynthesis, we first must have a brief look at the various modes of photosynthetic carbon gain that have evolved.

We then need to distinguish their impact in relation to the spatiotemporal dynamics of the occupation of space, where we move from macrohabitats within larger ecosystems and biomes across to sizeable plant communities and eventually to microhabitats in mosaic-like structures of vegetation. Individual species may show various degrees of plasticity, i.e., intraspecific diversification, expressing modes of photosynthesis with certain modifications or different modes of photosynthesis at different times under different conditions. From functional plasticity within individual species to functional diversity between various different species with their occupation of micro- and macrohabitats and the mosaic of microhabitats within macrohabitats, we move along a gradient of increasing complexity (Fig. 1). Complexity is increasing with the increasing number of degrees of freedom of

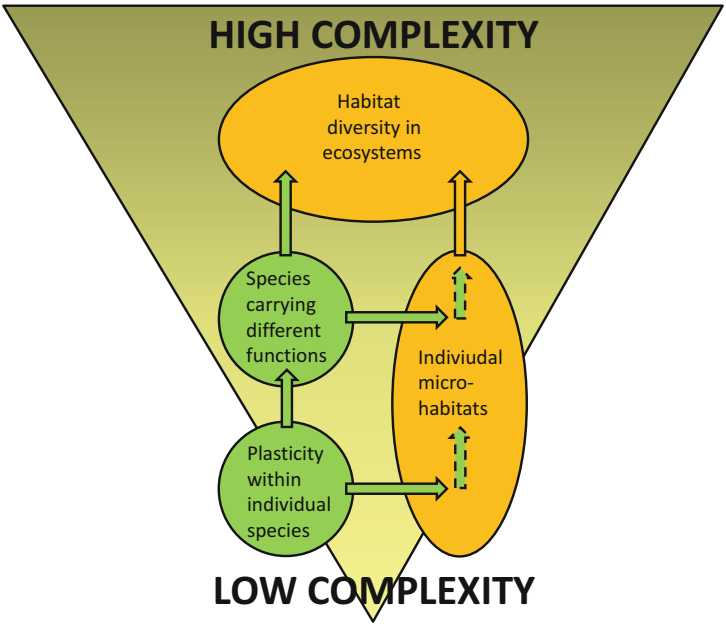

Fig. 1 Complexity increases in a network-like manner from intraspecific plasticity and interspecific functional diversity as species occupy individual microhabitats or a diversity of habitats in ecosystems consisting of a diversity of microhabitats. The lowest degree of complexity we have with individual species endowed with intraspecific plasticity in individual microhabitats. A higher degree of complexity is attained at the microhabitat level with a combination of species carrying different functions, i.e., interspecific diversity. Diversity in ecosystems is established by integrating species with different functions and/or individual microhabitats with a diversity of species, so that still higher degrees of complexity are reached

performance of systems given by the plasticity and diversity of their components, here the plant species in the habitats and ecosystems.

We know that biodiversity is the basis of sustainability and stability (Tilman et al. 1996, 2001, 2006; Hector et al. 1999; Schläpfer and Schmid 1999; Roscher et al. 2005; Balvanera et al. 2006; Griffin et al. 2009; Weigelt et al. 2009; Proulx et al. 2010; Körner 2012; Loreau and Mazancourt 2013; Oliver et al. 2015; Souza and Lüttge 2015; Lüttge 2016). Biodiversity implies complexity. With this in mind, the question of how we may explain and understand diversity broadens itself. Is it not much more than establishment of individual plants carrying different functional traits and contributing to functional diversity? Is it rather an evolution of habitats toward a complexity which supports sustainment? Sustainment includes an idea of stability. However, since all systems of life are open non-steady-state systems with a continuous flow of matter and energy through them, this must be a very dynamic stability. The idea of habitat evolution is currently of high actual relevance in view of changes of climate which affect the occupation of habitats by plants and where the complexity of biodiversity opens avenues for changing plant distribution.

2 Modes of Photosynthetic Carbon Gain

The principal modes of photosynthesis are listed in Table 1.

Table 1 Modes of photosynthesis

Mode of photosynthesis	Basic mechanism	Sub-modes	Timing of light reactions and CO_2 assimilation
C_3	Primary CO_2 fixation by RUBISCO[a]		Simultaneous
C_4	Primary CO_2 fixation by PEPC[b], CO_2 pumping to RUBISCO via malate or aspartate	C_3/C_4 intermediate	Simultaneous
C_2	Primary CO_2 fixation and O_2 fixation by RUBISCO, CO_2 pumping to RUBISCO via respiratory glycolate		Simultaneous
CAM[c]	Primary CO_2 fixation by PEPC, CO_2 pumping to RUBISCO via malate or citrate	Idling, cycling, C_3/CAM intermediate	Separated during nighttime and daytime, respectively

[a]Ribulosebisphosphate carboxylase/oxigenase
[b]Phospho*enol*pyruvate carboxylase
[c]Crassulacean acid metabolism

2.1 C_3 Photosynthesis

The evolution of green autotrophic organisms began with C_3 photosynthesis. Primary fixation of CO_2 is mediated by the enzyme ribulosebisphosphate carboxylase/oxigenase (RUBISCO). Light reactions of photosynthetic electron transport providing the reducing power and the ATP energy for the reduction and assimilation of CO_2 must occur simultaneously in the light period. When RUBISCO evolved 3.5×10^9 years ago, atmospheric CO_2 concentrations were extremely high at 5–10 bar. The concentrations of O_2 were negligible and only began to rise with the evolution of O_2-producing photosynthesis. $2.0–1.5 \times 10^9$ years ago when photosynthesis already had fully evolved, atmospheric O_2 concentrations were only 0.2% (Berner 1994; Matyssek and Lüttge 2013). These conditions at its early stages have not exerted selective pressure for high affinity to the substrate CO_2 of RUBISCO, and throughout its evolution to date, it has preserved a low affinity to CO_2 and a structurally given affinity to oxygen. This is quantified for RUBISCOs of different species by the specificity factor with the ratios of V_{max} (maximum rates) and K_m (Michaelis constants) for the reaction with O_2 and CO_2, respectively (Spreitzer and Salvucci 2002; Heldt and Piechulla 2008; Lüttge 2011). The reaction with O_2 is responsible for photorespiration.

C_3 photosynthesis is the basic and most broadly expressed mode. The other modes listed in Table 1 have evolved from it. Of particular ecophysiological relevance is the fact that at a current atmospheric CO_2 concentration of about 0.04%, RUBISCO is working much below substrate saturation and there is photorespiration with its demand of energy and the loss of previously fixed carbon. C_3 photosynthesis shows plasticity of ecophysiological performance. Plastic reactions occur in response to the water factor with stomatal regulation of the conductivity for water vapor (g_{H2O}) and the associated water-use efficiency (WUE). Expression of a midday repression of photosynthesis with stomatal closure often is an answer to stress given by low availability of water and high incident irradiance creating a high evaporative demand of the atmosphere (Schulze et al. 1974, 1975a, b; Tenhunen et al. 1984; Pathre et al. 1998; Franco and Lüttge 2002). Plasticity also adapts and/or acclimates plants to light regimes by the development of sun and shade types, respectively. Moreover, there is variable susceptibility to photoinhibition and oxidative stress by over-excitation of the photosynthetic apparatus (Osmond and Grace 1995). Overall the performance of C_3 photosynthesis inherently has a large potential of functional ecophysiological diversity.

2.2 C_4 Photosynthesis

There are some water plants performing C_3 photosynthesis which have evolved CO_2-concentrating mechanisms (CCM) to balance the low CO_2 affinity of RUBISCO (see special issue: Functional Plant Biology 2002). In prokaryotic

cyanobacteria, CO_2 is captured catalytically via redox-dependent mechanisms in the thylakoid membranes as HCO_3^- and/or by HCO_3^- transporters and concentrated in the protein structures of so-called carboxysomes where carboanhydrase regenerates CO_2. In the chloroplasts of eukaryotic algae, pyrenoids which are rich in RUBISCO and other photosynthetic enzymes play a similar role. However, higher land plants have evolved most sophisticated mechanisms of CCM. This began about 300–250 million years ago under the selective pressure of low CO_2 concentrations when the atmosphere had reached CO_2 levels as we have them also to date (lower than 0.04%). A very major CCM is the C_4 photosynthesis of angiosperms (see special issue: Journal of Experimental Botany 2017).

In C_4 photosynthesis, the primary fixation of CO_2 is not by RUBISCO with the C_3 product of phosphoglyceric acid but by phospho*enol*pyruvate carboxylase (PEPC), which has a 60-fold higher affinity to its substrate HCO_3^- than RUBISCO has to its substrate CO_2 (Spreitzer and Salvucci 2002; Heldt and Piechulla 2008; Lüttge 2010, 2011). With subsequent enzymatic steps, this generates a C_4 product mainly of malate or also of aspartate. An essential basis of the CCM is an anatomical differentiation in leaves, the so-called Kranz anatomy. There are specialized cells of an outer so-called mesophyll layer and an inner so-called bundle sheath layer. They can surround individual veins or also around leaves the entire system of veins (Lauterbach et al. 2017; Schüssler et al. 2017). PEPC operates in the mesophyll cells and concentrates carbon due to its high HCO_3^- affinity. In the bundle sheath, RUBISCO is operating for assimilation and eventual reduction of CO_2. The carbon pumping toward RUBISCO is achieved by transport of malate (aspartate) from the mesophyll to the bundle sheath cell layer, where by different decarboxylation reactions, CO_2 is made available to RUBISCO at elevated concentrations which are independent of the CO_2 concentration in the air. Anatomical variation or diversity among C_4 plants is given by the different types of Kranz anatomy. C_4 photosynthesis can even be performed by single cells with distinguished compartments with a dimorphism of chloroplasts when the cells are large enough allowing a path of diffusion between them (Jurić et al. 2017). Biochemical variation or diversity is given by the formation of malate or aspartate after primary fixation of CO_2 and by different types of the decarboxylation reaction for secondary fixation of CO_2 by RUBISCO and further assimilation.

The ecophysiological advantages of the CCM of C_4 photosynthesis are threefold: (1) it suppresses photorespiration, (2) it increases nitrogen-use efficiency (NUE), and (3) it increases water-use efficiency (WUE). In C_3 plants at ambient CO_2 concentrations, the CO_2 concentration at RUBISCO is about 6 μmol l^{-1}. In C_4 plants in the bundle sheath, it is much higher, which allows RUBISCO to operate at CO_2 saturation and prevents the reaction with O_2 and hence photorespiration and the loss of carbon and the consumption of energy associated with it. This is a special advantage at warm temperatures above 25°C because the ratio of O_2 fixation/CO_2 fixation by RUBISCO increases with temperature. The CCM allows effective operation of CO_2 assimilation in the bundle sheath at lower levels of the RUBISCO enzyme, i.e., lower investment of nitrogen. With the supply of CO_2 to RUBISCO via the CCM, stomatal regulation can reduce transpiratory loss of water vapor, while

CO_2 acquisition still remains sufficient. In this way WUE is improved. Thus, C_4 plants prefer sites with high irradiance, high temperature, and low water and have a particularly high productivity of new biomass. For these reasons, there is intensive research which is also motivated by the phenomenon of climate change and global warming and the hope to be able to engineer traits of C_4 photosynthesis into crop plants (Huang et al. 2002; Surridge 2002; Mitchell and Sheehy 2006; Hibberd et al. 2008; Li et al. 2017). We have meanwhile an enormous amount of information about C_4 photosynthesis including the molecular biology of its structural requirements and its enzymology (see special issue: Journal of Experimental Botany 2017).

2.3 C_2 Photosynthesis

A sub-mode of C_4 photosynthesis has been termed C_3/C_4 intermediate photosynthesis (Table 1). By contrast to crassulacean acid metabolism (CAM) where C_3/CAM intermediate performance implies shifts between the two modes in time (see Sect. 2.4), C_3/C_4 intermediate photosynthesis shares features of both modes. The major players are not C_3 or C_4 compounds but the C_2 compounds glycolate and glycine. A more appropriate term therefore is C_2 photosynthesis (Bellasio 2017). The C_4-like feature of it is compartmentation between mesophyll and bundle sheath layers, i.e., an anatomical and not a biochemical trait. The C_3-like feature is reaction of RUBISCO with CO_2 and O_2 in the mesophyll cells. The latter leads to photorespiratory production of glycolate from which glycine is formed in the peroxisomes of the mesophyll. Glycine is transported into the bundle sheath where in the mitochondria one CO_2 and one serine are produced from two glycines. The CO_2 is concentrated at RUBISCO. The glycine is shuttled to the Calvin cycle in the bundle sheath chloroplasts via formation of hydroxypyruvate and glycerate. The biochemical C_2 pathway is simpler than the C_4 pathway, and it is argued that in evolution C_2 photosynthesis was a step toward C_4 photosynthesis.

The ecophysiological advantage of C_2 photosynthesis is that it prevents photorespiratory loss of carbon recapturing carbon skeletons from glycolate/glycine and that it also has a CCM function by generating CO_2 in the bundle sheath.

2.4 Crassulacean Acid Metabolism (CAM)

CAM is the only one of the modes where primary fixation of CO_2 and the light reactions of photosynthetic electron transport do not occur simultaneously. In C_3 photosynthesis, both processes are intimately connected. In C_4 and C_2 photosynthesis, the connection is given by the transport of metabolites between mesophyll and bundle sheath cells. However, in CAM plants primary CO_2 fixation occurs in the dark and is separated in time from CO_2 assimilation in the light.

In fully expressed CAM, we distinguish four phases (Osmond 1978). Nocturnal primary CO_2 fixation is mediated by PEPC. It is called phase I. Organic acids produced, mostly malic acid but in many cases also citric acid, are stored in the cell sap vacuoles overnight. In a transitory phase II in the morning, activity of PEPC is downregulated and RUBISCO upregulated. Then stomata close in phase III. Organic acids are remobilized from the vacuoles and decarboxylated. This is the CCM of CAM. Internal CO_2 concentrations established amount to 2-fold to 60-fold of ambient atmospheric levels (Lüttge 2002, 2004). When all the nocturnally stored organic acid is consumed, stomata can open in the later afternoon, and CO_2 can be taken up, fixed by RUBISCO, and assimilated entirely like in a C_3 photosynthesis fashion. This is phase IV of CAM.

Like C_4 photosynthesis, the CCM of CAM also evolved around 300–250 million years ago when atmospheric CO_2 concentrations were low. However, the predominant ecological advantage of CAM in extant plants is saving water. Nocturnal carbon gain in phase I and stomatal closure in phase III during the hottest parts of the day when the driving force and demand for transpiration would be highest highly reduce transpiratory loss of water vapor. A major advantage of CAM is also the enormous intrinsic plasticity of the expression of its features (Lüttge 2006). With increasing water problems, first the stomatal opening during phases II and IV is suppressed. Then the expression of phase I with stomatal opening is reduced. Simultaneously organic acid can still be produced by re-fixation of respiratory CO_2 by PEPC so that organic acids produced and stored can originate partly from atmospheric and from internally recycled CO_2, respectively. This can culminate in continuous stomatal closure day and night while carbon metabolism idles around internal respiratory liberation and photosynthetic re-fixation of CO_2. This mode is called CAM-idling. It reduces loss of water vapor to the minimal cuticular transpiration. A milder variant is CAM cycling where also respiratory CO_2 is re-fixed during the night but stomata open during the day when CO_2 coming from stored organic acid supplements CO_2 taken up from the atmosphere. CO_2 recycling reduces nocturnal loss of carbon and has aspects of CCM. A still higher degree of plasticity is expressed in many plant species that have been found to be genuinely C_3/CAM intermediate. They can switch between full C_3 photosynthesis and full CAM. This may be related to development, like in the annual halophyte *Mesembryanthemum crystallinum* L., where salinity stress induces CAM and this is not fully reversible (Winter and von Willert 1972; Ratajczak et al. 1994). In other species, it may be driven dynamically by environmental factors and be fully reversible with repeated switches between the two modes, like in the perennial leaves of species of *Clusia* (Lüttge 2006, 2007a, b, c, 2008b).

We have noted above that C_4 photosynthesis is much more productive than C_3 photosynthesis. Conversely CAM is inferior and its productivity is low and mostly relates to expression of phase IV (Green and Williams 1982; Acevedo et al. 1983; Luo and Nobel 1993; Nobel 1996; Lüttge 2004, 2010). It provides adaptation to extreme stress of low water supply. With its extraordinary plasticity, i.e., expression of phases, idling and cycling, and C_3/CAM intermediacy, it adapts to multifactorial stress (Lüttge 2004, 2010) and episodic stress. It is not a strategy for productivity but a strategy for survival.

2.5 *Dialectics of Feedback: Environment Shapes Species; Species Shape Environment*

The comprehensive survey of modes of photosynthesis above and in Table 1 and their adaptive ecophysiological benefits, although just a rather abbreviated summary of much general knowledge based on textbooks, appeared to be necessary for allowing us now to set out for fathoming their relations to establishment of plants in habitats. Evidently, by evolutionary pressure for natural selection, the environment shapes species. Conversely, with Hegelian dialectics, we can hold that species shape the environment. This extends considering evolution from creating species diversity toward creating habitat diversity and further on to emerging integrated systems at higher scale levels of communities, ecosystems, biomes, and eventually the entire biosphere (Lüttge 2012; Matyssek and Lüttge 2013) named Gaia by Lovelock (1979, 2009). Lovelock (2010) even suspects that mental engrossment of evolution might have been modified if Darwin had included Gaia as part of his reasoning.

However, for the nonlinear dialectic feedback between environment and species, we now better move on to considering this at three specific ecological scales, i.e., at the scale of the macrohabitats of large ecosystems up to biomes, at the scale of more selected plant communities, and at the scale of microhabitats of chessboard-like vegetation mosaics. With the dialectics of the diversity of functions and the diversity of niches given, we must reflect if we are really asking the right question, when in comparative ecophysiology of photosynthesis, we search for adaptive traits in plants to explain niche establishment. We shall see that this is particularly difficult for a mosaic of micro-sites. A synthesis does not appear to be at hand, caveat in and challenge for better understanding biodiversity in the future.

3 Habitats

3.1 *Macrohabitats: Ecosystems up to Biomes*

Macrohabitats are large ecosystems. Globally the most embracing ones are the zonobiomes as defined by Walter and Breckle (1984) and also recently surveyed in this series in the context of how plants shape the terrestrial environment on Earth (Lüttge 2016). To which extent does the diversity of modes of photosynthesis contribute to the establishment of vegetation in these global zones?

3.1.1 Dominant C_3 Photosynthesis

As it has originally evolved, C_3 photosynthesis remains globally by far the most dominant mode. Since C_4 photosynthesis is absent among tall trees (Sage et al. 1999;

Sage and Pearcy 2000) and CAM among bona fide trees with secondary growth only occurs in the neotropical genus *Clusia* (Lüttge 2007a, b, c), the extended vegetation of forests on Earth is a C_3 domain. This covers large zonobiomes, such as the vast boreal belt of conifers and the entire cold-temperate boreal zonobiome VIII, the temperate nemoral zonobiome VI with summer green deciduous forest, and the warm temperate zonobiome V with temperate rainforests. The huge zonobiome I of evergreen tropical rainforests also needs to be mentioned here, although we must note that at the levels of communities (Sect. 3.2) and micro-sites (Sect. 3.3), also CAM plants make a significant contribution. The tropical zonobiome II characterized by summer rain is shared by C_3 and C_4 photosynthesis. It bears various types of tropical forests with C_3 photosynthesis and extended savannas dominated by C_4 grasses. In the cold Arctic and Antarctic zonobiomes, C_3 photosynthesis is the only mode expressed, and this is also so for marine photosynthetic organisms if we exclude some isolated examples of individual species where it is assumed that they may show some C_4- or CAM-like features. Anatomical and morphological diversity is extremely large among the C_3 plants in these zonobiomes. Functional diversity more closely related to photosynthesis becomes pertinent at the community and microhabitat levels (Sects. 3.2 and 3.3).

3.1.2 Dominant C_4 Photosynthesis

Biome dominants among the C_4 species are listed by Sage (2016). Some of these I would rather prefer to place at the level of selected communities (Sect. 3.2.1). Nevertheless, C_4 species adapted to warm climates "dominate semi-arid and arid landscapes of low-to-mid latitudes" (Sage and Stata 2015). It is evident that C_4 grasses and sedges dominate the landscapes and determine the ecophysiological functioning of savannas, e.g., the Serengeti, in the tropical zonobiome II as well as of steppes in Asia and of prairies in North America in the arid temperate continental zonobiome VI to such an extent that they clearly are zonobiome markers. As argued by Sage and Stata (2015) without the ecological impact of the C_4 plants, these biomes may not exist at all. This is due to a competition of C_4 grasses, sedges, and eudicotyledons which prefer hot open environments, with C_3 woody species. Some shrubby woody C_4 species do exist, but there are no real C_4 trees (Sage et al. 1999; Sage and Pearcy 2000). C_4 plants have a low tolerance of shade. In the open habitats, they are better adapted to factors which suppress woodlands.

3.1.3 Prominent Contribution of CAM

With CAM being a rather perfect adaptation to limited supply of water, we might expect that CAM plants dominate dry zonobiomes such as zonobiome III with desert climate. However, this is not the case as we shall see in Sect. 3.2.2. There is no entire zonobiome greatly dominated by CAM plants. Perplexingly and counter intuitively, the only zonobiome where CAM plants make a very prominent contribution is

zonobiome I of evergreen tropical rainforests. This is due to the fact that these forests are very rich in epiphytes. Lacking soil contact, these plants are particularly exposed to water-supply stress, and CAM supports their establishment on branches and in canopies of trees. Of all vascular epiphytes, 57% are CAM plants, which may make up close to 30% of the entire leaf biomass in some tropical rainforests (Sect. 3.3.1; Lüttge 2004, 2010).

3.1.4 Conclusion

For the globally dominant C_3 photosynthesis, it is readily recognized that plants with this mode shape entire zonobiomes (Sect. 3.1.1). Geographically large-scale dominance is much more restricted for C_4 photosynthesis, i.e., to some savannas of the zonobiome II (Sect. 3.1.2). It is even more limited for CAM within zonobiome I (Sect. 3.1.3). The situation is very different at the lower scale of the community level. Due to habitat diversity within the zonobiomes, communities are selected, where both C_4 plants and CAM plants can characterize the vegetation.

3.2 Selected Communities

3.2.1 C_4 Communities

C_4 communities are tabulated by Sage (2016: Table 4). Besides the grassland communities of zonobiomes II and VI (Sect. 3.1.2), we find high abundance of C_4 species in azonal vegetation of some pedobiomes which are more determined by characteristics of the substratum than by the phytogeographic zone. Such communities are those of marshlands. They include saline marshes because many C_4 species express halophyte traits. Particularly coastal salt marshes can be mentioned here. The abundance of C_4 species across the European continent is correlated with temperature and the degree of aridity (Pyankov et al. 2010).

3.2.2 CAM Communities

Although CAM is the very adaptation to scarcity of water with its nocturnal carbon acquisition and to high irradiance with its CCM reducing photoinhibition, CAM plants do not occur or are extremely rare in very harsh deserts. This is due to succulence. Generally CAM plants are leaf or stem succulent because the nocturnal accumulation of organic acids requires rather large central cell sap vacuoles. This goes hand in hand with the storage of water (Lüttge 1986). With this CAM plants can overcome many weeks and even months without rain by CAM idling and using their internal water reserves with only minimal loss by cuticular transpiration. However, then they regularly need to refill the water reserves. Therefore they depend on

recurrent rainy seasons. They are absent from the harsh deserts of Asia, Australia, and the inner Sahara. In the extreme desert of the Negev in Palestine with the legendary 7 years of drought of the bible, the only CAM plant is *Caralluma negevensis* Zohary, which grows in protected mini-niches in the shade of rocks and is supplied with moisture by early morning dew (Lange et al. 1975). For a different reason, CAM succulents are also rare in the grasslands of savannas. Ellenberg (1981) points out that this is due to competition with grasses, including C_4 species, which rapidly tend to overgrow the CAM species with their lower productivity and growth rates. Conversely, in semideserts such as those of the southwestern USA and Mexico, the physiognomy of extended landscapes can be determined by stem-succulent Cactaceae and leaf-succulent Agavaceae which are CAM plants (Lüttge 2010). In Venezuela we find communities of large columnar cacti which – if we are permitted to call these tall plants "trees" – we may consider as "cactus forests" (Vareschi 1980). Semi-deciduous, deciduous, and shrub forests in the neotropics may have a dense ground cover of the CAM species *Bromelia humilis* Jacq. (Lüttge 2004, 2008a). In all of these cases, we can speak of typical CAM communities.

Besides that noted, there is a very large variety of plant communities with abundant CAM species as listed and described in some detail by Lüttge (2004, 2010). The epiphytic habitat of tropical rainforests was already mentioned (Sect. 3.1.3), where looking at microhabitats will show us interesting features of sympatric occurrence of C_3 and CAM plants (Sect. 3.3). In addition to the other communities already referred to above, there are CAM plants on lava fields, in salinas, in coastal sand dune restingas, and on inselbergs. In the restingas, the CAM species *Clusia hilariana* Schltdl. (Sect. 3.2.3.2) makes up more than 40% of the green coverage, so that the vegetation physiognomy has been named "*Clusia* scrub" (Dias and Scarano 2007; Martins et al. 2007) and CAM coverage dominates C_3 coverage. Another community with dominant CAM is the xerophytic thorn-bush vegetation in the south and southwest of Madagascar with a rich diversity of CAM-performing succulents of the Didiereaceae, Asclepiadaceae, Euphorbiaceae, Cucurbitaceae, Mesembryanthemaceae, Orchidaceae, and Vitaceae (Kluge et al. 1995).

3.2.3 Selected Variety of Communities with C_3 Species and CAM or C_4 Species Immersed

At the large scale of biomes, patterns of habitat occupation of plants related to modes and capacity of photosynthesis are clearly emerging (Sect. 3.1). To a certain degree at the scale of communities, C_4- or CAM-dominated vegetation types are also expressed in many cases (Sects. 3.2.1 and 3.2.2). However, the situation becomes increasingly more difficult and complex, and correlations dissipate at lower scaling levels. The C_3 vegetation of our planet is so immensely diverse that it is impossible to summarize patterns by a reasonable selection of particular communities. Within the aims of this article to assess relations of photosynthetic functional diversity and habitat diversity, it is only possible to try and advance with some biased choice.

I shall do it on the background of some work in which I was involved myself especially in the tropics of Venezuela and Brazil.

Coastal Sand Plains

At the Caribbean coast in northern Venezuela near the municipality of Chichiriviche, we find an alluvial sand plain supporting a diversity of plant communities (Medina et al. 1989). The open sand plain is covered by fresh water during heavy rains in the rainy season and by a thick salt crust in the dry season. Vegetation units are:

- Flooded areas with fresh water accumulated during the wet season
- A belt with small halophytes
- Grassland elevated between 5 and 10 cm from the flat of the sand plain
- Small vegetation islands of 3–10 m in diameter and 10–40 cm higher than the flat
- Deciduous woodland

(Medina et al. 1989). What about modes of photosynthesis in this geographically localized diversity of vegetation types?

C_3 photosynthesis. Most of the plant species found are C_3 plants. Obviously together with other traits for adaptation or acclimation, C_3 photosynthesis evidently is the most suitable mode under the variety of conditions in these coastal sand plains. Plants with C_4 photosynthesis and CAM are immersed in the C_3 vegetation.

C_4 photosynthesis. Small halophytes are the evergreen C_3 species *Batis maritima* L. and *Sesuvium portulacastrum* L. The C_4 plant *Portulaca rubricaulis* H. B. K. is growing sympatrically with them displaying a similar life-form but shedding its leaves in the dry season (Lüttge et al. 1989b). *Sporobolus* is a genus of C_4 plants. The grass *Sporobolus virginicus* (L.) Kunth is distributed over several of the vegetation units (Medina et al. 1989). *Portulaca rubricaulis* and *S. virginicus* are the only C_4 species in the entire community.

Crassulacean acid metabolism. In the grassland, we find some isolated stem-succulent columnar cacti performing CAM. On the small vegetation islands, scattered members of all subfamilies of the CAM-performing Cactaceae, i.e., Pereskioideae, Opuntioideae and Ceroideae, can be encountered (Lüttge et al. 1989a). The columnar cacti insulate themselves from salinity because they are giving up their absorptive fine roots during the dry season (Lüttge et al. 1989a). The CAM species *Tillandsia flexuosa* Sw. and *Schomburgkia humboldtiana* Reichb. grow epiphytically (Griffiths et al. 1989).

The terrestrial CAM bromeliad *Bromelia humilis* Jacq. is spatially prominent as it may build up more extended and denser layers (Lee et al. 1989). It forms tanks with overlapping leaf bases of its rosettes serving as phytotelmata to store rainwater and produces tank roots for absorption of water and nutrients. Soil roots may also participate in absorption, but they are normally not much developed, and the plants rather lie loosely on the ground. *Bromelia humilis* shows vigorous clonal growth by ramets, and it expresses three different phenotypes spanning different vegetation units. Large green rosettes grow in the semi-shade of the deciduous forest. Small

stunted rosettes with brightly yellow leaves are formed toward the open sand plain and on the vegetation islands. In between there are pale green rosettes of an intermediate size. The large green rosettes are productive and show net growth, while the yellow rosettes are just replacing necrotic leaves dying off at the bottom of the rosettes (Lee et al. 1989).

The various CAM species in the habitats of the sand plain are avoiders of the salinity stress by having only loose (*B. humilis*) or no soil contact (epiphytes) or by sacrificing their fine roots in the dry season (cacti). CAM with internal CO_2 recycling up to full CAM-idling allows all these salinity avoiders overcoming periods of water shortage.

It appears that C_4 photosynthesis and CAM are features of a few individual species niching themselves in among the communities of C_3 plants and not so much adaptations for dominant habitat occupation. That *B. humilis* can form denser and somewhat extended mats of plants may be rather due to its capability of clonal growth although CAM obviously is a survival strategy in the yellow individuals of the clones.

Atlantic Rainforest Complex

Synecology is based on habitat and species comparisons. To assess and depict the synecological and ecophysiological structure of landscapes at the community level, we aimed to draw matrices for a variety of habitats with ecophysiological data for a substantial number of species. For this we chose sites around the Atlantic rainforest of Brazil (Scarano and Lüttge 2004, 2005; Duarte et al. 2005; Lüttge and Scarano 2007). The montane Atlantic rainforest of Brazil is one of the 25 global hot-spots of species biodiversity (Ribeiro et al. 2009, 2011). It is bordered by a number of communities which build up the habitat diversity of the rainforest complex:

- Coastal sand dune vegetation of moist, dry, and intermediate restingas
- Dry sand dune forests
- Swamp forests
- Semi-deciduous dry forests
- Inselbergs
- High-altitude vegetation

(Lüttge and Scarano 2004, 2007; Duarte et al. 2005; Lüttge 2005; Scarano et al. 2005a; Gessler et al. 2008).

The Atlantic forest grows on tertiary ground. In phytogeographical history, species from the Atlantic forest have migrated into the younger vegetation types marginal to it and especially into the restingas (Rizzini 1979; Araujo 2000; Lüttge 2006). These are sandy coastal plains that date from the Quaternary which have hardly any endemic species. A comparison of the bushes of *Clusia hilariana* Schltdl. and *Andira legalis* (Vell.) Toledo having CAM and C_3 photosynthesis, respectively, and the taproot C_3-hemicryptophyte *Allagoptera arenaria* (Gomes) O. Ktze. suggested that morphological and physiological diversity interacted in supporting niche occupation in the restingas (Gessler et al. 2008). Among the species of the

genus *Clusia*, especially CAM plants were most successful to colonize restingas. These are *C. spiritu-sanctensis* G. Mariz & B. Weinberg, *C. hilariana* Schltdl., *C. fluminensis* Pl. & Tr., and *C. lanceolata* Camb. (Roberts et al. 1996; Reinert et al. 1997; Zaluar and Scarano 2000; Scarano 2002; Scarano et al. 2005a, b; Lüttge 2007c). There is also the C_3 species *C. parviflora* Saldanha & Engl. but less abundant and associated with semi-shade. For the understanding of biodiversity, it is noteworthy that on the restingas the *Clusia* species from the Atlantic rainforest are considered as nurse plants which by interactive facilitation have functioned as a basis for the establishment of plant diversity (Scarano 2002).

In several of these communities marginal to the Atlantic rainforest ranging from moist to rather dry conditions, a representative number of species have been studied (Scarano et al. 2001, 2005a, b; Duarte et al. 2005; Gessler et al. 2005a, b). C_3 plants and some CAM plants mix in the communities sympatrically. Specialists and generalists having broad and narrow habitat-type preference, respectively, were compared. Based on fingerprinting of the ecophysiologically relevant photosynthetic parameters of saturating irradiance ($PPFD_{sat}$) and light-saturated electron transport rate (ETR_{sat}), matrices of habitat occupation were drawn (Lüttge 2005; Lüttge and Scarano 2007). Specialists are limited to particular sites. Generalists can be equipped by a broad ecophysiological optimum or by plasticity of acclimation (Lüttge 2005). No generalizations could be perceived, however. Generalists and specialists were not forming groups of similarly performing plants. They did not always differ in ecophysiological behavior (Lüttge 2005; Duarte et al. 2005). Sometimes, however, noteworthily not always, ecophysiological performance and local abundance corresponded. While no abstractions about correlations of specific ecophysiological performance and site or habitat occupation can be derived, nevertheless a clear albeit somewhat dissipating conclusion emerges. In addition to the floristic diversity of species and the morphological diversity of life-forms, we have an overwhelming functional diversity of ecophysiological behavior including not only interspecific adaptations but also intraspecific variations of acclimation in time and space (Scarano et al. 2005a; Lüttge and Scarano 2007). One example which became obvious when designing the matrices was given by mapping the relations between photosynthetic capacity and water-use efficiency (WUE). Photosynthetic capacity was quantified by the rate of maximum photosynthetic electron transport (ETR_{sat}), and WUE was derived from carbon isotope discrimination during transpiration ($\Delta^{13}C$). From straightforward functional relations, one would expect high ETR_{sat} to be correlated with low WUE and vice versa, which in fact was observed in many cases. However, similarly correlations of high ETR_{sat}/high WUE and low ETR_{sat}/low WUE were also realized without any clear relations to site characteristics (Lüttge 2005). The different types of diversity multiply and create complexity in a completely nonlinear fashion (Fig. 2):

$$\text{Complexity } \Xi \text{ Species } \times \text{ Life Forms } \times \text{ Functions}$$

Inspection of niching in microhabitats (Sect. 3.3) will further support this notion of diversity and complexity.

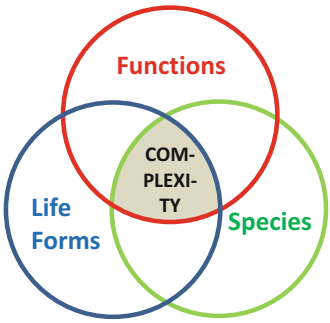

Fig. 2 The diversities of species, life-forms, and functions overlap in a nonlinear fashion creating complexity

3.3 Microhabitats

3.3.1 Epiphytes

Evergreen tropical rainforests constitute the huge zonobiome I of C_3 vegetation (Sect. 3.1.1). However, they also house the majority of the known CAM plants. CAM being an adaption to stress by limited water supply, this at a first glance appears counterintuitive. The major groups of desert succulents are the stem-succulent cacti and the leaf-succulent agaves, with about 1,500 and 300 species, respectively, almost all of them, i.e., about 1,800 species, are CAM plants. However, these are highly outnumbered by CAM epiphytes. About 10% of all vascular plants are epiphytes, i.e., approximately 23,500 species. These are predominantly Orchidaceae with about 19,000 species and Bromeliaceae with about 2,500 species; half of them, i.e., a total of about 10,700 species, are CAM plants. Fifty-seven percent of the epiphytes are CAM plants (Lüttge 1989, 2004; Zotz and Hietz 2001). The ecophysiological reason is that the supply of water is the dominating environmental problem in the epiphytic habitats to which CAM is the best suited adaptation (Zotz and Hietz 2001). Hence, we might consider this epiphyte vegetation as CAM communities. However, canopies of individual trees and isolated branches of trees have the characteristics of micro-sites.

Pittendrigh (1948) has made a census of all the about 70 bromeliad species of Trinidad and classified them as exposure group, sun group, and shaded group. Subsequently their mode of photosynthesis, C_3 and CAM, respectively, was determined, and their ecophysiology was studied (Griffiths and Smith 1983; Smith et al. 1985; Plant, Cell and Environment 1986). The relative distribution of C_3 and CAM bromeliads on the island geographically depends on rainfall, irradiance (cf. Pittendrigh's groups), and altitude. It increases with rainfall and altitude up to the upper montane rainforest while sharply declining in the subalpine rainforest (Smith 1989). There is a clear tendency for the CAM species to be centered on the drier sites and the C_3 species on the wetter sites. However, it is important to note that there is considerable overlap. In many sites both CAM and C_3 bromeliads of similar life-forms are found (Smith et al. 1986; Lüttge 1987). In some cases on the same tree

branch, a CAM and a C_3 bromeliad can be observed side by side where they look so similar that – if they are not flowering – species identification can best be made by mode of photosynthesis. They directly compete for space on their tree branch. We realize the diversity of modes of photosynthesis present but cannot deduce a specificity of prevalent site occupation at this microhabitat level.

3.3.2 Vegetation Mosaics

In a tropical vegetation mosaic, four species were compared which belong to the two subfamilies which were earlier distinguished in the Clusiaceae, i.e., Clusioideae and Kielmeyeroideae, and which are now considered different families, i.e., Clusiaceae and Calophyllaceae, respectively (Gustafsson et al. 2007; Cardoso et al. 2017), namely, *Clusia criuva* Camb. and *Clusia arrudea* Planchon & Triana ex. Engl. in the former and *Kielmeyera coriacea* Mart. & Zucc. and *Calophyllum brasiliense* Camb. in the latter family. The vegetation mosaic at the municipality of Tiradentes, Minas Gerais, Brazil, is made up of closely co-located habitats such as:

- Rupestrian grassland (rocky savanna)
- Cerrado/savanna
- Small rock outcrops
- Riverine and gallery forest alongside a small river

Among the four taxonomically closely related species, *K. coriacea* and *C. brasiliense* were C_3 plants, *C. criuva* was C_3/CAM intermediate, and *C. arrudea* had the capacity of CAM cycling (de Mattos et al. 2019). *Clusia criuva* mainly performed C_3 photosynthesis with midday depressions, and *C. arrudea* also made little use of the CAM-cycling option. It appeared that plastic performance of C_3 photosynthesis was the profitable choice in a diversity of habitats with a variety of conditions and CAM-type modes remained an escape for severe stress. Diversity of modes of photosynthesis and their plastic expression among the plants create various options, but ecophysiological performance with respect to photosynthesis did not explain distinct distributions in the microhabitats.

3.3.3 Atlantic Rainforest Micro-Sites

Besides investigations in the communities around the Atlantic rainforests of Brazil and influenced by it (Sect. 3.2.3), studies were also performed in the rainforest itself, namely, in the Santa Lúcia Biological Station, municipality of Santa Teresa, state of Espírito Santo, southeastern Brazil (Lüttge et al. 2015). The station protects 488 ha of montane and submontane ombrophilous dense forest at 600–900 m a.s.l.. The rainforest community is not homogenous. Three predominant vegetation physiognomies are:

- Forest on sharp hills

– Forest on flat lands mostly by the river Timbuí
– Granitic-gneissic rock outcrops

(Thomaz and Monteiro 1997). The forest structure covers a diversity of micro-sites. Ecophysiology of photosynthesis was studied in the sub-sites of hill forest and rock outcrops and the ecotones between them and in the riverine forest. Two groups of species were chosen. One group consisted of species widely distributed among the sub-sites, namely, *Clusia aemygdioi* Gomes da Silva & B. Weinberg, *Clusia intermedia* G. Mariz, and *Tibouchina heteromalla* (C. Don) Cogn. The other group contained species locally restricted to sub-sites, namely, *Clusia marizii* Gomes da Silva & B. Weinberg, *Clusia spiritu-sanctensis* G. Mariz & B. Weinberg, and *Kielmeyera occhioniana* Saddi. *Clusia spiritu-sanctensis* was an obligate CAM plant; the other three species were C_3 plants.

Compared to the restricted species, the widespread species were not more flexible and did not show superior performance in terms of ETR_{sat} irrespective of habitats. The widespread species *C. aemygdioi* and *C. intermedia* were equally plastic in their light use performance. Although *C. intermedia* was inferior in photosynthetic capacity expressed by ETR_{sat}, both species were equally abundant at a sun-exposed rock outcrop site. The C_3 species displayed high plasticity of acclimation producing sun- and shade-type plants. The CAM species *C. spiritu-sanctensis* showed very flexible expression of the CAM phases. It occurred both on the drier rock outcrops and in the moister and more shaded riverine forest, where it made much use of phase IV of CAM and was quite productive. Thus, CAM did not predetermine habitat distribution.

Overall, quite clearly ecophysiological performance of photosynthesis was not sufficient for explaining the local distribution of the plants among the micro-sites. Reproductive output including asexual and clonal reproduction to the extent that it determines propagation may have participated in modulating the diversity of species in the diversity of micro-sites. Spatiotemporal events such as arrival at sites and time of occupation of sites possibly may have been more important than a putative site-specific ecophysiological performance.

4 Competition and Facilitation: Interactions Between Plants with Different Modes of Photosynthesis in Their Habitat

Among the small number of great hypotheses which currently guide our thinking in ecology, we have the stress-gradient hypothesis (SGH) and the growth-differentiation balance (GDB) hypothesis. The SGH deals with the balance between facilitation and competition (Lüttge 2016; Souza et al. 2016). It says that when stress is severe, facilitation rules the relations among plants, while competition prevails under low stress and high affluence (Bertness and Callaway 1994; Callaway and Walker 1997; Lin et al. 2012; Callaway 2013; Dangles et al. 2013). The GDB

considers the effects of trade-off between the efforts for growth and defense, respectively, and the allocation of resources to either of the two (Loomis 1953; Herms and Mattson 1992; Matyssek et al. 2002, 2005, 2012). Defense is important for maintenance, and growth is the basis of competition (Cahill 2013; Souza and Lüttge 2015). Both hypotheses address competition, and therefore they have direct relations to our question of how functional photosynthetic diversity may determine occupation of space and establishment of plants in diverse sites. Competition is an essential aspect when we try to understand the mechanisms of site occupation. It occurs between individual species and also between entire vegetation types.

We have seen above that at large-scale habitats, we may have dominant C_3 or C_4 vegetation and prominent contributions of CAM, respectively (Sects. 3.1.1, 3.1.2, and 3.1.3). We noted competition between C_4 grasses and C_3 trees. At large scales competition between entire vegetation types, i.e., between grasslands and forest (Sect. 3.1.2) or between two different growth forms with different modes of photosynthesis, C_4 and C_3 grasses and CAM succulents, is effective (Sect. 3.2.2). At finer scales we have found it increasingly difficult to recognize one more or less dominant mode of photosynthesis (Sect. 3.3). Instead we faced competition between individual plants with different functions. We often see different modes of photosynthesis in very similar life-forms of plants growing strictly sympatrically side by side, such as the C_3 halophytes with the C_4 halophyte *P. rubricaulis* (Sect. 3.2.3) or epiphytic bromeliads with C_3 photosynthesis and CAM, respectively (Sect. 3.3.1). In Panama it was observed that CAM bromeliads of the genus *Aechmea* must have recently radiated into the extremely wet cloud forests (Pierce et al. 2002). Crassulacean acid metabolism with the flexible expression of the CAM phases must have preadapted them for the development of some competitive advantages over the C_3 bromeliads. It allows carbon acquisition not only during the day but also during the night. Therefore, in daily courses where leaves are frequently wet inhibiting CO_2 diffusion at the surface, it makes carbon acquisition more flexible. In the Andes of southern Ecuador, competition between the widely used tropical C_4 pasture grass *Setaria sphacelata* (Schumach.) Stapf & C. E. Hubb. ex M. B. Moss and the C_3 bracken fern *Pteridium* sp. infesting the pastures was studied, and their canopy photosynthesis was modeled (Silva et al. 2012; Beck et al. 2013). The grass showed better growth at dry and warm weather, whereas the fern performed better under wet and cool conditions so that the dominance of the fern increased at higher altitudes. In a Brazilian swamp forest, a C_3 bromeliad *Nidularium innocentii* Lemaire and a CAM bromeliad *Nidularium procerum* Lindm. were separated at micro-sites where they showed clonal growth (Scarano et al. 1997, 1999; de Freitas et al. 2003). Forming mono-specific stands, *N. innocentii* dominates in deep shade of temporarily flooded sections of the forest, while *N. procerum* covers permanently flooded areas under open canopies. However, in principle both species can actually adapt to and grow in either of the two types of patches. At the boundaries of their zones where the clones meet, they intermingle and compete for establishment in the space (Grams and Lüttge 2011).

Many examples of sympatric C_3 and CAM plants of similar morphotypes are found in the genus *Clusia* (Lüttge 1999). In an open secondary savanna in northern Venezuela, we observed *C. multiflora* H.B.K. with C_3 photosynthesis and

C. minor L. with C_3/CAM intermediate photosynthesis to form bushes together growing in intimate mutual contact and competing for space directly (Franco et al. 1994; Grams et al. 1997). Both were similarly occupying the sun-exposed site irrespective of the mode of photosynthesis. It was seen that due to the plasticity inherent in CAM, *C. minor* was also able to establish itself in the semi-shade of a nearby deciduous dry forest which was not achieved by the obligate C_3 species *C. multiflora* (Herzog et al. 1999b). In the Brazilian restingas, the CAM species *C. hilariana* was also capable of growing successfully under a fairly large range of irradiance levels (Franco et al. 1996). In the karstic Sierra de San Luis, in the state of Falcon, northern Venezuela, the C_3 *C. multiflora* performed well side by side with three different CAM *Clusia*s (Popp et al. 1987; Franco et al. 1994; Haag-Kerwer et al. 1996). In coastal communities of rock outcrops and restingas in Brazil, both the C_3 species *C. parviflora* Saldanha & Engl. and the CAM species *C. hilariana* effectively adjust their photochemical efficiency to environmental conditions and diurnal variations of stress (de Mattos et al. 1997). CAM not even may always be superior at exposed sites and may not always provide better photoprotection at high irradiance (Herzog et al. 1999a).

Much more work on such competition is needed to better understand habitat occupation by competing species with different modes of photosynthesis and different plasticity of performing specific modes. Approaches should be plantation experiments including transplantation protocols. Where, due to the high complexity of ecosystems, the specific comparison of particular communities and micro-sites bears limitations of understanding, studying gradients is a promising alternative (Fiedler and Beck 2008; Beck 2008). In such studies, it would be important to include field measurements of photosynthetic parameters as for the above example of *S. sphacelata* (C_4) and *Pteridium* (C_3). In an afro-montane forest in Ethiopia, gradients of the distribution of two sympatric C_3 species *Lobelia gibberoa* Hemsl and *Solanecio gigas* (Vatke) C. Jeffrey of the same giant rosette-type life-form along the northern and southern flanks of a small valley were studied (Lüttge et al. 2001). In this case niche occupation was independent of photosynthetic performance which was very similar in both species. The control of water relations was more important allowing *S. gigas* to grow on both flanks and restricting *L. gibberoa* more to the moister northern flank, so that *S. gigas* had a competitive advantage at the southern slope.

An example of facilitation at the community level is given by the restingas in Brazil. On the coastal sand dune plains, *Clusia* species especially the obligate CAM species *C. hilariana* migrating in from the Atlantic rainforest function as nurse plants (Pimentel et al. 2007). They are starting vegetation islands gradually building up plant diversity (Scarano 2002, 2009; Dias and Scarano 2007; Brandão Correia et al. 2010; Lüttge 2013). The seedlings of *Clusia* trees may germinate within the tanks of bromeliads which are often also CAM species (Scarano 2002, 2009). These bromeliads first function as nurse plants for the *Clusia* seedlings which eventually overgrow them. Facilitation switches to competition, and the bromeliads are restricted to the edges of the developing diverse vegetation (Brandão Correia et al. 2010).

5 Conclusions: Understanding Biodiversity?

Biodiversity is a feature of evolution. From the basic concept that natural selection drives evolution, we realize that species are well adapted to their habitats but not perfectly. Adaptation is a dynamic process. Its motive forces are inherent in natural selection under the pressure of abiotic environmental conditions as well as biotic competition. This allows, and even asks for, dynamic structures with biodiversity offering selection a choice. In the quadruped scheme (Souza and Lüttge 2015), plasticity, diversity, and complexity join in creating and sustaining dynamic stability. We know, for example, quite well that biodiversity supports ecosystem functioning and productivity (for references, see Sect. 1).

Both plasticity and diversity imply complexity. Understanding biodiversity means understanding complexity. Integration of building blocks or modules and their self-organization leads to the emergence and establishment of systems with increased complexity (Lüttge 2012; Beck 2019). It is particularly well illustrated by the example of the restingas (Sect. 3.2.3) where arrival and early site occupation may lead to an assembly of species driven in by chance, and where selective self-organization with sequential events of successions create complexity, in which biodiversity plays a large role. In this vein it is not particular traits, such as the modes of photosynthesis surveyed in this essay, which selectively determine site occupation. Looking for this, we may ask the wrong question. This is particularly so regarding the microhabitats where we can directly follow the immediate sympatric competition of plants with fundamentally different ecophysiological modes of photosynthesis. Under the premise that space also is a fundamental resource (Grams and Lüttge 2011), this is a scenario of contention of modes of photosynthesis during and after the arrival of species carrying them.

The expression that "diversity creates diversity" (Beck 2019) is a decisive statement (Scherber et al. 2010; Ebeling et al. 2011). It is related to the diversity of stress conditions and also addresses the distinction between performance optimizing productivity (productivity optimum) and ecological niche occupation (ecological optimum), respectively. Using microcosms Grime et al. (1987) demonstrated that high richness of species occurred within a window of stress conditions. Under severe stress, only a few very specifically adapted species survive. At low stress and high affluence, a limited number of highly competitive and productive species in the SGH context become dominant. However, in between, under medium stress creative plasticity and variability are unfolding where biodiversity allows a multiplicity of reactions under the ecological conditions given. This obviously is also one of the reasons why in the moist tropical forests, we have the hot-spots of the highest biodiversity on Earth, because a multifactorial stress is given there by network-like interactions of many different stress factors of medium intensity rather than single severe conditions (Lüttge 2004, 2008a; Souza and Lüttge 2015).

Therefore, in conclusion, the diversity of arrivals at sites governed by chance, the establishment at dynamically variable conditions, and the hype that "diversity creates diversity" appear best leading us to an understanding of why so often we

find that ecophysiological performance per se, such as photosynthesis, is insufficient for explaining local distribution of plants and habitat occupation.

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When the Tree Let Us See the Forest: Systems Biology and Natural Variation Studies in Forest Species

Luis Valledor, María Carbó, Laura Lamelas, Mónica Escandón, Francisco Javier Colina, María Jesús Cañal, and Mónica Meijón

Contents

Abstract Systems biology has emerged in the last years as a comprehensive set of tools which provides rich biological information by integrating different cell organization levels (genome, epigenome, transcripts, proteins, and metabolites) and providing not only the relationships and correlations among them but also giving the possibility of modeling how and why cells and tissues are plastic to different environments. To perform this characterization, several analytical methods (NGS, mass spectrometry) and statistical and modeling tools must be used. Contrary to classic targeted approaches, the untargeted analyses of a plant system far from introducing noise or scattering the focus of the research can provide new candidates, pathways, or counterintuitive mechanisms otherwise undetectable. In this chapter,

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All authors contributed equally.

L. Valledor, M. Carbó, L. Lamelas, M. Escandón, F. J. Colina, M. J. Cañal, and M. Meijón (✉)
Plant Physiology, Department B.O.S., Faculty of Biology, University of Oviedo, Oviedo, Asturias, Spain
e-mail: meijonmonica@uniovi.es

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we briefly describe methodology and applications of systems biology by using three different studies: natural variation of the metabolome of *Pinus pinaster* and its relation to different geographic origins and wood quality traits and how *Pinus radiata* adapt to thermal or ultraviolet stresses. These examples illustrate how systems biology can contribute toward the full understanding of the biological processes mediating tree stress adaption and development, and forest ecology, besides constituting a powerful tool for breeders toward the selection of specific donors for specific environments, or model how new mixtures of genotypes will impact over forestry demanding traits.

Keywords Environment effects, Epigenomics, Integrative analysis, Metabolomics, Proteomics, Transcriptomics

1 Introduction

Forests provide wood material and fundamental ecosystem services for mankind including preservation of biodiversity, carbon cycling, climate regulation, and preservation of water quality and soils (Bonan 2008). However, human-induced climate change represents a fundamental worldwide challenge for plantations and natural forests. Current models of vegetation dynamics predict profound landscape alterations affecting not only natural forests but also plantations due to an increased average temperature and extreme drought and/or heat periods. Besides reducing growth during these periods, stressful conditions also affect the following years and increase the possibility of pest and pathogen attack leading to increased mortality (Aspinwall et al. 2015).

These changes in natural and managed forest landscapes will have a major impact over environment (conservation, CO_2 capture) and productivity, endangering the sustainability of forest use, in a period in which the required production and consumption of wood products and wood energy are expected to increase largely, following historical trends (Aspinwall et al. 2015). Therefore, in order to reach future sustainability of forest ecosystems and supply the demand of wood products, a dual approach focusing on both characterizing the mechanisms exhibited by the most tolerant individuals/populations in natural forests and improving the productivity, health, and performance of plantations through breeding programs considering the modeled future scenarios of climate change.

Breeding strategies over the last decades employed phenotypes, QTLs, or classic biomarkers for selecting individual elite trees. This approach demonstrated to be excellent for improving simple traits; however it lacks power when dealing with complex traits, as plant-environment interactions and adaptive processes. Recently, more advanced genetic tools as GWAS analyses (Meijón et al. 2014) have been proposed and effectively exploited in model species to understand the consequences of variation found in nature. Natural phenotypic variation can be conceptualized as an orthogonal dimension of biology. Just as an individual's DNA sequence can be

mapped onto a reference genome, natural variation in gene expression, protein function, and molecular interactions can be mapped onto cellular networks elucidated in reference lines of organisms (Gasch et al. 2016). The processes by which genetic variation of complex traits is generated and maintained in populations have been treated for a long time in abstract and statistical terms. For non-model species, such as the vast majority of trees, this approach is even more difficult to apply due to their large genomes of these species and the low number of available genetic markers which reduce statistical power of this approach. As a consequence, as yet, quantitative genetics has provided limited insights into our understanding of the molecular bases of phenotypic variation in tree species (Landry and Rifkin 2012). However, understanding how the different populations from one plant species, and in particular trees, have been adapted over the centuries to grow in different environmental conditions is crucial for understanding the molecular basis of a multitude plant physiological processes.

Approaches based on systems biology offer a comprehensive view of plant systems (Fig. 1). Technologies such as transcriptomics, proteomics, and metabolomics were developed with the aim to analyze molecular data of living systems on a genome scale. This leads to genome-scale, dynamic molecular data in combination with a genomic template. The ultimate goal is to derive a mathematical model of metabolism that is driven by genome data and correctly predicts the phenotype and ecophysiology of the plant (Sheth and Thaker 2014).

In the last 10 years, three major developments have transformed system biology: affordable genome sequencing, the OMICS revolution, and computer-assisted theoretical biology and modeling including the rapid development of the Internet into a knowledge platform and scientific database as well as bioinformatic tools that allow the integration and analysis of complex datasets (Weckwerth 2011). First, genome sequencing has led to a repertoire of plant genome sequences which still has to be explored in its depth, starting with *Arabidopsis thaliana* as the first plant model system. Many achievements since the first release of the *Arabidopsis* genome sequence have justified all the efforts that try to understand a non-crop plant thoroughly. Since then, many other plants, even forest species, such as *Populus trichocarpa*, *Quercus robur*, or *Norway spruce*, have been sequenced or are in the process of being sequenced (Tuskan et al. 2006; Plomion et al. 2018; Nystedt et al. 2013). Second, advances in mass spectrometry and transcriptome sequencing have enabled the analysis of cellular proteins, metabolites, and transcripts in unimaginable scale with thousands of biomolecules analyzed from a single injection. The use of next-generation mass spectrometers allows peptide or metabolite characterization without precedent in terms of speed, resolution, dynamic range, and accuracy, being possible the analysis of full proteomes (Nagaraj et al. 2012) and metabolomes (Meijón et al. 2016). In the same way, RNA sequencing (RNA-Seq) has distinct advantages over previous approaches and revolutionized our understanding of the complex and dynamic nature of the transcriptome without a mandatory prior need of a reference genome (Kukurba and Montgomery 2015).

Finally, the integration of multiple omics approach is a challenge task for multiple reasons: amount of heterogeneity data available from the different levels of complexity and the inherent artifacts owing to the different omics platforms employed

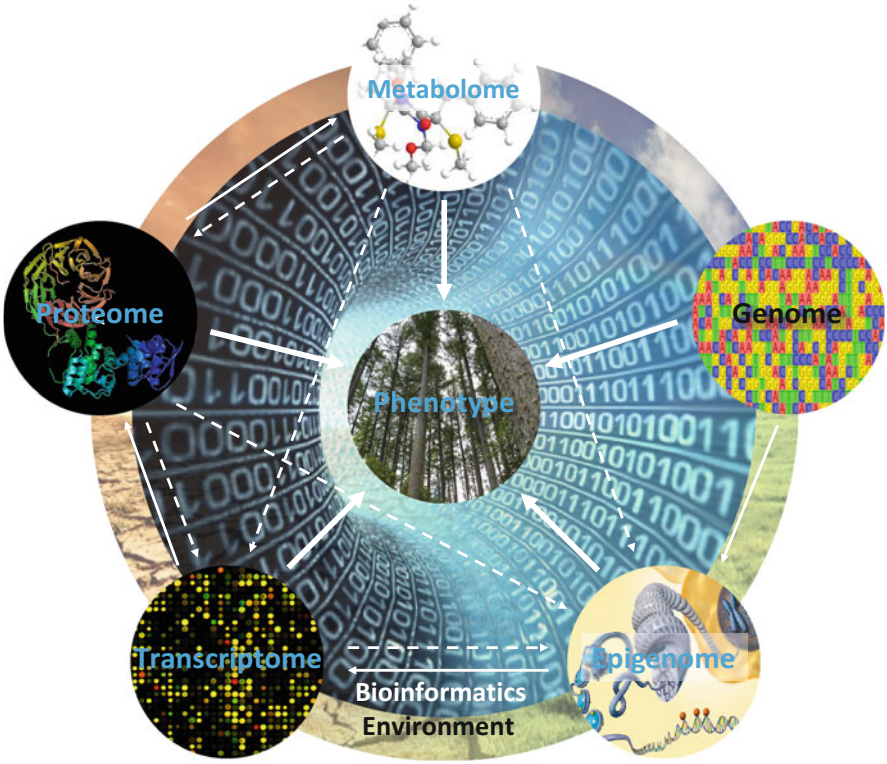

Fig. 1 Systems biology aims to predict phenotype based on the integration of the different omic levels and environmental factors by using specific bioinformatic tools for allowing not only data integration but also predicting interactions between biomolecules, gene function, and regulatory networks going beyond the mere description of potential biochemical pathways defined by the genes present in specific genomes

such as variation between manufacturers and omics technologies (Singh et al. 2016). Data heterogeneity is therefore a major obstacle to combining multiple omic studies (Fan et al. 2014). Fortunately, given the huge amount of heterogeneity data that is generated, bioinformatics, statistical analyses, and specific integration methods have been developed in the last years in order to extract and determine the most relevant information in biological terms (Singh et al. 2016).

But, how these advances can be applied to forest ecology and productivity research? In this chapter, current impact of systems biology in plant science and how to apply these technologies using natural variation and combining molecular biology tools in non-model species to validate the final hypothesis will be illustrated. The comprehensive and untargeted looks into molecular and biochemical networks of individual trees that is showed by this approach, contrarily to classic targeted analyses, allow us to go further and decipher how trees interact with the environment and have evolved to adaption to specific ecological niches, this information being relevant to ensure sustainability of natural forests and increase the productivity of plantations.

2 Combining Natural Variation and Systems Biology, Why?

Since the definition of the "central dogma of molecular biology" in 1958, most biological questions have been studied following a reductionistic approach focusing only on the functions of individual genes, generally a small number at a time. Furthermore, most of the times, the set of analyzed genes or gene products has been previously defined (reductionistic and targeted approach). These kinds of approaches, trying to explain "the whole plant" by employing a dramatically low number of variables, have been proved to be extremely useful, since all efforts taken to understand the function of each individual gene have been greatly beneficial to explain how a plant works. In theory, by understanding gene function, we can better define biochemical pathways and responses to environment. However, these approaches fail when studying complex traits such environmental responses or counterintuitive regulatory networks, in consequence mostly providing partial and sometimes biased understanding of phenotypic variation consequence, for example, of the imposition of a stress situation. Plant systems are complex, redundant, and greatly regulated so characterizing only some genes is not enough to predict plant behavior and metabolism most of the times. In other words, a plant system is much more than its components, and its properties cannot be fully modeled from isolated parts.

In the 2000s, the first plant large genome sequencing project, Arabidopsis Genome Initiative (1001 Genomes Consortium 2016), provided a complete catalog of individual genes and proteins for making possible to take the first steps toward understanding a plant system. Later, the advance in next-generation sequencing platforms allowed the (re)sequencing of plant genomes. Obviously, having a "catalog of parts" of our system is not enough, being also necessary to understand how these different elements interact. Thus, an integrative approach to investigate how genes, transcripts, proteins, and metabolites interact with each other in space and time is paramount to systems biology (Weckwerth 2011).

The availability of genome sequences allows, by employing next-generation sequencing platforms or mass spectrometry-based techniques, the quantification of transcripts, proteins, and metabolites present in a cell/tissue (Liu et al. 2012; Zhang et al. 2013; Kuehnbaum and Britz-McKibbin 2013). Co-expression analyses, definition of biomolecules interactions, and modeling based on available enzymatic reactions databases allow an unprecedented capability to model and understand a core plant system and its response to different growth situations (Cramer et al. 2011).

The consideration of natural variation, the variation of individual populations of the same species adapted to different environmental conditions, within this workflow gives us an unprecedented analytical power to describe not only individual plant systems but also how these systems specifically adapt to a wide range of environments and stress situations (Benfey and Mitchell-Olds 2008). Initial steps were again carried out in *Arabidopsis* by employing genome-wide association studies (GWAS) (1001 Genomes Consortium 2016). Nonetheless, current analytical workflows

(Pascual et al. 2017; Escandón et al. 2017) allow the inclusion of new layers to the system (environmental conditions and natural variations) without the requirement of extensive resequencing projects combining them with proteomics, transcriptomics, and/or metabolomics making this suitable to be applied in forest species, almost all of them recalcitrant, with a low representation in genetic databases (Meijón et al. 2016) and with long generation times, which difficult the use of most of the genetic-based approaches such as GWAS or extensive crossings as in model organisms. As it is explained below, this approach has been proved fruitful to define environmental adaptive mechanisms, its relation to specific environmental niches, and wood quality traits. These works demonstrated that individual trees not only allow us to see the forest from a systems perspective but also predict its adaptive capabilities and productivity.

Consequently, modern systems biology is already contributing to a radical transformation of molecular life sciences (Yuan et al. 2008). Nevertheless, untargeted holistic hypotheses established by system biology should be validated following classic molecular biology, genetics, and physiology in further experiments but contrary to reductionist approaches, the new candidates to be validated are strongly supported by extensive network analyses and have been proved to be those elements of the system less biased to explain the studied biological phenomena. Systems biology and molecular biology work hand-in-hand to provide a complete picture of how and why plant physiology is the way it is (Stevens 2004).

2.1 How?

2.1.1 Using Omics Technology in Non-model Species

In the last decade, research has been mostly focused on the study of model species, which have been crucial for the in-depth study of cellular and molecular life processes (Armengaud et al. 2014). Model species have been chosen, in most of the cases, for their easiness of study, small genomes, availability of mutants or protocols, facility of cultivation, or agronomic importance. After *Arabidopsis*, rice, corn, or alfalfa have been sequenced, and its genomes were richly annotated knowing the function of a large proportion of the genome. On the other hand, forest trees cannot be considered as a model due to its large generation times and complexity, abundance of many molecules which interfere with molecular protocols, lack of mutants, and complex and large genomes. Genome size and complexity has delayed, compared to herbs or monocots, the availability of tree genomes, specially oaks and conifers. Even for tree sequenced species, databases are often poorly structurally and functionally annotated (Buts et al. 2014).

This lack of available sequences and/or annotations is a major limitation for performing omic analyses since transcripts and proteins are identified and quantified based on genome information. In recent years, new workflows have been described coping with these limitations by integrating available sequences in public

repositories (both classic and RNA-Seq data) (Romero-Rodríguez et al. 2014; López-Hidalgo et al. 2018).

Transcriptomics was greatly stimulated with the development of a powerful and innovative sequencing tool in Human Genome Project (2001). RNA-Seq provides accurate information about gene expression, quantifying tens of thousands of genes in a single run, with costs being reduced each year, and these experiments are now affordable for a large number of researchers. In brief, this technique works as follows: RNA of the samples is fragmented and retrotranscribed, and then all of the fragments are amplified and sequenced independently (millions of reads per sample). Each sequence is then aligned to a reference genome/transcriptome and quantified by counting the number of reads per gene. If reference sequence is not available, there are algorithms which allow a previous de novo assembly of the transcriptome. Furthermore, RNA-Seq improves the detection and assignment of peptides in proteomics experiments following a so-called proteogenomic approach (Bryant et al. 2016; Zargar et al. 2017; Zhu et al. 2017) since using databases generated from cDNA that contain fewer irrelevant entries, noncoding sequences, and incorrect splice variants compared to DNA (Armengaud et al. 2014).

Nowadays, proteins can be easily identified and quantified by using a bottom-up proteomic approach in many plant systems (Jorrín-Novo et al. 2015). The use of next-generation spectrometers such as Orbitrap or qTOF, coupled to liquid chromatography separation systems, allowed peptide characterization without precedent in terms of speed, resolution, dynamic range, and accuracy, being possible the analysis of full proteomes (Nagaraj et al. 2012). Proteins are first digested into peptides, and these are then identified based on their masses and fragmentation patterns which are compared to sequence databases. These ions corresponding to the peptides are also quantified, and proteins present in the sample are then reconstructed based on the identified peptides (for a review Zhang et al. 2013). As it was explained above, the use of species-specific databases greatly improves the number of identified proteins compared when using standard databases (Romero-Rodríguez et al. 2014).

As in proteomics, advances in mass spectrometry have enabled the analysis of cellular metabolites in unthinkable scale (Patti et al. 2012; De Vos et al. 2007). But contrary to proteins, which are usually easy to extract from tissues and handle, the major challenge of metabolomics is related to the chemical complexity of the metabolome, the biological variance inherent in most living organisms, and the dynamic range limitations of most instrumental approaches (Sumner et al. 2003). To reduce and/or to circumvent these problems is mandatory the use of selective extraction and parallel analyses using a combination of technologies to obtain the most comprehensive visualization of the metabolome (Gehlenborg et al. 2010). Metabolites are also quantified by chromatographic peak height or area and identified by comparing retention time and mass to standards, or by making an interpretation of MSn fragmentation patterns.

2.1.2 Data Integration and Bioinformatic Tools

In systems biology, each advance of high-throughput techniques is usually geared toward obtaining a greater amount of molecular data and, at the same time, increasing its quantitative sensibility. The analysis of these data was often considered the bottleneck of omic studies (Bino et al. 2004; Ritchie et al. 2015); to overcome this issue, novel bioinformatic tools are constantly being developed or updated to fulfil ongoing demand, and therefore bioinformatics is an exponentially growing field (Manzoni et al. 2016; Rajasundaram and Selbig 2016).

Multi-omics approach is based on the overlap of different information layers and allows us to discover and unravel the complex mechanisms and their interplay in plant systems as well as track the molecular variability among individuals and link it to phenotype differences (Fukushima et al. 2014; Großkinsky et al. 2018). The integration of these omic layers is nowadays one of the most powerful strategies to deeply understand and analyze a wide variety of biological processes from a holistic point of view (Mochida and Shinozaki 2011). However, obtaining this global picture may be challenging especially in non-model species and require the use of bioinformatic tools and statistical analyses.

One of the major targets of systems biology is to use these statistical methods to establish data-driven models that are able to predict the nonlinear behavior of the system (Weckwerth 2011). Therefore, it becomes crucial to establish protocols which are not only concerned about post-extraction data integration but rather provide a means to extract all molecular levels from a single sample (Roume et al. 2013; Weckwerth et al. 2004), especially when samples show high biological variation and molecular fluctuation or are limited in amount such as single cells, specific tissue, or fluids. Consequently, the different analyzed molecules (metabolites, DNA, RNA, and proteins) should be obtained from the same tissue to avoid bias and time effects. The extraction of all these molecules from the same sample is crucial for the subsequent integrative analysis, since we will avoid including bias and time effects (Roume et al. 2013; Weckwerth et al. 2004). There are numerous protocols that have sought this objective of multiple extraction (Gunnigle et al. 2014; Roume et al. 2013; Sambrook and Russell 2006; Weckwerth et al. 2004; Xiong et al. 2011), but few have been successfully tested on recalcitrant species (such as forest species). However, recently, Valledor et al. (2014) have developed a protocol that can be considered as universal, since it works in a broad range of organisms (*Cyanothece*, *Chlamydomonas*, *Lemna*, *Arabidopsis*, *Oryza*, *Populus*, *Pinus*, and *Eucalyptus*) requiring minimum lab equipment and being considerably cheaper than other available alternatives.

Metabolomics, proteomics, and transcriptomics raw data often come from three main platforms, microarray, NGS, and MS, and so must be preprocessed according to their origin (Kim and Tagkopoulos 2018). Before starting with data integration, it is essential to manage initial data heterogeneity (Fukushima et al. 2009). Multiple bioinformatic tools can be used to manage omics data, and some of the more useful are summarized in Table 1 and described below. For MS-based experiments, the

Table 1 Summary of the bioinformatic tools available for omics processing data cited in this chapter

Tool	Function	Description	Reference
R	Data preprocessing, statistical and network analyses	Open-source software with a great variety of available packages for a complete omics analysis (igraph, KNN, RF, mixOmics, etc.). Although it requires programming skills	http://www.R-project.org. Rohart et al. (2017), Hastie et al. (2017), and Liaw and Wiener (2002)
Python	Data preprocessing, statistical and network analyses	Open-source software with a great variety of available packages for a complete omics analysis (igraph, NetworkX, etc.). Although it requires programming skills	http://www.python.org. Fisch et al. (2015) and Ebrahim et al. (2013)
Perseus	Data preprocessing and statistical analyses	Open-source software. Useful and user-friendly but limited analysis options available	Tyanova et al. (2016)
Cytoscape	Network operations, analysis, and depiction	Open-source software. Allows network analysis and visual settings configuration	Shannon et al. (2003)
STRING	Biological networks representation	Open-source software. Contains several species biological information. Available as online tool and also as Cytoscape plug-in	Jensen et al. (2009)
BiNGO	Network bio-analysis	Open-source software, for further network analysis in terms of GO annotations. Available as a Cytoscape plug-in	Maere et al. (2005)
MAKER-P	Plant genome annotation	Open-source software. Designed for enhanced novel plant genome annotation	Campbell et al. (2014)
Cufflinks	Transcriptome assembly	Open-source software. Widely used for transcriptome assembly and relative transcript quantification	Trapnell et al. (2010)

most common data pre-treatments are missing values imputation, filtration, normalization, and transformation (Fukushima et al. 2009; Gardinassi et al. 2017; Van den Berg et al. 2006). To ease data imputation, there are several R packages such as KNN imputation (Hastie et al. 2017) and RF (Liaw and Wiener 2002), a random forest-based algorithm, to assess not available values. R- and Python-based methods are generally considered of high difficulty, while other tools as Perseus (Tyanova et al. 2016) are user-friendlier.

Integrative analyses are usually depicted as interaction networks which allow establishing relationships based on clustering algorithms to find out co-expression patterns among the different omic levels studied (Moreno-Risueno et al. 2010) and/or correlations as sparse partial least square (sPLS) implemented in mixOmics

R package (Rohart et al. 2017). One of the most extended network visualization tools is Cytoscape (Shannon et al. 2003), which has extra plug-ins as STRING (Jensen et al. 2009) and BiNGO (Maere et al. 2005) to enhance its functionality by combining already known biological information with omics-derived mathematical correlations; for more details, see Gligorijević and Pržulj (2015).

In forest species, an integrative data analysis enables to go further when compared with single-omics approaches. In Mahesh et al. (2018), an integrated genomic, transcriptomic, and proteomic multilayer analysis was used to improve the genome assembly and annotation for *Santalum album*.

Furthermore, these authors used MAKER-P tool (Campbell et al. 2014) to predict coding genes and merged them with Cufflinks (Trapnell et al. 2010) based transcriptome. Cufflinks allows the de novo transcriptome assembly; in addition, they used proteomic information to validate the expression of predicted genes, following a proteogenomics strategy to enhance the annotation yield. Another example of a multi-omics integrative analysis is described in Wang et al. (2018), where is provided a detailed pathway of the regulation of lignin by using transgenic *P. trichocarpa* seedlings and integrating genomic, transcriptomic, proteomic, fluxomic (study of metabolic fluxes), and phenomic (e.g., wood traits) levels of regulation.

The hypothetical networks determined by multi-omics integrative approach should be validated using molecular biology in further experiments, where it is possible to take advantage of accumulated knowledge about model species.

2.1.3 Validation of Systems Biology-Based Discoveries

As in model species, omics assays also encompass particularly high-throughput variable screening ones in non-model biological targets. DNA sequencing and MS-based techniques vertebrate these assays measuring hundreds to millions of different nature variables at a time. These statistical methods described above are able to identify most relevant variables within complex change landscapes. Some of them are also capable of integrating different origin data, identifying correlations and clusters between their variables. All these ease the harnessing of data, improving thereafter the experimental system response panomics image comprehension and eventually the discovery of novel biomarkers/targets/interactions. Once discovered their promising applications and taking into account the subsequent importance of omics findings, it is also important to mind the drawbacks of these methods. In other words, omics data treatment algorithms burdens make post hoc candidate validation mandatory.

Validation encompasses a plethora of techniques where the focus is on the independent verification of the found candidates/correlations in the experimental system under the same tested conditions. This is reasonably easy in model species but carry limitations in non-model ones. Between these limitations are the incomplete or non-sequenced genomes, poorly annotated databases, and lack of genetics, proteomics, and metabolomics resources assistance, in which relies validation. All

these make non-model systems blacker boxes than model ones, where validation is still possible but at a higher cost, giving usually a final system chiaroscuro with many unknown elements. Circumventing the obvious need to solve non-model species limitations, nowadays validation in non-model species requires a final translation into model species systems (Fig. 2). This allows the knowledge gained in non-models to become contextualized in the frame of better known systems. Parallelisms are established across species allowing the better understanding of the original research model while addressing the potential identity of many of the unknown elements and highlighting the unique ones. Furthermore, this

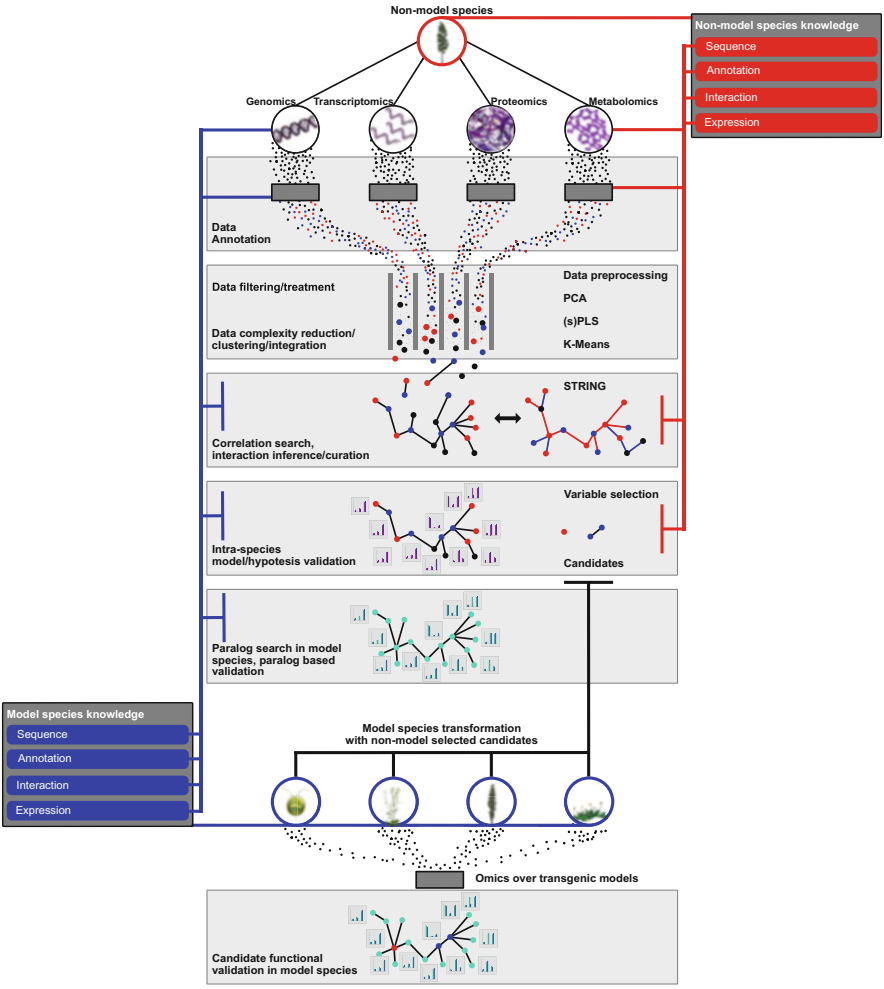

Fig. 2 Workflow of omics approach. Integration of omic datasets facilitates hypothesis generation and prediction of plant physiology processes, and further hypothesis validation will be necessary by using classic molecular biology tools

parallelization not only validates but turns findings more accessible for further applications driving novel research and strategies in other species, setting the basis for multiple crop improvement, and leading the path into new pathways introduction. Comparison also helps into evolutive explanations.

Although omics research in non-model species lack most of the model species resources, these species knowledge still comprises a useful tool for non-models. Most non-model omics rely in some moment on parallelisms between these systems. Among these are conserved domain search/enrichment, paralog search, homology assisted correlation validation, and correlation-based function assignment. Non-model systems annotation enrichment/completion and correlation validation rely as in model ones on function, domain, and interaction databases as KEGG (Kanehisa et al. 2017), Gene Ontology (GO) (Ashburner et al. 2000), MapMan (Thimm et al. 2004), InterProScan (Jones et al. 2014), Prosite (Sigrist et al. 2013), and STRING (Szklarczyk et al. 2015). Omics works use this enrichment, easing system interpretation and validation. Pascual et al. (2016) work constitute an exceptional example of annotation enrichment. Correlation inference benefits also from model species data. Co-expression networks functional enrichment with known interactants within the model addresses its power to find valid correlations while suggesting potential functions to unknown interacting nodes. STRING constitutes one of the most complete databases for this purpose. Roustan et al. (2017) identified key signaling elements into *Chlamydomonas reinhardtii* nitrogen starvation response combining correlation network functional and STRING interaction enrichment. On a higher validation level, paralogs are also used to validate selected candidates behavior giving information about their shared or alternative function in model systems. Zorrilla-Fontanesi et al. (2016) described in banana osmotic stress resistance, identifying and characterizing *Musa* spp. stress resistance candidate gene paralogs in *Arabidopsis* as continuation of a classical RT-qPCR-based intra-species validation of the original candidate gene set. *Arabidopsis*-based validation highlighted banana stress response specifics with *Musa* unique gene duplications discovery through paralog search and gene specialization processes description through paralog expression pattern comparison.

The most stringent validation method goes through the introduction of the selected omic candidate into a close model species. This procedure ratifies gene function into a better characterized system. Traditionally, transgenics has provided a powerful tool for cross-species gene functional description and validation. Non-model candidates coming from non-omics works expression into model systems have recently become the center of multiple omics works. This is the case of the *Physcomitrella patens* gene *PpENA1*, a traditional stress resistance candidate in the moss (Lunde et al. 2007) whose stress relation was validated in rice in an omic assay (Jacobs et al. 2011). However, omics itself constitute an excellent candidate provider being transgenics and model species their best validation systems.

There are multiple examples of omics candidates related with multiple cues which after their characterization and validation in the original species they were transferred to model species. In an abiotic stress, transcriptomic work in the red algae *Pyropia tenera* (Im et al. 2017), *PtDRG2* and *PtFUT* were proposed as stress

resistance candidates. Both genes were validated in the microalgae model *Chlamydomonas reinhardtii* which have no paralogs validating their stress-related role (Im et al. 2017; Wi et al. 2018). The same strategy was selected in *Cicer arietinum* (chickpea), where an EST analysis under drought stress gave *CarNAC3*, a stress resistance candidate (Gao et al. 2008). This gene was validated into the same species (Peng et al. 2009) and confirmed afterward in the forestry model *Populus trichocarpa* in which the same stress link was observed (Movahedi et al. 2015). Non-model forestry species have also centered omics studies highlighting *Populus euphratica*. After the transcriptomic characterization of this species drought response (Tang et al. 2013), *bHLH TF PebHLH35* was identified as stress candidate validating afterward their function in *Arabidopsis* through the induced drought resistance (Dong et al. 2014).

Although non-model omics have on model species a necessary support for validation, first ones also provide candidates leading non-model research. A microarray on *Arabidopsis* lignin synthesis mutants (Rogers et al. 2005) found Dof TFs related with lignin synthesis. This encouraged the identification of the *Arabidopsis* Dof *P. pinaster* paralogs and its characterization by Rueda-López et al. (2008). Some of these pine genes were transferred to *Arabidopsis* whose mutants starred a transcriptomics assay also by Rueda-López et al. (2015) validating their function through the substantial lignin increase into their tissues. Validation of this gene function in *Arabidopsis* encouraged its further validation through its transfer to poplar observing similar results (Rueda-López et al. 2017).

However, although this type of works, using model species to validate omics hypothesis, is beginning to be carried out, it is still a very little explored approach, despite its great potential.

3 What We Have Learned from Omics in Forest Species

Systems biology has risen as a direct result of the limitation of conventional reductionist biology to understand complex phenomena emerging as a result of dynamic and multiscale biological interactions. However, as it has been described in the previous sections, by applying statistical and bioinformatic tools, systems biologists integrate the elementary processes into a coherent description that allows them to predict and characterize the systems-level properties and behavior of complex biological phenomena. As the field of systems biology matures, it is beginning to see practical answers to real biological problems. It is timely to step back and review what we learnt about tree physiology from a systems biology approach by presenting several case studies in plants. However, in most of the cases presented, still it is necessary that further molecular biological studies validate established networks and physiological hypothesis.

In the current context of global change, it has become mandatory to analyze and understand the adaptation mechanisms of plants, particularly the key forest species that maintain our ecosystems and forests. The identification of several components

of multigene regulatory networks that regulate complex developmental process such as the adaptation to new environmental conditions is arduous especially by using a classic molecular biology approach, which typically only identifies single regulatory elements. It is at this point where omic integrative studies take great relevance.

3.1 *Metabolome Analysis of Natural Variation in* P. pinaster *Reveals Drought Resistance Is Related to Tree Origin*

In our first example, metabolome role in adaptation process and phenotypic diversity was studied by new approach combining system biology and natural variation studies in *P. pinaster* (Meijón et al. 2016). The results of this work showed that it is possible to cluster *P. pinaster* populations on the basis of their metabolites in relation to the environmental conditions of the origin of the population (Fig. 3a, b), aridity of the provenance being identified as the key to the clustering. Moreover, by using two complementary MS techniques (GC-MS and LC-Orbitrap-MS) and integrative analyses based on clustering algorithms to find out co-expression patterns among metabolome, and environmental and growth data of the populations, it was possible to establish that secondary metabolites, in particular flavonoid compounds, are essential to *P. pinaster* adaptation to drought environment (Fig. 3c), while primary metabolites are related to organ differentiation. Ultimately, these data

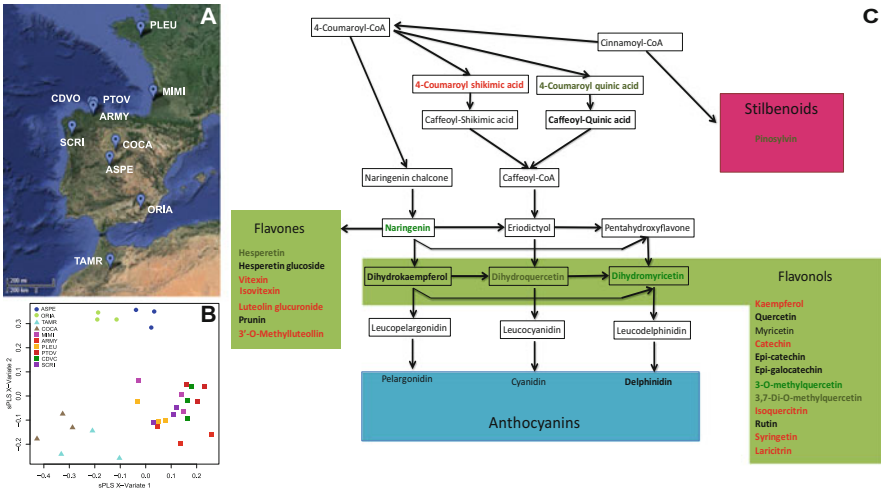

Fig. 3 (**a**) Localization of *Pinus pinaster* provenances. (**b**) Sparse partial least squares discriminant analysis (sPLS-DA) of polar and nonpolar metabolites of needles. Different colors show populations analyzed. (**c**) Summary of flavonoid pathways. Words in bold indicate metabolites identified in needles, red represents high levels in populations from arid areas (ORIA, TAMR, ASPE, COCA), and green high levels in populations from non-arid areas (CADV, ARMY, MIMI, PLEU, PTVO, SCRI). Adapted from Meijón et al. (2016)

provide a valuable step forward in our understanding of the adaptation processes and genetic and phenotypic diversity found in *P. pinaster*. This work represents the first study of natural variation in conifers and the genetic effect on the metabolome related to local adaptation.

3.2 *Integrative Omics Approach Allowed to Unmask Possible Heat Resistance Biomarkers in* P. radiata

The integration of transcriptomic, proteomic, physiological, and metabolomics data also allowed the description of complete pathways involved in the process of acclimation to heat stress in *P. radiata* (Escandón et al. 2017, 2018). Although heat-stress acclimation is still in its infancy in the forestry species, the results obtained through these works were promising not only in defining the mechanisms behind high-temperature response in *P. radiata* but also providing novel heat tolerance candidate biomarkers. In these works, *P. radiata* plants were exposed to temperatures within a range mimicking future scenario based on current models of heat increase. Metabolomics analyses, using complementary mass spectrometry techniques, allowed the reliable quantification of more than 2,000 metabolites. The analysis of identified metabolites and highlighter metabolic pathways across heat time exposure revealed the dynamism of the metabolome in relation to high-temperature response in *P. radiata* (Escandón et al. 2018), identifying the existence of a turning point (on day 3) at which *P. radiata* plants changed from an initial stress response program (shorter-term response) to an acclimation one (longer-term response) (Fig. 4a).

Furthermore, the integration of metabolome and physiological measurements suggested a complex metabolic pathway interaction network related to heat-stress response. Cytokinins, fatty acid metabolism, and flavonoid and terpenoid biosynthesis were revealed as the most important pathways involved in heat-stress response in *P. radiata*, with zeatin riboside (ZR) and isopentenyl adenosine (iPA) as the key hormones coordinating these multiple and complex interactions (Fig. 4b).

When the shorter-term response was studied in detail by exploring the proteome and targeted transcriptome, new information was revealed (Escandón et al. 2017). Integrative analysis of metabolome and proteome showed a complex molecular interaction network including three main physiological functions: (1) hormone subnetwork, where fatty acids, flavonoids, and hormones presented a key role; (2) oxidoreductase subnetwork, including several dehydrogenase and peroxidase proteins; and (3) heat-shock protein subnetwork, with numerous proteins that contain an HSP20 domain, all of which seem to be overexpressed at transcriptional level.

omics approach beyond pinpointing the basic mechanisms underlying physiological reaction of *P. radiata* during heat response allowed to unmask several possible candidates to be used as biomarkers of heat resistance in breeding programs, some

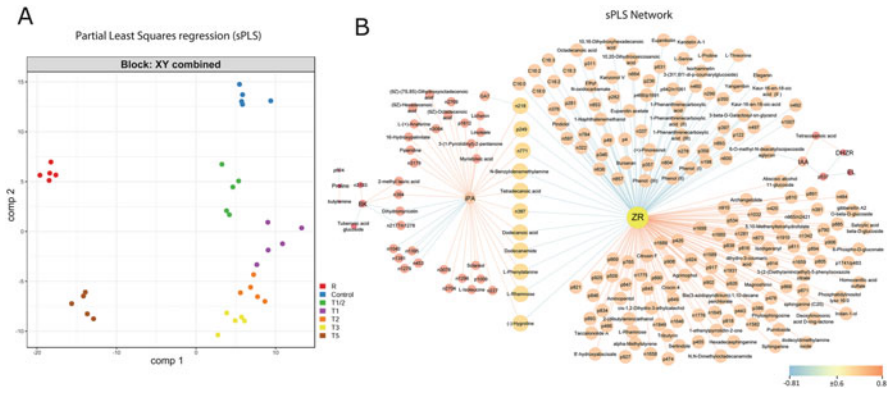

Fig. 4 Integrative analysis of metabolome and physiological parameters during heat treatment – taken from Escandón et al. (2018). (**a**) Classification of the different samples according to sPLS. Components 1 and 2 allowed the clustering of treatments analyzed according to shorter-term (T1/2, T1, T2, T3) and longer-term (T5 and R) heat-stress responses. (**b**) Interaction networks constructed after sPLS analysis using metabolites as the predictor matrix and physiological measurements as the response matrix. Edge color represents the correlation value. Only those correlations equal or higher, in absolute value, than 0.6 are shown. Color nodes reflect the amount of control that this node exerts over the interactions of other nodes in the network (higher control = lighter color). Abbreviations: *EL* electrolyte leakage, *ZR* zeatin riboside, *iPA* isopentenyl adenosine, *IAA* indol-3-acetic acid, *DHZR* dihydrozeatin riboside, *BK* castasterone, *GA7* gibberellin 7, *C* control, *R* recovered, *T1/2* 3 h after 40°C on day 1, *T1* 6-h heat exposure on day 1, *T2* day 2, *T3* day 3, *T5* day 5

proteins as PHO1, TRANSCRIPTION FACTOR APFI, and MITOCHONDRIAL SMALL HEAT SHOCK PROTEIN and crucial metabolites, such as L-phenylalanine, hexadecanoic acid, and dihydromyricetin (Escandón et al. 2017, 2018).

Clearly, integrative omics approach is being revealed essential to identify the molecular mechanisms involved in abiotic response in non-model species.

3.3 Last Step: Combining Omics Data and Molecular Biological Tools to Validate Candidates

The last example, focused on studying UV irradiation effects in *P. radiata* (Pascual et al. 2017), has gone beyond, and some of the key candidates identified are being validated by using molecular biological tools and model species.

The study of the mechanisms leading to adaptation of UV in a species of great economic importance like *P. radiata* is also essential in the current scenario of increasing UV radiation. Pascual et al. (2017) applied UV treatment in a time-dependent manner to *P. radiata* plants. The combined metabolome and proteome analysis was complemented with measurements of physiological parameters and gene expression showing sparse PLS analysis a complex interaction networks at

molecular and physiological level (Fig. 5). In summary, results showed that early responses prevented phototoxicity by reducing photosystem activity and the electron transfer chain together with the accumulation of photoprotectors and photorespiration. Apart from the reduction in photosynthesis as consequence of the direct UV damage on the photosystems, the whole primary metabolism was rearranged to deal with the oxidative stress while minimizing ROS production. New protein kinases and proteases related to signaling, coordination, and regulation of UV stress responses were identified.

A CHLOROPLASTIC SENSOR KINASE (CSK) raised as one of the most interesting candidates for further research about the molecular mechanisms by which it is related to UV stress. The performance of functional studies following a mutant approach has additional difficulties in tree and non-model species like *P. radiata*. The slow growth rates, as well as the lack of mutant collections and the very time-consuming process of mutant generation, are the main issues to deal with. Raised candidate proteins were further studied by taking advantage of the collections of mutants existing for the model plant *Arabidopsis thaliana* to study the UV stress response mechanisms mediated by chloroplast kinases. Currently, we are performing UV and oxidative stress sensitivity assays using a partial silencing mutant (Fig. 5), and preliminary data seem to show that mutants are less sensitive to UV light than *wt* line (unpublished), and phenotypes can be rescued by overexpressing pine kinases sequences in *Arabidopsis*, validating this way not only the function but also the conserved role of these proteins.

4 Concluding Remarks and Future Perspectives

The combination of two orthogonal perspectives, natural phenotypic variation and integrative high-throughput analyses of RNAs, proteins, and/or metabolites of a single cell, organ, or organism can reveal profound insights into how cells function, by pointing to individual genes, proteins, or other elements that vary functionally and by revealing mechanistic plasticity across different genetic backgrounds. This gives us an unprecedented capability to understand how cellular networks function and can adapt to different environments, characterizing priming or long-term genetically encoded adaptive processes. Application of these knowledge and technology, translational systems biology, will give us the possibility of improving tree species in an unprecedented way being capable not only of predicting the behavior of a genotype in future scenarios but also providing targets to engineer specific metabolic pathways understanding how these changes will impact over the whole system. All together systems biology will allow us to go deeper into the biological understanding of basic biological processes mediating speciation and adaption and also give us the possibility of tailor-made design of the forests of the future, ensuring sustainability, profitability, and in consequence conservation of one of the most precious natural resources as forests are.

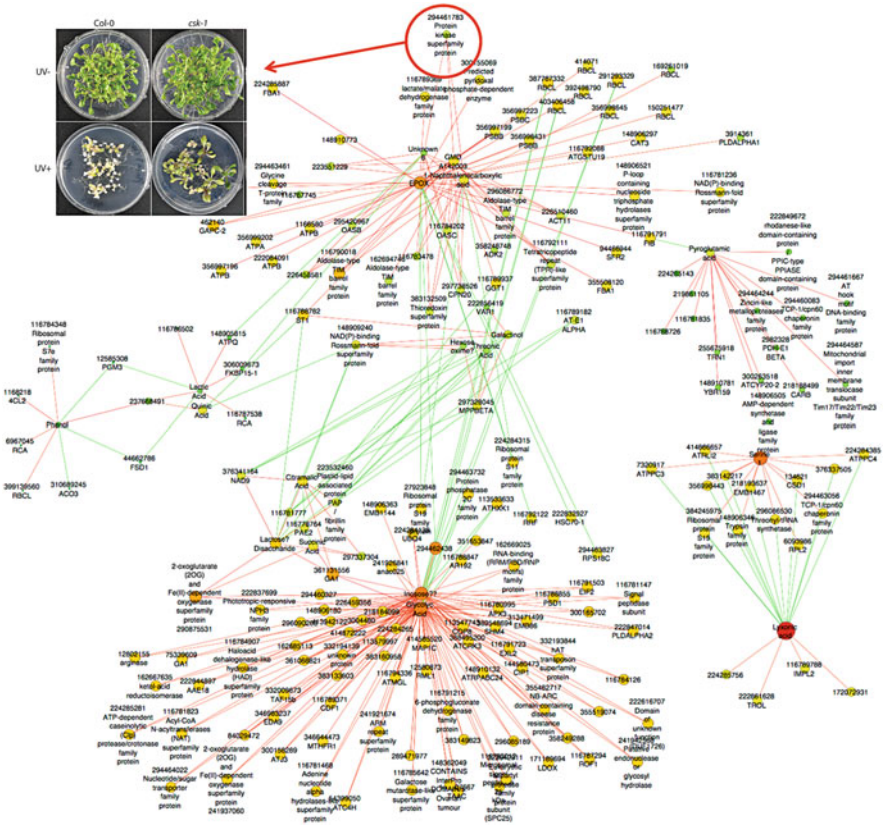

Fig. 5 sPLS-based network constructed using quantified proteins as a predictive matrix for the changes observed in the metabolome and the physiological measures. Red edges represent positive correlations, while the green ones represent negative correlation values. Only those correlations equal or higher, in absolute value, than 0.8 are shown. The network includes two main clusters, respectively, center in EPOX, 1-naphthalenecarboxylic acid and a couple of unknown metabolites, and in glycolic acid. The first is positively correlated to several ATPases and photosynthesis-related proteins, like RBCL or PSBB. On the contrary, glycolate is negatively correlated to those photosynthesis-related proteins while positively correlates with photorespiration and photorespiration-related enzymes, like SHM4, CAT2, or COP8. Furthermore, there are several light-mediated signaling elements consistent with the known light-dependent regulation of photorespiration. Other minor clusters represented in the network are centered in pyroglutamic acid, a sub-product of protein degradation, a main process in UV stress response, and in other metabolites related to stress, like phenol or lyxonic acid. It also highlights the presence all over the network of ribosomal proteins and several signaling proteins (kinases, phosphatases, and GTPases), most of them revealed here to be relevant for stress response, but not known their signaling pathway/s. In the upper corner of the figure is showed UV sensitivity assay using *wt* plants of *Arabidopsis thaliana* and *cks* mutant strong candidates identified in sPLS network. Pictures were taken 10 days after the day of the treatment with UV (0.25 W m^{-2} during 30 min). Adapted from Pascual et al. (2017)

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The Ecological Importance of Winter in Temperate, Boreal, and Arctic Ecosystems in Times of Climate Change

J. Kreyling

Contents

Abstract Climate warming is strongest in winter and in northern ecosystems. Ecological and biogeochemical impacts, however, depend mainly on soil temperatures. Climate warming can contrastingly affect winter soil conditions across northern biomes due to the crucial importance of snow cover: Increasing winter precipitation results in soil warming in the arctic, while midwinter snowmelt events can induce more severe soil frost in arctic and boreal ecosystems. Cold-temperate ecosystems are projected to experience increased soil frost due to strongly reduced snow cover no longer insulating the soil against still cold air temperatures. In cool-temperate ecosystems, warming eventually causes the complete loss of soil frost. Both pathways, soil warming and soil cooling, have important implications for ecology and biosphere-atmosphere feedbacks: In arctic and boreal ecosystems,

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J. Kreyling (✉)
Experimental Plant Ecology, University of Greifswald, Greifswald, Germany
e-mail: juergen.kreyling@uni-greifswald.de

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increased decomposition and mineralization allow for enhanced primary production, but midwinter melting followed by frost and/or rain-on-snow events might counteract this trend. More variable surface temperatures can damage primary production, and colder soil temperatures, due to reduced snow cover, can significantly decrease decomposition in cold-temperate ecosystems. For cool-temperate ecosystems, wetter winters could result in nutrient leaching, and altered dormancy patterns could cause increased frost damage despite air warming. In summary, winter processes are clearly relevant for the biosphere-atmosphere feedback, and even the sign of this feedback, i.e., ecosystems acting as carbon sink or as carbon source, depends on winter processes in temperate, boreal, and arctic ecosystems. This review concludes that current knowledge is not sufficient to quantify this feedback with satisfactory certainty. Important processes and the key uncertainties are identified, e.g., synchronicity in above- versus belowground growing season; temporal hierarchies in ecological processes such as the role of root damage and root activity for decomposition of soil organic matter ("priming"); or shifts in plant species composition due to winter climate change determining primary production as well as litter quantity and decomposability. Evidently, sound projections of future ecosystem functioning and biotic feedbacks to climate change require a comprehensive understanding of winter ecological processes, which have so far too often been neglected.

1 Winter Climate Change

1.1 Air Temperature

Climate records and climate models consistently project polar amplification in air temperature warming of the Northern Hemisphere, implying increased rates of warming from tropical to polar regions (Stocker 2014). Furthermore, warming in winter is expected to exceed warming in all other seasons (Stocker 2014). Winter warming will exceed annual warming for arctic (app. 4.4 and 3.4 K, respectively), boreal (app. 3.8 and 3.0 K, respectively), and temperate (app. 2.7 and 2.6 K, respectively) ecosystems until 2100 according to 42 CMIP5 global models for the RCP4.5 scenarios (Stocker 2014). However, occasional cold winter extremes will continue to occur despite this general warming trend (Pachauri and Mayer 2015). For Europe, such events might even increase in frequency as future warming of the Polar Ocean might increase advection of polar air masses, causing cold extremes over Europe (Petoukhov and Semenov 2010; Yang and Christensen 2012).

1.2 Precipitation

Winter precipitation is generally expected to increase, again, with largest increases toward the poles (Stocker 2014). For Northern Asia, for instance, this increase is

projected to be more than 50%, whereas summer precipitation is projected to hardly change (Stocker 2014).

Ecologically more important than a general increase in precipitation amount, however, are shifts in the form of precipitation. Snowfall is declining at the geographical margins of seasonal snowfall occurrence but increasing toward regions with colder winters in North America (Kunkel et al. 2009). Likewise, snowfall in Canada is increasing toward the North and decreasing toward the South of the country (Mekis and Vincent 2011). Similar patterns are reported for Europe and Japan (Scherrer and Appenzeller 2006; Takeuchi et al. 2008). Taken together, winters in temperate regions are expected to become wetter, while winters in boreal and arctic regions are expected to become snowier.

1.3 Snow

More than half (55%) of the land area of the Northern Hemisphere is influenced by seasonal snow cover (Zhang et al. 2003). Snow cover, however, is declining by 5.3 days per decade since winter 1972–1973 (Choi et al. 2010). Largest decreases occur in regions with winter mean air temperatures in the range of −5 to +5°C around the mid-latitudinal coastal margins of the continents (Brown and Mote 2009). Snow cover will continue to further shrink by 7% for RCP2.6 and by 25% in RCP8.5 by the end of the twenty-first century for the multi-model average, with strongest changes in advancing spring snowmelt (Brown and Mote 2009; Brutel-Vuilmet et al. 2013).

Snow depth and the snow water equivalent, however, are more sensitive to snowfall amount than snow cover (Brown and Mote 2009). For Eurasia, for instance, winter snow accumulation is currently increasing, but snowmelt is occurring earlier and quicker (Bulygina et al. 2010). With regard to maximum snow depth, model projections are mixed and come with little confidence (Stocker 2014). According to available models, snow depth will increase or only marginally decrease in the coldest regions, while annual maximum snow depth will decrease strongly closer to the southern limit of the (formerly) seasonally snow-covered area (Brutel-Vuilmet et al. 2013; Demaria et al. 2016; Estilow et al. 2015; Raisanen 2008).

1.4 Soil Temperature

Many relevant ecological processes are driven by winter soil temperatures, e.g., biogeochemistry or overwintering of organisms (Kreyling 2010). Despite this high ecological importance, soil temperatures are not even considered in the reports of the Intergovernmental Panel on Climate Change (IPCC) apart from the projected degradation of permafrost (Stocker 2014). Soil temperature shifts with climate change are complex, because soil temperature depends in nontrivial ways both on air temperature and precipitation changes as soils are protected from air frost events if

the insulating snow cover is sufficiently deep and continuous (Groffman et al. 2001; Sturm et al. 1997). This insulating power of snow is well known and can prevent soils from freezing temperatures even considering winter conditions in boreal regions (Isard and Schaetzl 1998). Already 30–40 cm of powder snow can effectively decouple air and soil temperature (Sturm et al. 1997). However, the insulating power of snow cover changes with the degradation of the snow. Compaction, for instance, can completely remove its insulation power, and soils under ski pistes are consequently much colder than outside the prepared areas (Rixen et al. 2004; Steinbauer et al. 2017).

Decreased insulation by snow cover could expose soils to cold air temperatures either overnight or during cold snaps, resulting in the apparent paradox of "colder soils in a warmer world" (Groffman et al. 2001). In light of the ongoing and projected changes in air temperature and snow cover/depth outlined above, this phenomenon is to be expected for regions where air temperatures continue to drop below zero degrees centigrade and snow cover is missing, i.e., focus on the temperate regions formerly experiencing continuous snow cover but now falling out of the seasonally snow-covered area (from here on referred to as cold-temperate). Here, e.g., in Southern Canada, the Northeast United States, or southern Scandinavia and northeastern Central Europe, soil minimum temperatures may actually become colder, and frequency of freeze-thaw cycles is increasing with climate change (Brown and DeGaetano 2011; Campbell et al. 2010; Henry 2008). Further south (from here on referred to as cool-temperate), e.g., across large parts of Central Europe, the increase in air temperature makes more than up for the decreasing snow cover, and soil minimum temperatures are rising even stronger than mean air temperatures, while soil freeze-thaw cycles are becoming less frequent (Kreyling and Henry 2011).

Boreal and arctic regions might experience soil warming due to the insulating effect of increased snow cover (Iijima et al. 2010). Here, however, temporal variability of winter weather may become ecologically important if widespread snowmelt events during winter increase in frequency, e.g., due to more frequent occurrence of warm air masses over some Arctic regions in winter (Visbeck et al. 2001).

Another ecologically relevant scenario of winter precipitation change are increases in the frequency of rain-on-snow events, which can cause the formation of massive and gas-impermeable ice covers (Bieniek et al. 2018) and reduce the insulation capacity of the snow cover (Martz et al. 2016).

2 Ecosystem Responses to Winter Climate Change

Winter processes can have substantial ecological effects on seasonally snow-covered ecosystems, as originally demonstrated for the Arctic (Clein and Schimel 1995). We have shown that winter climate can have stronger effects on dissolved and gaseous carbon (C) emissions of a wet boreal forest than summer climate (Haei et al. 2013). Likewise, temperate ecosystems are strongly shaped by seasonal cycles, with the

winter season being a major ecological filter and driver (Campbell et al. 2005; Kreyling 2010). Snow provides a relatively mild subnivean microclimate for plants, animals, and soil beneath (Kausrud et al. 2008; Pauli et al. 2013). Changes in snow cover, consequently, affect ecological processes. In particular, soil freezing in response to decreased snow cover has been identified as crucial parameter which can disrupt soil microbial activity (Bolter et al. 2005; Yanai et al. 2004), damage plant roots (Tierney et al. 2001; Weih and Karlsson 2002), and lead to increased soil nitrogen (N) leaching (Joseph and Henry 2009), increased soil trace gas losses (Matzner and Borken 2008), decreased plant productivity (Schuerings et al. 2014), and plant mortality (Schaberg et al. 2008; Buma et al. 2017). Freezing can also affect soil physical processes directly by breaking up soil aggregates (Oztas and Fayetorbay 2003) and by reducing soil water infiltration (Iwata et al. 2010).

Despite the fact that studies focusing on winter ecology report its high relevance, comparably few ecological studies on the effects of climate change consider winter at all (Fig. 1). More than two thirds of studies on ecological responses to climate change in arctic ecosystems, where the importance of winter is hardly arguable, ignore winter effects, and this share further increases for boreal and temperate ecosystems (Fig. 1). This pattern appeared to change in 2010, when a strong increase in climate impact studies in temperate and arctic ecosystems took winter into account (Fig. 2). However, this increase did not sustain and the number of studies considering winter rather stagnated, in particular compared to the overall number of scientific papers on climate change effects in these biomes (inserted panel in Fig. 2). The following review of ecological responses to winter climate change is therefore fragmentary. Primary production has been studied in some detail, but these studies rather demonstrate high complexity in the relevant processes than a coherent trend (Sect. 2.1). There is also quite some knowledge available on soil biotic activity,

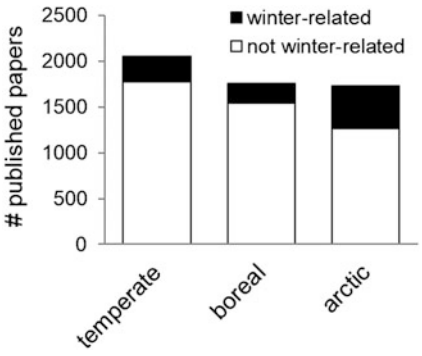

Fig. 1 Published research on biogeochemical and ecological impacts of climate change separated by papers dealing with effects of winter climate change (search string in ISI Web of Science: =((winter or frost or snow or freez*) and ("climate change" or "climatic change" or "global warming" or "climate warming") and (biogeochemi* or "primary product*" or "biomass" or "decomposition" or "mineralization") and (boreal or arctic or temperate)); articles only) versus climate change in other seasons (search string in ISI Web of Science identical to the one above but without "(winter or frost or snow or freez*) and")

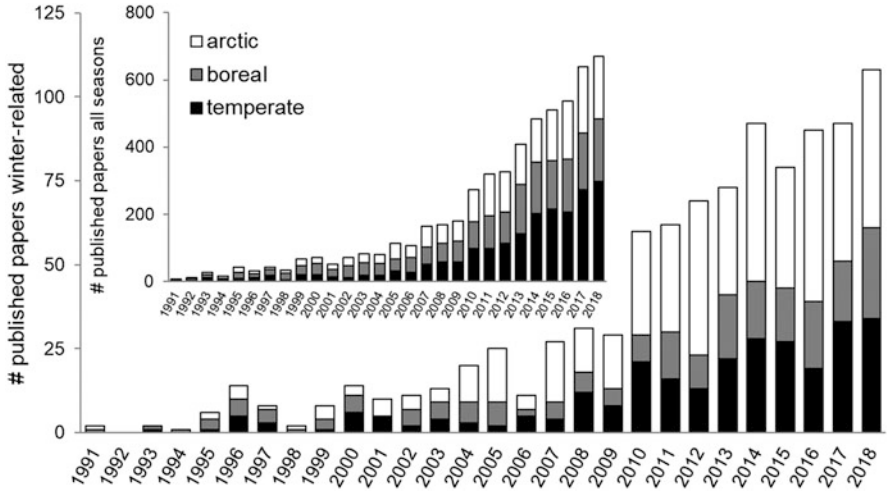

Fig. 2 Temporal development of published research on biogeochemical and ecological impacts of climate change separated by papers dealing with effects of winter climate change (main graph) versus climate change effects in all seasons (inlay). Search strings in ISI Web of Science identical to Fig. 1

decomposition, and mineralization in response to the expected changes in winter climate (Sect. 2.2). Little is published about nutrient leaching (Sect. 2.3), and a critical lack of knowledge exists on the interplay of these three key ecological aspects. The net effects of (winter) climate change on the C cycle, i.e., the biotic feedback to climate change, can therefore hardly be assessed up to now (Sect. 3).

2.1 Primary Production

The general expectation of increased primary production with warming is used to explain the greening or "shrubbification" of the Arctic over the past decades (Elmendorf et al. 2012). This expectation appears sound as temperate, boreal, and arctic plant species are clearly temperature-limited over winter (Larcher 2003) and as elongation of the growing season increases primary production (Slayback et al. 2003). Furthermore, there is experimental evidence that increased snow depth enhances plant growth of arctic shrubs (Krab et al. 2018). However, the recently described "arctic browning" (Phoenix and Bjerke 2016) warns against naïve extrapolation of the "greening" trend. Increased fire frequency (Bret-Harte et al. 2013) or pest outbreaks (Netherer and Schopf 2010) are potential explanations for this reversed greening in some regions and at specific times. Winter processes offer other explanations, as increased productivity with longer and warmer growing seasons may be counteracted when (1) winter climate change leads to increased soil freezing, when (2) warmer autumns or warm spells during winter lead to

premature dormancy release and increased winter and spring frost damage, when (3) winter warm spells expose plant organs to subsequent frost by melting the insulating snow cover, when (4) winter rain events lead to ice encasement, or when (5) wetter winters delay spring phenology.

1. It is well documented that increased depth and duration of soil freezing caused by snowpack reductions adversely impact root vitality (Comerford et al. 2013; Kreyling et al. 2012a; Reinmann and Templer 2018; Tierney et al. 2001), photosynthesis (Göbel et al. 2019), shoot elongation (Comerford et al. 2013), and N uptake by trees (Campbell et al. 2014). Root frost damage can even lead to regional dieback of ecologically and economically important tree species, as the example of *Chamaecyparis nootkatensis* (yellow cedar) in the Pacific Northwest implies (Schaberg et al. 2008; Buma et al. 2017). For the cold-temperate regions where soil frost is expected to increase in frequency and magnitude due to reduced snow cover, growth and C sequestration in the deciduous forests might be most adversely impacted by this projected shift in winter climate (Reinmann et al. 2019). Here, reduced stem growth might be explained as a passive consequence of reduced nutrient availability. Alternatively and more likely in light of the below described increase in nutrient availability (Sect. 2.2) with soil frost, C resources might be actively shifted belowground to facilitate compensatory root growth later in the year, which is a common consequence of increased root damage due to soil frost (Gaul et al. 2008; Sorensen et al. 2016). Studies on root-stem resource allocation (e.g., studies on within-tree dynamics of nonstructural carbohydrates (Hartmann and Trumbore 2016)) that could distinguish between these two alternative mechanistic explanations are so far lacking.

   Changes in the frequency of soil frost events, i.e., the number of freeze-thaw cycles, are another aspect of altered winter insulation of soils, yet they appear to only have minor or transient effects on plant performance (Kreyling et al. 2010, 2012b; Larsen et al. 2007). Taken together, lethal and sublethal root and shoot damage are expected to slow down or even reverse the projected increase in primary productivity in regions with increased incidence of soil frost events due to permanently or temporarily reduced snow cover.
2. Temperate species can incur freezing damage at temperatures in the range of −5 to −10°C but survive much colder temperatures in winter after successful hardening in a dormant state (Noshiro and Sakai 1979). With climate change, plants may be particularly vulnerable to frost in late winter and early spring because of premature dehardening (Augspurger 2013; Gu et al. 2008; Rigby and Porporato 2008; Liu et al. 2018; Montwé et al. 2018). Such premature dehardening can be caused by several interrelated effects such as cold acclimation occurring later in autumn, under shorter photoperiod and lower light intensity, all affecting the energy partitioning between growth, built-up of reserves, and cold acclimation that can further be influenced by elevated atmospheric CO_2 concentrations (Rapacz et al. 2014). Temperate deciduous tree species differ considerably in budburst forcing requirements and photoperiod sensitivity (Malyshev et al. 2018) and, consequently, in winter (Kreyling et al. 2015a) and

spring (Muffler et al. 2016) frost tolerance, indicating that winter climate changes also have the potential to affect competitive balances between co-occurring species. Generally, spring damage is potentially more relevant than winter damage because woody species have the ability to adjust winter frost tolerance to ambient conditions dynamically, while frost tolerance around budburst is minimal and nonadaptable (Vitra et al. 2017). Midwinter warming events, however, can break dormancy in temperate (Kreyling et al. 2015b), boreal (Ogren 1996), and arctic (Bokhorst et al. 2010b) plants, and early spring warming increases forcing conditions leading to budburst followed by late frost events (Augspurger 2013). Annual wood increment of cool-temperate deciduous trees can be decreased by more than 50% due to single frost events after leaf-out (Príncipe et al. 2017).

Note, though, that winter dormancy is a complex phenomenon as dormancy depth depends not only on forcing temperatures but also on chilling sums, meaning that plants require a certain amount of cold temperatures for dormancy release (Laube et al. 2014). Consequently, warmer winters could also lead to unfulfilled chilling requirements. Until now reported in only one extreme case, winter warming can consequently even lead to strongly delayed spring phenology because of unfulfilled chilling requirements and unbroken dormancy (Yu et al. 2010).

Furthermore, winter temperature and moisture are critical drivers for seed dormancy and germination, and global climate change alters these cues and can compromise seedling emergence and vigor (Walck et al. 2011). Yet, few studies have tested the importance of snow cover and winter temperature on seed survival, germinability, seedling establishment, and growth in response to climate change. First evidence suggests that post-winter seed germination decreases with reduced snow cover in trees that disperse seeds in summer or fall (Drescher and Thomas 2013). Likewise, seed mortality increases with decreasing snow cover over winter for *Fagus crenata* (Homma et al. 1999).

3. Extreme winter warming events leading to snowmelt leave plant shoots vulnerable to cold air temperatures subsequent to the warming event even in arctic ecosystems (Bokhorst et al. 2009, 2011). Again, susceptibility is species-specific, and evergreen plants are more vulnerable than graminoids or deciduous species to such extreme temperature variabilities in the arctic (Bokhorst et al. 2018). Here, subsequent frost damage is not driven by dormancy break but rather by exposing plant organs to frost events which usually are protected by the insulating snow cover. A coordinated distributed experiment at 13 temperate grassland sites implies that negative effects of snow removal on biomass production increase sharply toward colder sites, with up to 25% reduced productivity at cold-temperate sites and no visible effects at cool-temperate sites (Henry et al. 2018). We have further found comparable effects of short-term (2–5 days) winter warming pulses in temperate ecosystems, where at least some species reacted with strongly reduced biomass production, although the increased susceptibility in this case might also be caused by dormancy break (Schuerings et al. 2014).

Observational studies support the findings of these experimental studies: Boreal understory plant species show clear differences in their snow cover

preferences (Rasmus et al. 2011), emphasizing the regulatory importance of altered snow cover for species composition. Snow cover manipulations consequently affect species occurrence patterns in boreal (Kreyling et al. 2012a) and, likewise, in steppe (Chi et al. 2018) ecosystems.

4. Ice encasement harms plants by anoxia, accumulation of CO_2, ethanol, lactic acid, and ethylene (Gudleifsson 1994, 2010). More frequent occurrences of rain-on-snow events and midwinter thaw events therefore result in increased damage by ice encasement (Bélanger et al. 2002; Tompkins et al. 2004). However, plants differ in their vulnerability to ice encasement with juvenile conifer trees (Domisch et al. 2018) and alpine plants (Bjerke et al. 2017) reacting quite sensitively, while arctic willow and arctic graminoids appear rather tolerant (Bjerke et al. 2018).

   Extreme aboveground ice encasement furthermore occurs in the form of ice storms, i.e., supercooled rainfall during freezing air temperatures leading to ice coating of aboveground plant organs and, due to the sheer weight, to branch and crown loss of forest trees (Rustad and Campbell 2012). Frequency of such events is projected to increase, at least for the temperate zone of western North America (Cheng et al. 2007).
5. The start date of the growing season can be delayed by increasing winter precipitation (Vaganov et al. 1999). It has recently been shown that extraordinarily wet winters delay the start of the growing season by several days over a wide range of boreal forests due to colder soil temperatures related to both the increase in snowmelt heat flux and reduced absorption of solar radiation, which are proportional to the amount of winter precipitation (Yun et al. 2018).

Taken together, several negative feedbacks to primary production are hidden in projected winter climate changes, and their importance and relative effect sizes are hardly known, in particular in a multi-year perspective. However, evidence suggests the surprising conclusion that we should expect more plant frost damage in a warmer world and that the effects of winter climate change on primary production are not yet studied to a degree that allows for sound predictions into the future.

2.2 Soil Biotic Activity, Litter Decomposition, and Mineralization

Generally, soil warming leads to increased soil biotic activity and mineralization (Rustad et al. 2001). Accordingly, transplanting intact soil cores to warmer sites results in strongly increased nitrogen mineralization with presence or absence of a continuous snow cover mediating this effect (Wang et al. 2016). Zero degrees Celsius represents an important threshold for abrupt changes in microbial activity and substrate usage because of decreasing availability of liquid water (Mikan et al. 2002). However, much colder temperatures are typically required to cause microbial lysis, and microbial growth can continue below freezing (McMahon et al. 2009).

Nevertheless, the sublethal effects of freezing on soil microorganisms are not well understood, and the length of freezing, the number of freeze-thaw cycles, and the rate of freezing can all increase cell damage for a given freezing intensity (Elliott and Henry 2009; Vestgarden and Austnes 2009). In addition, for soil microorganisms that survive freezing and desiccation over winter by accumulating osmolytes, thawing can potentially cause mortality via osmotic shock, caused by exposure to melt water from snow (Jefferies et al. 2010).

Increased snow depth in arctic ecosystems has been shown to decrease richness and lead to a turnover of ectomycorrhizal fungal communities, potentially stimulating C and N mobilization and primary production (Morgado et al. 2016). Increased microbial activity in warmer soils over winter will also increase soil respiration in arctic (Mikan et al. 2002), boreal (Öquist and Laudon 2008), and temperate systems (Muhr et al. 2009; Schuerings et al. 2013). Reduced insulation due to absent snow cover, e.g., after extreme winter warming events, however, can lead to colder soils and strongly reduced annual decomposition rates, as, e.g., shown for a wet boreal forest (Kreyling et al. 2013). Soil microbial biomass, rates of soil C mineralization, and heterotrophic soil respiration in northern temperate hardwood forests, however, do not appear to be altered by reductions in snowpack and increases in soil freezing (Sorensen et al. 2016; Steinweg et al. 2008). Yet, in another snow removal experiment in the same ecosystem, soil respiration increased in response to induced soil frost, probably because of root mortality providing easily decomposable organic matter which might lead to priming and increased SOM degradation (Reinmann and Templer 2018). Infrared warming of a temperate grassland resulted in absence of snow cover, more variable soil temperatures, and unaltered decomposition rates (Walter et al. 2013). Increased soil temperature variability, in particular soil freeze-thaw cycles, adversely affects soil microbial biomass and increases available N and fuel N_2O emissions according to a meta-analysis on 47 available studies (Song et al. 2017). Winter litter decomposition, however, is remarkably unaffected by midwinter warming events leading to snowmelt in arctic ecosystems (Bokhorst et al. 2010a). Methane emissions in response to winter climate change appear less well studied. In an arctic moist tundra, it has been shown that long-term increases in snow accumulation can lead to markedly increased methane emissions, implying a positive feedback to climate warming due to the strong greenhouse capacity of methane (Blanc-Betes et al. 2016).

Taken together, soil warming increases soil biotic activity and litter decomposition. It needs be noted, though, that effect sizes diminish over time (Romero-Olivares et al. 2017). Soil cooling, however, can have various consequences: decreasing soil biotic activity and litter decomposition due to temperature limitation, showing no effect, or even increasing soil biotic activity due to increased supply of easily decomposable necromass. Increased soil temperature variability generally reduces soil biotic biomass but leads to increased soil nutrient availability (Comerford et al. 2013).

2.3 Nutrient Leaching

Increased N leaching following soil freezing has been explained by decreased root uptake due to lethal or sublethal root damage (Campbell et al. 2014; Matzner and Borken 2008). However, the projected increase in winter rain for the temperate regions and in total winter precipitation for arctic regions could further counteract the positive effects of soil warming on tree growth through increased mineralization as the mineralized nutrients might be leached out with the downward flow of the additional water, thereby getting lost for primary production (Bowles et al. 2018). Apart from this general and theoretical expectation, there is surprisingly little literature on this question. Roots occurring in deeper soil layers after winter soil warming have been interpreted as indirect indicator for N leaching (Schuerings et al. 2013). The expectation of increased nutrient leaching might also require fine-tuning to acknowledge potentially negative feedbacks. For instance, the supply of easily decomposable material might decrease with less winter root damage (Tierney et al. 2001) and a reduction in soil aggregate disruption (Oztas and Fayetorbay 2003) for regions with increasing thickness of snow cover and warming soils. Furthermore, despite evidence for increased N leaching losses in response to increased soil frost in some temperate systems (Fitzhugh et al. 2001), the opposite effect has been reported in others (Hentschel et al. 2008). Snow removal leading to colder soils with more frequent freeze-thaw cycles have furthermore been shown to increase ammonium availability but not nitrate loss in a cool-temperate forest in Northern Japan (Shibata et al. 2013). Finally, winter warming pulses can trigger N leaching with plant community composition largely controlling the amount of leaching (Kreyling et al. 2015b).

Overall, responses of soil N retention to winter climate change will depend on the so far understudied balance between plant uptake responses and N leaching losses (Sanders-DeMott et al. 2018), and future projections are hardly possible with the current state of knowledge.

3 Feedbacks from Ecosystems to Climate

Feedbacks in the C cycle between the atmosphere and the terrestrial biosphere remain a considerable source of uncertainty for both global climate projections and predictions of the terrestrial net primary productivity (NPP; Heimann and Reichstein 2008). Empirical studies and the models that rely on them for parameterization often use only growing season climate conditions to explore relationships between climate and plant growth or ecosystem NPP (Friedlingstein et al. 2006; Sanders-DeMott et al. 2017). Concerning northern forest ecosystems (i.e., cold-temperate and boreal forests), satellite data, model projections, and in situ observations imply that C uptake rates have increased due to winter warming that has lengthened the growing season in recent decades (Xia et al. 2014). A decrease in

winter snow cover restraining C emissions to the atmosphere due to colder soil temperatures has been shown to cause a negative feedback to global warming for cold-temperate and boreal forests according to Eddy-covariance C flux data (Yu et al. 2016). Still, the strength and temporal variability of such a potential C sink appears uncertain due to potentially confounding effects regulating primary production described above (Sect. 2.1). The large uncertainty in soil C dynamics between multiple models and experiments (Sulman et al. 2018) emphasizes the conclusion that a better understanding of feedbacks between the biosphere and the atmosphere is crucial for both the projections of realized climate change and the ecological impacts of this climate change. Below, I develop hypotheses for biosphere-atmosphere feedbacks which await rigorous testing and point out key uncertainties (summarized in Fig. 3). These hypotheses are based on very simplistic comparisons between trends in C gain through primary production (Sect. 2.1), trends in C loss through decomposition (Sect. 2.2), and potentially limiting effects of increased nutrient leaching (Sect. 2.3) in view of the projected key changes in ecologically relevant climatic parameters (Sect. 1).

3.1 Arctic

Strongly increasing air temperatures lengthen the growing season. Increased snow cover and air warming lead to soil warming. Based on these trends, decomposition and mineralization over winter increase. A shift in the species composition toward more productive species enhances primary production and suppresses nutrient leaching. The net feedback to the climate system depends on relative effect sizes

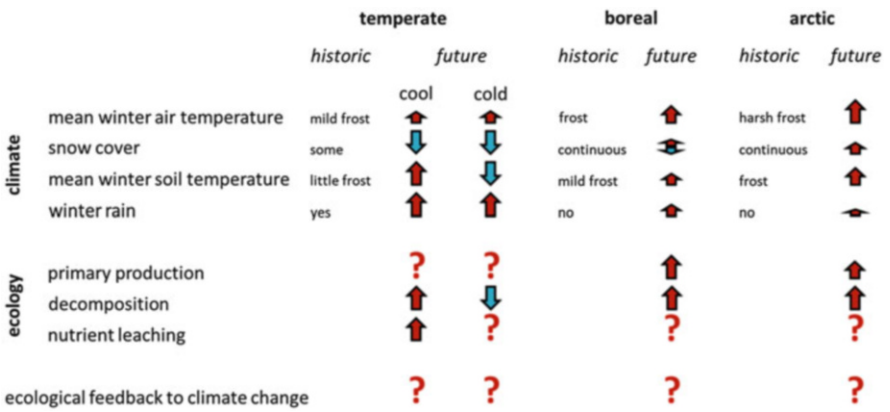

Fig. 3 Winter climate change of ecologically relevant parameters, their hypothetical effects on ecosystem functioning, and potential feedbacks to the climate systems separated by biomes

of increased decomposition in comparison to increased production, with the latter being complicated by several potentially negative balances such as increased climatic variability resulting in more frequent midwinter melting and rain-on-snow events which damage plant organs and can lead to dieback. The role of winter climate change on the net feedback of the biosphere to climate change is currently unclear for arctic ecosystems.

3.2 Boreal

Increasing air temperatures lengthen the growing season. Together with slightly increased or unaltered snow cover, air warming leads to slight soil warming. Based on these trends, decomposition and mineralization increase. Primary production increases. Nutrient leaching increases mainly during extreme events which limit primary production, e.g., due to increased climatic variability resulting in more frequent midwinter melting or rain-on-snow events which damage plant organs and can lead to plant dieback. Plant community composition is altered by these winter climate changes; its feedback on decomposition and nutrient leaching is unclear. The role of winter climate change on the net feedback of the biosphere to climate change is currently unclear for boreal ecosystems.

3.3 Temperate

Temperate ecosystems move toward opposing winter climate regimes. Cold-temperate ecosystems are influenced by the apparent paradox of "colder soils in a warmer world" (Groffman et al. 2001) due to air temperatures still being cold while soils being no longer protected by an insulating snow cover. This leads to reduced decomposition rates over winter and reduced primary production due to (sub)lethal root damage which further allows for increased nutrient leaching. Primary production is further limited by more frost damage to aboveground plant organs because of reduced frost tolerance and earlier leaf-out despite increased climatic variability leading to damaging frost events mainly in spring.

Cool-temperate systems are no longer snow-covered and air warming exceeds the cooling capacity of a missing snow cover for soil temperature. Consequently, air and soil temperatures increase, lengthening the growing season, increasing primary production and increasing decomposition rates. Markedly more winter rainfall,

however, leads to nutrient leaching which limits primary production. Effect sizes here are unknown. Primary production is further limited by more frost damage to aboveground plant organs because of reduced frost tolerance and earlier leaf-out despite increased climatic variability leading to damaging frost events mainly in spring.

The role of winter climate change on the net feedback of the biosphere to climate change is currently unclear for temperate ecosystems.

4 Summary and Key Uncertainties

Up to now, winter climate change effects on key ecological processes, i.e., primary production, decomposition, and nutrient leaching, are understudied in temperate, boreal, and arctic ecosystems. Surprisingly, uncertainties even about directions of change are largest for cold-temperate ecosystems. Change in snow cover and its effect on soil temperatures have been identified as key aspect in winter climate change. Clearly, better projections of soil temperature and climatic variability are needed from climate models in order to better predict ecological feedback loops to climate change and inform experiments about realistic manipulations (see Fig. 4 for potential experimental designs). Generally, experimental and observational studies need to shed light on temporal hierarchies in ecological processes, such as the role of root damage and root activity for decomposition of soil organic matter ("priming"; Kuzyakov 2010) or shifts in plant species composition due to winter climate change determining primary production (Krab et al. 2018) as well as litter quantity and decomposability (Cornelissen et al. 2007). Primary production is furthermore strongly determined by phenology, and a sound understanding of changes in the growing season needs to acknowledge not only the obvious but also the "hidden" aspects, i.e., explore the (missing) synchronicity between above- and belowground growth (e.g., Blume-Werry et al. 2016). Another major source of uncertainties are biotic interactions (Makoto et al. 2014) including phenological mismatches (Allstadt et al. 2015) and altered herbivory patterns (Tsunoda et al. 2018). Finally, global change drivers interact and first evidence suggests that this interaction is rarely additive, as shown for instance for the interaction between snow depth changes and N deposition (Vankoughnett and Henry 2014). Evidently, sound projections of future ecosystem functioning and biotic feedbacks to climate change require a comprehensive understanding of winter ecological processes, which have so far been too often neglected.

Fig. 4 Examples for winter climate change manipulation experiments. Top left, soil warming by heating wires in the ground to avoid soil frost come with the drawback that soil warming in the absence of air warming is unrealistic (Schuerings et al. 2013); top right, ecosystem warming by infrared heaters and warming cables on the ground allow for simulation of winter warming extremes as they are able to melt even thick snowpacks quickly (Kreyling et al. 2015b); bottom left, aboveground infrared warming mimics a warmer climate with realistic consequences on snow cover and soil temperature variation (Walter et al. 2013); bottom right, snow-out shelters reduce winter soil insulation while not altering water and nutrient input as the snow accumulated on the top melts through the perforated plastic sheets upon melting events (displayed study unpublished but see Kreyling et al. 2012a for comparable setup). Combining snow-out shelters with realistic changes in winter precipitation amount and/or air temperature warming is a promising approach to test for winter climate change effects in temperate ecosystems

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