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Ulrich Lüttge
Wolfram Beyschlag
Burkhard Büdel
Dennis Francis
Editors

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Part I
Review

A Half-Century Adventure in the Dynamics of Living Systems

Michel Thellier

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Abstract In response to the question "What is life", molecular biology has provided knowledge concerning the structure and function of the constituents of living systems. However, there still remains the point about understanding the dynamics of the processes involved in the functioning of the system. In our contribution to this quest, we began by some methodological improvements (especially

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concerning stable as well as radioactive isotopic tracers, ionic interactions and electrode measurements) and their possible applications to scientific or practical problems. Enzymatic reactions, fluxes of solutes and signalling processes play a crucial role in the dynamics of living systems. We have studied several non-usual cases of enzyme kinetics, particularly the functioning of those proteins that assemble when participating in a task and disassemble when the task is over (functioning-dependent structures or FDSs), and we have found that these FDSs could induce original regulatory properties in metabolic pathways. By studying fluxes of solutes through artificial (enzyme-grafted gel slabs) or real (frog skin) barriers, we have compared apparent kinetic parameters of the system with the real molecular parameters, and we have shown that increasing the complexity of a system may permit to evaluate parameters of the system that cannot be obtained using a conventional, reductionist approach. Concerning the transport of solutes between cells and their external medium, we have proposed to substitute a formalism derived from non-equilibrium thermodynamics for the classical combination of rectangular hyperbolas; in this interpretation, the important parameter is equivalent to a conductance; moreover we introduce a "symmetry-criterion" that is especially well adapted to discriminate active from passive exchanges between cells and exterior (while the Ussing's flux ratio equation remains the easiest way to discriminate active from passive exchanges through an epithelium). Plants are sensitive to a number of stimuli, biotic or non-biotic, traumatic or non-traumatic. Simplified systems (such as foliar discs or cell suspension cultures) have permitted us to study some cell responses to stimuli. With entire plants, we show that migration, storage and recall of information can also take place, and that a plant can recall stored information several times. From all that, we come to the conclusion that an important characteristic of living beings is that not only the processes within them are dynamic but that even their structure is dynamic for a part.

Keywords Active vs. passive transports • Enzyme-grafted gel slabs • Enzyme kinetics • Flux-ration equation • Functioning-dependent structures • Information recall • Information storage • Isotopic tracers • Plants • Solute fluxes • Stable isotopes • Symmetry-criterion

Abbreviations and Symbols

ACC	1-aminocyclopropane-1-carboxylic acid
FDS	Functioning-dependent structure
MAAC	Malonyl-1-aminocyclopropane-1-carboxylic acid
M–M	Michaelis–Menten
NCR	Neutron capture radiography
RCL function	The function enabling plants to recall stored information
SIMS	Secondary ion mass spectrometry
SPICE	Simulation programme with integrated circuit emphasis
STO function	The function permitting plants to store morphogenetic information

1 What is Life?

During centuries, a "vital strength" was assumed to have conferred on inanimate matter the property of becoming alive and of continuing to promote the spontaneous generation of living organisms from non-living material; however, not everyone agreed with this. The controversy continued to increase until the nineteenth century when all the experiments purporting to prove spontaneous generation were shown to be erroneous [see, for instance, Spallanzani (1787) and Pennetier (1907)]. Chemists succeeded in the abiotic synthesis of organic compounds (thus demonstrating that the substances produced by living processes and by artificial synthesis were not different in essence) and the theories of vitalism and spontaneous generation were abandoned.

Even before the old beliefs were rejected, a new approach, often termed "reductionism", had begun to be developed. The aim was no longer to obtain a global interpretation of life, but to describe biological objects at the molecular, cytological and histological levels and to study how the structure of each object endowed it with its particular function. This resulted in the acquisition of fundamental biological knowledge and in the development of innovative agricultural, medical and industrial applications. However, it was also clear that the reductionist approach was not sufficient to give a fully satisfactory answer to the question "*What is life?*"

This answer entailed understanding not only the *function* but also the integrated *functioning* of biological objects. The availability of isotopic tracers, the increasing performance of computers and conceptual advances (e.g. the "*Catastrophe theory*" by René Thom, the "*Dissipative structures*" of Ilya Prigogine and the general theories of non-equilibrium thermodynamics and of dynamical systems) progressively provided researchers with scientific tools more efficient than those available to the physiologists of the previous centuries.

Our group has used these tools to address the problem of the nature of life [97, 108] in four complementary ways: (a) methodological improvements (and their practical applications), (b) kinetics of enzyme-catalysed reactions under unusual conditions (including the case of "functioning-dependent structures" and their role in cell regulation), (c) solute fluxes and transmembrane transports, (d) signalling and response to stimuli. We have used plants as experimental models in most cases. I have collaborated with many co-workers. Their contributions have always been valuable and sometimes essential. Their names appear in the list of our major publications, which rank in chronological order in the Appendix.

2 Methods and Methodological Improvements

2.1 Radioactive and Stable Tracers, the NCR and SIMS Techniques

My interest in the use of radioactive isotopes for autoradiography has been marginal [75], but I have made an extensive use of radioactive tracers for the measurement of

exchanges of inorganic ions and other solutes. The radioactive isotopes of a few elements of biological interest (such as Li, B, N and O) are, however, unusable because of their very short half-lives; but these elements have a stable isotope with a high cross-section for a nuclear reaction with thermal neutrons. The general expression of a nuclear reaction, X(x, y)Y, means that a radiation, x, interacts with a nuclide, X, to form another nuclide, Y, with the emission of one or several radiations, y. For the four isotopes under consideration, the nuclear reactions of interest are $^{6}Li(n,\alpha)^{3}H$, $^{10}B(n,\alpha)^{7}Li$ $^{14}N(n,p)^{14}C$ and $^{17}O(n,\alpha)^{14}C$, in which n is a thermal neutron, p a proton and α a helium (^{4}He) nucleus. Following previous authors (Fick 1951; Hillert 1951; Faraggi et al. 1952), we have used these reactions for the imaging and the measurement of unidirectional fluxes of Li, B, N and O [1–2, 9, 12–13, 43–44, 58, 61, 67, 89]. The technique, which has been termed NCR for "Neutron-capture radiography" (Larsson et al. 1984), consists of sandwiching the sample between a quartz slide and a cellulose-nitrate film and irradiating the whole arrangement with thermal neutrons. When a radiation hits the film, it leaves a damage trail that can be enlarged by treatment with NaOH or KOH into a track visible by optical microscopy. The lateral resolution is of the order of a few micrometers; we have rendered the technique quantitative and we have contributed to show how to discriminate from one another the tracks originating from different nuclear reactions.

In the Secondary ion mass spectrometry (SIMS) method, which is also termed "Ionic analysis", the specimen is bombarded by a narrow beam of ions (the "primary ions") and the "secondary ions" that are sputtered from the specimen surface are collected, sorted by mass-spectrometry and arranged for building an image of the nuclide distribution at the specimen surface (Castaing and Slodzian 1962). Under the leadership of Camille Ripoll, the SIMS section of our laboratory was equipped initially with an IMS4F instrument [26, 55, 72, 80, 88, 120]. The machine was able to detect and image stable as well as radioactive isotopes of almost all chemical elements; several different secondary ions were detected quasi-simultaneously; the mass resolution allowed to discriminate secondary ions even when they were with the same mass number; the lateral resolution was of the order of 300 nm. In a collaboration with CAMECA (France) and Oxford Instruments (UK), our group has created a device permitting to study frozen-hydrated specimens [99, 113, 114], thus limiting the risk for mobile substances to be mobilised or lost during sample preparation. More recently, we have been equipped with a NanoSIMS50 instrument (Hillion et al. 1997) that can detect and image several different secondary ions strictly simultaneously with a lateral resolution of 50 nm.

For any system, the influx (or inflow), J_j^{ei}, of a solute, S_j, from exterior, e, to interior, i, is the unidirectional rate of S_j entry. An easy way to measure J_j^{ei} consists of labelling S_j in the external medium with a radioactive or a stable tracer, S_j*, and measuring the progressive accumulation of S_j* in the internal medium. Conversely, when an appropriate pre-treatment has permitted to label S_j with S_j* in the internal medium, one may follow the outflux (or outflow), J_j^{ei}, of S_j (i.e. the unidirectional rate of S_j exit) by measuring the progressive accumulation of S_j* in the external medium. The net flux (or net flow), J_j, of S_j is written

$$J_j = J_j^{ei} - J_j^{ie} \tag{1}$$

2.2 Ionic Interactions, Ionic Condensation, Microelectrodes

Inorganic ions (also termed "small ions") interact with one another and with the non-mobile distributions of electric charges borne by macromolecules [73]. With Maurice Demarty [15, 21, 42, 82], we have studied the binding of small cations with the anionic sites existing in plant cell walls. We have determined capacities of calcium fixation, saturation kinetics, dissociation constants and ionic selectivities (e.g. Ca^{2+}/Na^{+}, Ca^{2+}/K^{+} or Na^{+}/K^{+}). Using diffusion measurements in films made of isolated cell walls embedded in cellulose nitrate, we have also measured the mobility of the main cations (Na^{+}, K^{+}, Ca^{2+}, Mg^{2+}).

The inorganic cations present in living material are usually considered to be either free or bound; but this is an oversimplification, especially because it neglects the phenomenon of "ionic condensation" (Manning 1969, 1996). In the presence of polymers arranged in a linear distribution of non-mobile electric charges, some of the free counterions (ions with a sign opposite to that of the fixed charges) "condense" on the polymers at a critical value of the polymer charge density. This phenomenon resembles a phase transition. The condensed counterions are delocalised and can diffuse along the polymers, but they cannot leave their close vicinity. The divalent ions condense before the monovalent, the trivalent before the divalent, etc. Since the charge-density of the aligned polymers is easily increased or decreased (e.g. following the action of enzymes such as kinases and phosphatases), this can control the relative concentrations of free and condensed counterions; reciprocally, the calcium condensation/decondensation can change the activity of calcium-dependent kinases and phosphatases and consequently modulate the density of the non-mobile aligned charges. With Camille Ripoll, we have envisaged the possibility that these processes have a role in signal transduction [109].

With Jean-Paul Lassalles [47, 50, 52], we have determined sub-cellular electrical characteristics by use of microelectrodes and we have analysed the electrical noise to study transmembrane ion transports. The method was suited to the determination of vacuole ATPase activities, the electric resistance of cell membranes, the characteristics of passive ionic channels and the effect of introducing inorganic ions or pharmacological agents into the vacuole.

2.3 Practical Applications

Apart from using the above techniques for our own researches, we have been solicited by other teams for collaborations concerning diverse scientific or practical problems.

Using NCR, we have quantitatively imaged boron in plants at the tissue and cell levels for agronomical purpose [30, 57, 77, 83, 98, 106]. A part of this work was done in Costa Rica with the support of the "International Atomic Energy Agency". In flax seedlings, boron concentrations were measured in the vacuoles and in the primary and secondary walls of cells in various tissues. In the foliar parenchyma of clover seedlings, boron was shown to come from seed reserves and root uptake in

approximate ratios of 50%/50% in the cytoplasm and 25%/75% in the cell walls; this suggests that apoplastic migration occurs. After foliar application, in some plant species (e.g. clover) most of the boron remained immobilised at the site of application; in other species (e.g. young coffee trees), boron was rapidly distributed in the entire plant; this discrepancy was explained by differences in the plant content of sorbitol complexes (Brown et al. 1999). Boron was remobilized from old leaves to younger tissues [30].

In boron neutron capture therapy (a method for cancer treatment), ^{10}B-loaded molecules are accumulated in the tumour cells, which they kill thanks to the ionising particles produced by the $^{10}B(n,\alpha)^{7}Li$ nuclear reaction under neutron irradiation. Using NCR, we have measured ^{10}B contents in the tumour and in the neighbouring healthy cells [63, 87, 89, 104].

Lithium administration has specific effects such as treating some mental disorders or causing teratologic abnormalities. Using NCR [19–20, 24, 28, 36, 49, 65], we were the first to show that (a) lithium was finely regionalised in the brain of normal adult mice, whereas it was almost homogeneously distributed in the brain of foetuses or of adult dismyelinating mutants, (b) Li equilibration between plasma and brain was very rapid, (c) in foetuses borne by Li-treated mice, lithium was concentrated in bones, heart, eyes and endocrine glands (which were also tissues sensitive to teratogenic Li effects) [46, 94] and (d) in cells, lithium seemed to accumulate close to the plasma membrane [81].

With SIMS, we have developed a method, based on the isotopic dilution $^{6}Li/^{7}Li$, for the determination of lithium concentrations in small liquid samples [60]. The same method can be extended to the determination of the concentration of any element possessing two stable isotopes or of any substance labelled with these isotopes. By combining the use of NCR and SIMS with that of a nuclear probe, we have contributed to show that the stable isotopes ^{14}N and ^{15}N can be used for the fine localisation and the labelling of nitrogen-containing substances, with a view to application to environmental, biological or agricultural projects [71, 74, 86, 92]. ^{15}N labelling was used for the study of NO pollution [102]. With the support of a doctoral fellowship for environment and energy control, we have used the stable isotopes ^{17}O and ^{18}O and their detection by NCR and SIMS for oxygen labelling, especially for the labelling of CO by ^{18}O in order to study the effect of CO pollution on pollen grains [100].

With our Australian Colleague Ross Jeffree, we have used SIMS for the depth profiling of manganese in shells of the bivalve *Hyridella* with a view to reconstitute the history of the pollutions by manganese rejects [91]. Again by SIMS depth profiling [69], we have determined ionic ratios in the cuticle of plant cells; by coupling the use of SIMS with a differential extraction of calcium and pectic substances in different wall areas [70], we have found that the binding of the pectic molecules with one another was mainly due to calcium bridges in the intercellular junctions of most cells and to covalent bonding in the internal part of the wall of the epidermal cells. Both types of bond were coexisting in the intercellular junctions of the cortical parenchyma.

In collaboration with Odile Morvan and with the support of a grant from the Technical Institute for Flax, we have shown that the differentiation of the secondary

walls of flax fibres was accompanied by a strong increase of the Na/Ca ratio [79]. In flax seeds [84], Ca and Mg were located within the protein bodies, Na was out of the protein bodies and K was scattered all over the seed tissues. With the support of a grant from the Ministry for teaching and research, we have observed a transient increase of calcium in beech cambium and phloem at the end of the period of quiescence [96].

With isolated cell walls of *Lemna* fronds, there was a high selectivity in favour of divalent vs. monovalent cations, whereas the selectivity between monovalent cations was always close to 1 [15]. In flax epidermal cell walls, calcium bridges bound galacturonic polysaccharides to one another [90]. Ionic condensation explained why the salt activity was low in the cell walls of plants adapted to saline habitats, in the presence of high external saline concentrations [25].

In brief. *Radioactive tracers are commonly employed for imaging by autoradiography and for the determination of unidirectional fluxes of solutes by isotopic labelling. Here we show that techniques such as "Neutron capture radiography" and "Secondary ion mass spectrometry" enable one to use stable isotopes for imaging and labelling purposes with capabilities comparable to those with radioisotopes. Moreover, these techniques can be used in cases when the chemical elements under study do not possess any radioactive isotope with a half-life suited to tracer studies. Ionic interactions are omnipresent in living tissues. We have adapted classical physico-chemical techniques to the study of the interactions taking place between non-mobile charges (e.g. charges borne by macromolecules or cell organites) and small inorganic ions. The role of "Ionic condensation" should not be underestimated, though usually neglected by biologists. Subcellular characteristics have been determined by use of microelectrodes. Apart from using these techniques in our main studies, we have adapted them to a number of practical applications to agronomical, ecological and medical problems.*

3 Enzyme-catalysed Reactions Under Non-classical conditions

3.1 Brief Reminder of Classical Enzyme Kinetics

In the case of Michaelis–Menten kinetics, each enzyme is monomeric and possesses a single catalytic site and no regulatory site. Under steady-state conditions and assuming a few simplifying hypotheses, the curve representing the reaction rate, v, as a function of the concentration, c_s, of the substrate, S, is a rectangular hyperbola

$$v = V_m \cdot c_s/(K_m + c_s) \tag{2}$$

V_m is the maximum rate of the reaction (enzyme saturated by the substrate), K_m is the "Michaelis constant" and $1/K_m$ corresponds approximately to the enzyme affinity for S. By contrast, allosteric enzymes (Monod et al. 1965) are polymeric and possess several catalytic sites and, possibly, one or several activatory or

inhibitory sites; assuming again a few simplifying hypotheses, the rate of functioning becomes a sigmoid function of c_s

$$v = V \cdot (c_s)^n / ((K)^n + (c_s)^n) \tag{3}$$

(Hill empirical equation) in which V, K and n are the parameters of the system. Due to the sigmoid form of the curve $\{c_s, v\}$, there is a narrow domain of c_s-values before and after which the enzyme activity is close to zero and quasi maximal, respectively. The binding of an activator (or of an inhibitor) displaces this threshold domain towards lower (or higher) c_s-values.

3.2 Non-usual Cases of Enzyme Kinetics

In cells, enzymatic reactions are often arranged in sequences, termed metabolic pathways, which transform an initial substrate into a final product through a series of intermediates. With Donald Mikulecky, we have studied a model sequence of four Michaelis–Menten enzymes followed by an allosteric enzyme. Using a simplified version of the SPICE programme (SPICE = Simulation programme with integrated circuit emphasis), we have calculated the time-courses of the concentrations of all the intermediates according to whether the initial substrate of the sequence was not or was an activator of the allosteric enzyme [78]. When the initial substrate is an activator, this is termed a feedforward control, a situation that occurs in the glycolytic pathway (Bali and Thomas 2001). In the feedforward case, a reversal of the direction of the reaction occurred before reaching equilibrium. The corresponding stationary states and their preceding transients were obtained by clamping the concentration of one intermediate. The same approach can be used for cases when a feedback control takes place or when oscillations or chaos occur.

Some enzymes can function in organic solvents (Butler 1979). We have studied the chymotrypsin-catalysed hydrolysis/synthesis of a peptide bond in mixtures of water and 1,4-butanediol, which is of the Michaelis–Menten type. Apparent parameters V_m^{app} and K_m^{app} were evaluated by adjusting a rectangular hyperbola to the experimental points $\{c_s, v\}$; real kinetic parameters, V_m and K_m were determined by taking into account the partition of the substrate between the catalytic site of the enzyme and the butanediol. Decreasing the water content from 100% to 20% caused the V_m^{app}-value to be reduced by a factor of 2 and the K_m^{app}-value to increase exponentially, while the V_m and K_m values were unchanged [76, 107].

When a Michaelis–Menten enzyme was immobilized in a gel slab bathing in aqueous solutions, different forms of $\{c_s, v\}$ curves (hyperbolic, sigmoid, dual-phasic, etc.) were observed, depending on the structural characteristics of the system (enzyme concentration within the gel, shape and dimensions of the gel phase, possible existence of pH gradients, etc.) [35]. In some cases, it was possible to adjust a combination of two rectangular hyperbolas to the experimental $\{c_s, v\}$

points, although a single enzyme was present. Even when a single rectangular hyperbola could be adjusted to the experimental points, the values of the apparent parameters, V_m^{app} and K_m^{app}, had no reason to be identical with the real enzymatic parameters, V_m and K_m.

3.3 *Functioning-Dependent Structures*

Proteins involved in metabolic or signalling pathways often assemble into complexes ranging from quasi-static to transient dynamic associations (Srere 1987; Ovadi 1988; Spirin and Mirny 2003). Hyperstructures represent a level of organisation intermediate between molecules and cells (Norris and Fishov 2001). At the interface between these two concepts [118, 121], we have introduced the notion of "Functioning-dependent structure" (FDS). An FDS is an assembly of proteins that associate with one another when performing a task but dissociate when the task is over [115]. Many different proteins are sometimes involved in those associations (Bobik 2006; Cheng et al. 2008); but two-enzyme complexes are observed in most cases. There are advantages inherent in such associations. Complexes of proteins often offer a better resistance to degradation by hydrolytic enzymes than free proteins do (Buchler et al. 2005). In an assembly of sequential enzymes, it is usually admitted (Srere 1987; Ovadi 1991; Winkel 2004), though sometimes disputed (Cornish-Bowden 1991; Wu et al. 1991), that the product of an enzyme is channelled to the catalytic site of the next enzyme without being liberated into the cellular medium. This protects labile reactants from degradation and protects the cellular medium from being poisoned by toxic reactants.

We have wondered whether the kinetics of FDS functioning might endow them with original regulatory abilities. To explore quantitatively this possibility, we have studied numerically the steady-state kinetics of a model system of two sequential monomeric enzymes, E_1 and E_2, according to whether they were free or associated in an FDS [115–116]. In this modelling, E_1 and E_2 possessed a single catalytic site, had no activator or inhibitor sites and catalysed reactions with a single substrate and a single product, i.e.

$$S_1 \overset{E_1}{\rightleftharpoons} S_2 \overset{E_2}{\rightleftharpoons} S_3 \tag{4}$$

The reactions involved are listed in Table 1. To take into account that an FDS is formed only when the enzymes are active, that is when they are engaged in enzyme–substrate complexes, the direct binding of E_1 with E_2

$$E_1 + E_2 \rightleftharpoons E_1E_2 \tag{5}$$

did not exist in the reaction scheme. The system thus comprised 17 different chemical species (free substances and complexes) and 29 reactions. The reactions

Table 1 Reactions involved in the model of FDS under study

Reaction number	Reaction	Rate constants	Equilibrium constant
1	$E_1 + S_1 \rightleftarrows E_1S_1$	k_{1f}, k_{1r}	K_1
2	$E_1 + S_2 \rightleftarrows E_1S_2$	k_{2f}, k_{2r}	K_2
3	$E_2 + S_2 \rightleftarrows E_2S_2$	k_{3f}, k_{3r}	K_3
4	$E_2 + S_3 \rightleftarrows E_2S_3$	k_{4f}, k_{4r}	K_4
5	$E_1S_1 + E_2 \rightleftarrows E_1S_1E_2$	k_{5f}, k_{5r}	K_5
6	$E_1S_2 + E_2 \rightleftarrows E_1S_2E_2$	k_{6f}, k_{6r}	K_6
7	$E_2S_2 + E_1 \rightleftarrows E_1E_2S_2$	k_{7f}, k_{7r}	K_7
8	$E_2S_3 + E_1 \rightleftarrows E_1E_2S_3$	k_{8f}, k_{8r}	K_8
9	$E_1S_1 \rightleftarrows E_1S_2$	k_{9f}, k_{9r}	K_9
10	$E_2S_2 \rightleftarrows E_2S_3$	k_{10f}, k_{10r}	K_{10}
11	$E_1S_1E_2 \rightleftarrows E_1S_2E_2$	k_{11f}, k_{11r}	K_{11}
12	$E_1S_2E_2 \rightleftarrows E_1E_2S_2$	k_{12f}, k_{12r}	K_{12}
13	$E_1E_2S_2 \rightleftarrows E_1E_2S_3$	k_{13f}, k_{13r}	K_{13}
14	$E_1S_1E_2 + S_2 \rightleftarrows E_1S_1E_2S_2$	k_{14f}, k_{14r}	K_{14}
15	$E_1S_2E_2 + S_2 \rightleftarrows E_1S_2E_2S_2$	k_{15f}, k_{15r}	K_{15}
16	$E_1S_2E_2 + S_3 \rightleftarrows E_1S_2E_2S_3$	k_{16f}, k_{16r}	K_{16}
17	$E_1E_2S_2 + S_1 \rightleftarrows E_1S_1E_2S_2$	k_{17f}, k_{17r}	K_{17}
18	$E_1E_2S_2 + S_2 \rightleftarrows E_1S_2E_2S_2$	k_{18f}, k_{18r}	K_{18}
19	$E_1S_1E_2 + S_3 \rightleftarrows E_1S_1E_2S_3$	k_{19f}, k_{19r}	K_{19}
20	$E_1E_2S_3 + S_1 \rightleftarrows E_1S_1E_2S_3$	k_{20f}, k_{20r}	K_{20}
21	$E_1E_2S_3 + S_2 \rightleftarrows E_1S_2E_2S_3$	k_{21f}, k_{21r}	K_{21}
22	$E_1S_1 + E_2S_2 \rightleftarrows E_1S_1E_2S_2$	k_{22f}, k_{22r}	K_{22}
23	$E_1S_1 + E_2S_3 \rightleftarrows E_1S_1E_2S_3$	k_{23f}, k_{23r}	K_{23}
24	$E_1S_2 + E_2S_2 \rightleftarrows E_1S_2E_2S_2$	k_{24f}, k_{24r}	K_{24}
25	$E_1S_2 + E_2S_3 \rightleftarrows E_1S_2E_2S_3$	k_{25f}, k_{25r}	K_{25}
26	$E_1S_1E_2S_2 \rightleftarrows E_1S_2E_2S_2$	k_{26f}, k_{26r}	K_{26}
27	$E_1S_1E_2S_2 \rightleftarrows E_1S_2E_2S_3$	k_{27f}, k_{27r}	K_{27}
28	$E_1S_2E_2S_2 \rightleftarrows E_1S_2E_2S_3$	k_{28f}, k_{28r}	K_{28}
29	$E_1S_1E_2S_3 \rightleftarrows E_1S_2E_2S_3$	k_{29f}, k_{29r}	K_{29}
Global	$S_1 \rightleftarrows S_3$	–	K

were characterised by their forward and reverse rate constants, k_{jf} and k_{jr}, and by their equilibrium constant, K_j, with

$$K_j = k_{jf}/k_{jr} \tag{6}$$

For easier analysis, the parameters and variable were expressed as dimensionless quantities. The definition of the dimensionless quantities imposed the relation

$$k_{1r} \equiv 1 \tag{7}$$

The equations of the system were obtained by writing down that (a) the total concentration of each enzyme, E_1 and E_2, was constant and (b) the variation of the concentration of each chemical species was given by the balance of the reactions

producing and consuming that species. Whatever the pathway from S_1 to S_3, the equilibrium constant, K, of the global reaction

$$S_1 \rightleftarrows S_3 \tag{8}$$

kept obviously the same value. This imposed the existence of relationships between the rate constants. Among the 58 rate constants of the system (Table 1), only 43 were independent and could be given an arbitrary value in the numerical calculations. The 15 other rate constants had to be calculated. We have chosen the set of constants

$$K_1, K_2, K_3, K_5, K_9, K_{10}, K_{11}, K_{12}, K_{13}, K_{15}, K_{17}, K_{27}, K_{29}, K \text{ and } k_{1r} \text{ to } k_{29r} \tag{9}$$

as our base of independent parameters. Under steady-state conditions of functioning, external mechanisms were assumed to maintain S_1 and S_3 at a constant and zero concentration, respectively; the concentrations of the other species had ceased to vary.

The numerical simulation consisted in varying systematically the values allocated to the independent parameters, calculating the corresponding values of the non-independent parameters and solving the equations of the system. For each set of parameter values, a curve $\{s_1, v\}$ (in which v was the dimensionless, steady-state rate of functioning and s_1 was the dimensionless concentration of S_1) was drawn in a few minutes. Testing three values (low, middle and high) for each independent parameter would have represented a total of approximately 10^{20} different cases to be numerically simulated. We used our intuition to limit the numerical simulations to a reasonable number. We have found sets of parameter values such that the $\{s_1, v\}$ curves obtained with an FDS presented extended linear or invariant parts, or were with a v-value appreciably above zero only in a limited domain of s_1-values (spike responses), or else exhibited step-like (sigmoid) or inverse-step responses [115]. In some cases, $\{s_1, v\}$ curves with two inflexion points, which conferred on them a "dual-phasic" aspect, were also observed; although their functional advantage is not clear, it is noteworthy that dual-phasic curves are often exhibited by both natural and artificial enzyme and transport systems (Sects. 3.2, 4.1.1 and 4.2.1).

Free enzymes also can generate linear and invariant responses [116] and the step responses are usually not as good as those obtained with allosteric enzymes (Sect. 3.1); but the spike and inverse-step responses complete nicely the panoply of regulatory functions that might exist in a metabolic pathway.

In brief. *Among the quasi-infinite number of chemical reactions potentially existing in a cell, only those catalysed by enzymes proceed at a rate compatible with life. A considerable number of investigations have thus been devoted by many authors to study the kinetics of enzyme-catalysed reactions: in the simplest case (enzymes of the "Michaelis–Menten" [M–M] type studied in diluted solutions* in vitro*), the kinetic curves have the shape of a rectangular hyperbola characterised by two parameters, V_m and K_m; in more complicated case (e.g. allosteric*

enzymes) the curve may become sigmoid, etc. Here, we have studied a few cases in which an enzyme was studied under more or less complex conditions. When an allosteric enzyme, inserted in a sequence of enzymes of the M–M type, was subjected to feedforward control (activation by a substrate found earlier in the sequence), a reversal of the direction of the reaction occurred before reaching equilibrium and the sequence behaved as an "anticipatory system". With enzymes of the M–M type studied in a partly aqueous/partly organic solution or under organised conditions, apparent parameters, V_m^{app} and K_m^{app}, could be determined by adjusting one or several rectangular hyperbolas to the kinetic data, but these apparent parameters were not equal to the real kinetic parameters, V_m and K_m; the number of hyperbolas adjusted to each kinetic curve was not representative of the number of enzymes involved; the existence of a sigmoid kinetic curve was not proof that an allosteric enzyme was present. The dynamic association of two sequential enzymes (Functioning-dependent structure [FDS]) occurring in an active metabolic pathway generated steady-state kinetics exhibiting the full range of basic input/output characteristics found in electronic circuits such as linearity, invariance, or the production of spike-, step- or inverse step-responses.

4 Fluxes of Solutes Exchanged by Biological Systems

4.1 Fluxes of Solutes Between Macroscopic Aqueous Compartments

In these experiments, an interface was separating two aqueous compartments, e and i (for exterior and interior). The compartments were large enough to allow the sampling of droplets for chemical analysis and the measurement of electric parameters.

4.1.1 Fluxes of Solutes Through an Enzyme-Grafted Gel Slab

Continuing the investigation described in the third paragraph of Sect. 3.2, we have examined a case [35] in which two enzymes of the Michaelis–Menten type, E_1 and E_2, inserted at random in a gel slab separating compartments e and i, were catalysing reactions such as

$$\begin{aligned} &\text{Enzyme } E_1\text{: } S + XY \rightarrow PX + Y \\ &\text{Enzyme } E_2\text{: } PX \rightarrow S + X \end{aligned} \tag{10}$$

At the start of the experiment, both compartments, e and i, contained equal concentrations of each solute (S, X, Y, XY and PX). The pH values in the

compartments were such that E_1 and E_2 were active only in layers of the gel slab, L_1 and L_2, facing e and i, respectively. As a consequence of the concentration profiles thus created in the gel slab, an uphill transport of *S* occurred from e to i at the expense of the energy provided by the splitting of XY. This mimicked a first-order active transport in Mitchell's classification (Mitchell 1967). Depending on the structural features of the system (pH difference between e and i, enzyme concentrations in the gel slab, thicknesses of L_1, L_2 and the inactive layer between them), the curve representing the rate, v, of the transport of *S* as a function of the concentration, c_s^e, of *S* in e exhibited a variety of shapes (e.g. hyperbolic, dual- or multi-phasic, sigmoid); moreover, when a combination of two rectangular hyperbolas could be adjusted to the experimental points $\{c_s^e, v\}$, no simple relationship was found to exist between the apparent parameters (V_{m1}^{app}, K_{m1}^{app}, V_{m2}^{app}, K_{m2}^{app},) calculated from the hyperbolas and the real parameters (V_{m1}, K_{m1}, V_{m2}, K_{m2}) of E_1 and E_2.

In another investigation [56, 59], a single enzyme subjected to appropriate structural constraints catalysed an uphill transport of a solute, S_j, driven by a pH gradient. This was equivalent to a second-order active transport (Mitchell 1967). In that case, again the apparent kinetic parameters of the transport were generally not representative of the real kinetic parameters of the enzyme involved in this system.

4.1.2 Fluxes of Solutes Through an Isolated Frog Skin

4.1.2.1 Ussing's Flux-Ratio Equation

The ionic concentration of the fresh water in which frogs bathe is much less than that of their internal medium. Therefore, they tend to lose salt by diffusion. Maintaining their homeostasis requires the operation of an active, inward pumping of ions. Using metabolic inhibitors is not sufficient to determine which ion(s) is/are actively pumped, because the inhibitor may give rise to secondary effects altering also the transport of passively exchanged ions. Ussing (1949, 1971) mounted isolated frog skins between two aqueous compartments, e (on the external side of the skin) and i (on the internal side), and he proposed to discriminate the ions passively and actively exchanged by use of a "flux-ratio equation". If S_j is an ion exchanged between e and i, verifying

$$R \cdot T \cdot \ln\left(J_j^{ei}/J_j^{ie}\right) = R \cdot T \cdot \ln\left(c_j^e/c_j^i\right) - z_j \cdot F \cdot (\Psi^i - \Psi^e) \tag{11}$$

means that S_j is passively transported. In (11), R, T and F have their usual thermodynamic signification, J_j^{ei} and J_j^{ie} are the inflow and outflow of S_j, c_j^e and c_j^i are the external and internal concentrations of S_j, z_j is the electric charge of S_j and Ψ^e and Ψ^i are the external and internal electric potentials). By contrast, when

$$R \cdot T \cdot \ln\left(J_j^{ei}/J_j^{ie}\right) > R \cdot T \cdot \ln\left(c_j^e/c_j^i\right) - z_j \cdot F \cdot (\Psi^i - \Psi^e) \tag{12}$$

or

$$R \cdot T \cdot \ln\left(J_j^{ei}/J_j^{ie}\right) < R \cdot T \cdot \ln\left(c_j^{e}/c_j^{i}\right) - z_j \cdot F \cdot (\Psi^i - \Psi^e) \tag{13}$$

this means that S_j is actively transported from e to i or i to e, respectively.

When samples of frog skin were mounted between Ringer's solutions, sodium was actively pumped inwards (relationship (12) satisfied with $S_j = Na^+$). Under short-circuiting conditions (i.e. imposing $\Psi^i - \Psi^e = 0$), the net flux of sodium expressed in μA, J_{Na} (1), was equal to the electric intensity measured through the skin. This was meaning that sodium was the only ion for which the distribution was dependent on an active pumping; all the other ions were passively distributed. The ionic pump was subsequently shown to be a Mg-dependent Na^+–K^+-ATPase, an enzyme that pumps Na^+ inwards and K^+ outwards (K^+ being immediately re-equilibrated between i and e by diffusion).

4.1.2.2 Lithium-Induced Electric Oscillations

Li^+ is the only ion that can replace Na^+ in the functioning of the ionic pump (Nagel 1977). In Ussing's experiments, the electric potential difference, $(\Psi^i - \Psi^e)$, was stable; but, if lithium was partially or totally substituted for sodium in compartment e, $(\Psi^i - \Psi^e)$ often became oscillatory (Takenaka 1936; Teorell 1954).

After studying the Li-induced oscillation experimentally [29], we modelled the skin epithelium [33] by two adjacent compartments, C_1 and C_2, and three interfaces, *a* (between the external medium and C_1), *b* (between C_1 and C_2) and *c* (between C_2 and the internal medium). Interface *a* was considered a membrane permeable to Na^+ and Li^+ but almost impermeable to K^+, interface *b* as a membrane bearing ATPases that pump Na^+ (or Li^+) from C_1 to C_2 and K^+ from C_2 to C_1, and interface *c* as a diffusive barrier. In the numerical simulation of the model, we were able to render it consistent with the experimental observations only when a certain number of properties were fulfilled. The first of this property was

$$V_1/V_2 \ll 1 \tag{14}$$

in which V_1 and V_2 are the volumes of C_1 and C_2 per unit surface area of epithelium. This means that C_1 has to be much smaller than C_2, thus excluding the possibility for C_1 and C_2 to correspond to the bulk of the cytoplasmic volume and to a few endoplasmic cisternae; C_1 was rather corresponding to cytoplasmic vacuoles transporting Na^+ or Li^+ ions and C_2 to intercellular spaces (most of the cell cytoplasm not being involved in Na^+ and Li^+ transport). Another requirement was

$$0 < \tau_1/\tau_2 < 1, \quad \text{with } 1 \text{ min } \leq \tau_1 \leq 5 \text{ min} \tag{15}$$

in which τ_1 and τ_2 are characteristic times corresponding to modifications of Li^+ in C_1 and K^+ in C_2, respectively. If ρ and μ represent the ratio of the active vs. the passive fluxes of Li^+ and K^+, respectively, the relationships

$$\rho \gg 1, \mu \gg 1, \quad \text{with } \rho \leq \mu \leq 2\rho \tag{16}$$

must also be satisfied, which means that ρ and μ have to be of the same order of magnitude. Finally, if P holds for the permeability coefficients of interfaces a, b and c for the ions Li^+ and K^+, these coefficients have to obey the relationships

$$P_{Li}{}^{a}/P_{K}{}^{c} \ll 1, P_{K}{}^{b}/P_{K}{}^{c} \ll 1 \tag{17}$$

By inducing an oscillatory behaviour (as a consequence of adding lithium in the external medium) ,we have thus yielded information on the values of a few variables (V_1, V_2, τ_1, τ_2, ρ, μ, $P_{Li}{}^{a}$, $P_{K}{}^{b}$ and $P_{K}{}^{c}$) that are characteristic of the system behaviour under natural conditions.

4.2 *Transport of Solutes by Plant Cells*

We term "cell transport", "transmembrane transport" or simply "transport" the exchange of solutes that takes place between the cells of entire plants or of separated tissues and their external medium. Solute transport must be distinguished from solute "migration", which corresponds to a movement of solutes at a lesser or greater distance within the plant. We term "carrier" any protein or assembly of proteins that catalyses the transmembrane transport of a solute. We have chosen cell suspension cultures or small aquatic plants as experimental models, because their exchanges of solutes were corresponding essentially to cell transport, while further solute migration was nil or extremely limited. We have revisited the conventional formulation of cell transport in order to get a Flow/Force description of the uptake of solutes.

4.2.1 Conventional Formulation of Cell Transport

The conventional formulation of cell transport was created for interpreting experimental measurements of the inflow, J_j^{ei}, of a solute S_j into a plant sample (e.g. a root system) as a function of the external concentration, c_j^e, of S_j. With a narrow range of c_j^e values, under steady-state conditions the distribution of points $\left\{c_j^e, J_j^{ei}\right\}$ often increases monotonically up to a plateau. Following a suggestion by Emmanuel Epstein (1953), a rectangular hyperbola

$$J_j^{ei} = V_{jm}^{app} \cdot c_j^e / \left(K_{jm}^{app} + c_j{}^e\right) \tag{18}$$

was adjusted to these points and was given the meaning of a system of the Michaelis–Menten type (Sect. 3.1). The apparent parameters, V_{jm}^{app} and $1/K_{jm}^{app}$, were assumed to correspond to the maximal inflow (carrier C_j saturated by S_j) and to an approximate value of the affinity of C_j for S_j. With a wide range of c_j^e-values, the distribution of points $\left\{c_j^e, J_j^{ei}\right\}$ often had a dual- or multi-phasic aspect, to which it was impossible to adjust a single hyperbola. When two or more hyperbolas could be adjusted to the experimental points, the conventional interpretation was that the transport process involved as many different carriers (C_{j1}, C_{j2}, etc.), the kinetic parameters of which were the apparent parameters (V_{jm1}^{app} and K_{jm1}^{app}, V_{jm2}^{app} and K_{jm2}^{app}, etc.) as calculated from the adjusted hyperbolas (Epstein 1966). When the distribution of experimental points exhibited a sigmoid shape, the carrier was assumed to be an allosteric protein (Glass 1976).

In brief, the conventional formulation aimed to derive precise molecular parameters from the measurement of fluxes into the whole, macroscopic system.

4.2.2 Flow/Force Formulation of Cell Transports

4.2.2.1 Principle of the Flow/Force Formulation

By contrast with the conventional formulation, the Flow/Force formulation aims to determine parameters characteristic of the overall functioning of a transport process, whatever the possible complexity of the underlying molecular mechanisms; i.e. it derives a macroscopic interpretation from the macroscopic flux measurements. Formerly, this approach was termed an "electrokinetic interpretation" because initially inferred from a formal analogy with classical electrokinetics [4–5], but it is in fact [6, 122] a consequence of non-equilibrium thermodynamics (Katchalsky and Curran 1965).

If a plant system (plant roots, water weeds, cell suspension culture, etc.) is left for a long time in a growth solution containing an invariable concentration, $^{\circ}c_j^e$, of a solute S_j, this plant system equilibrates with the external solution. The net flow, J_j, of S_j tends to become equal to zero [$J_j^{ei} = J_j^{ie}$ in (1)] when the growth of the system is negligible, while a non-nil J_j-value simply compensates for the dilution effect due to a non-negligible growth of the system. If larger or smaller values, c_j^e, of the external concentration of S_j are substituted for $^{\circ}c_j^e$, the net flow J_j is changed accordingly. Increasing or decreasing the external concentration can be accomplished by supplying the growth medium with an appropriate quantity of S_j or by diluting it with an appropriate volume of a solution lacking S_j but otherwise identical with the growth solution. According to non-equilibrium thermodynamics, as long as the c_j^e - values are not too different from $^{\circ}c_j^e$, J_j is a linear function of ln (c_j^e). When all calculations are done [122], this is written

$$J_j = J_j\left(c_j^e\right) = R \cdot T \cdot \lambda_j \cdot \ln\left(c_j^e/{}^{\circ}c_j^e\right) = L_j \cdot \ln\left(c_j^e/{}^{\circ}c_j^e\right) \tag{19}$$

when growth can be taken as negligible, or

$$J_j = J_j\left(c_j^e\right) = R \cdot T \cdot \lambda_j \cdot \ln\left(c_j^e / c_j\right) = L_j \cdot \ln\left(c_j{}^e / c_j\right) \tag{20}$$

in the general case (non-negligible growth), with

$$L_j = R \cdot T \cdot \lambda_j \tag{21}$$

In these equations, λ_j and L_j are constant parameters as long as c_j^e is not too different from $^\circ c_j^e$, ln (c_j) is the intercept of the straight line $\{\ln (c_j^e), J_j\}$ with the axis of abscissas and the other symbols have already been defined.

$R{\cdot}T{\cdot}\ln (1/{}^\circ c_j^e)$ (negligible growth) or $R{\cdot}T{\cdot}\ln (1/c_j)$ (non-negligible growth) correspond to the resulting effect of the terms other than c_j^e that contribute to the overall force driving the transport of S_j (e.g. internal concentration, c_j^i, of S_j, activity coefficients, possible coupling with metabolic reactions or with the transport of other solutes). The slope, L_j, of the linear approximation $\{\ln (c_j^e), J_j\}$ (and therefore also λ_j) represents the overall conductance of the plant system for the transport of S_j. We have verified that linear relationships $\{\ln (c_j^e), J_j\}$ could be adjusted, on ranges of c_j^e - values extending over two orders of magnitude or even more, to most of the published data relative to cellular transport [4–5, 122].

It may be noted that the Flow/Force formulation is simply, for a transmembrane transport process, the equivalent of what is Ohm's law for an electric process.

4.2.2.2 Arrhenius Plot: Significance of a Variation of Conductance

When changing the experimental conditions entails a modification of the conductance, λ_j, this may result from a quantitative or a qualitative change in the cell devices involved in S_j transport. A quantitative change may correspond to a modification of the number of S_j carriers per cell and a qualitative change to a modification of the specific activity of these carriers. To discriminate between these two possibilities, one may measure λ_j-values at different values of the absolute temperature T and draw graphs $\{1/T, \log \lambda_j\}$ (Arrhenius plots). If parallel or non-parallel straight lines can be adjusted to the points thereby obtained under the different experimental conditions, this means that the observed modification of conductance is purely quantitative or qualitative, respectively [5, 10, 122].

4.2.2.3 Active Versus Passive Transports: Use of the Flux-Ratio Equation

To test the active or passive character of the transport of a solute, S_j, through a membrane separating two sub-cellular compartments, we began by transposing the use of Ussing's flux-ratio equation (Sect. 4.1.2.1). In plant cells, the main compartments are the wall, w, the cytoplasm, c, and the vacuole, v; the membranes

are the plasmalemma (between w and c) and the tonoplast (between c and v). The relative volumes of compartments w, c and v and the surface areas of the membranes between them were determined in a morphometric study of the plant samples [38]. A compartmentalised model of the system was elaborated from those data. Compartment analysis (Atkins 1973) was used for flux determination [17, 32, 40–41].

The compartment analyses were carried out in six steps: (a) equilibration of the plant specimens with a growth medium containing a concentration, c_j^e, of a solute S_j labelled with an isotopic tracer, S_j*, (b) substitution of a fresh non-labelled external medium for the previous growth medium, (c) determination of the time-course of the exit of S_j* from the plant samples to the non-labelled external solution, (d) splitting of the overall curve of S_j*-exit into a series of exponential terms, (e) calculation of concentrations and fluxes (c_j^w, c_j^c, c_j^v, J_j^{ew}, J_j^{we}, J_j^{wc}, J_j^{cw}, J_j^{cv}, J_j^{vc}) from these exponential terms and using the compartmental modelling of the system, (f) evaluation or measurement of the electric potential differences, ($\Psi^w - \Psi^c$) and ($\Psi^c - \Psi^v$). Finally, according to whether

$$R \cdot T \cdot \ln\left(J_j^{wc}/J_j^{cw}\right) = R \cdot T \cdot \ln\left(c_j^w/c_j^c\right) - z_j \cdot F \cdot (\Psi^c - \Psi^w) \tag{22}$$

or

$$R \cdot T \cdot \ln\left(J_j^{wc}/J_j^{cw}\right) > R \cdot T \cdot \ln\left(c_j^w/c_j^c\right) - z_j \cdot F \cdot (\Psi^c - \Psi^w) \tag{23}$$

or else

$$R \cdot T \cdot \ln\left(J_j^{wc}/J_j^{cw}\right) < R \cdot T \cdot \ln\left(c_j^w/c_j^c\right) - z_j \cdot F \cdot (\Psi^c - \Psi^w) \tag{24}$$

it was concluded that, at the plasmalemma, S_j was passively transported (22), actively pumped inwards (23) or actively pumped outwards (24). Using the relationships

$$R \cdot T \cdot \ln\left(J_j^{cv}/J_j^{vc}\right) = R \cdot T \cdot \ln\left(c_j^c/c_j^v\right) - z_j \cdot F \cdot (\Psi^v - \Psi^c) \tag{25}$$

$$R \cdot T \cdot \ln\left(J_j^{cv}/J_j^{vc}\right) > R \cdot T \cdot \ln\left(c_j^c/c_j^v\right) - z_j \cdot F \cdot (\Psi^v - \Psi^c) \tag{26}$$

$$R \cdot T \cdot \ln\left(J_j^{cv}/J_j^{vc}\right) < R \cdot T \cdot \ln\left(c_j^c/c_j^v\right) - z_j \cdot F \cdot (\Psi^v - \Psi^c) \tag{27}$$

the same reasoning was held for the tonoplast. It is preferable (whenever possible) to substitute S_j-activities (a_j^w, a_j^c and a_j^v) for the concentrations c_j^w, c_j^c and c_j^v in relations (22)–(27). The reason is that the assumption

$$a_j^e/a_j^i = c_j^e/c_j^i \tag{28}$$

which was acceptable in the original Ussing's studies (in which both media, e and i, were diluted aqueous solutions) may become incorrect when e and i are sub-cellular compartments. We have taken into account the non-uniformity of the cells in the plant sample, the system growth, cell differentiation, the metabolism of S_j and possible shock effects occurring when changing the external medium [17, 32, 40, 48]. The drawbacks are that morphometric studies and compartment analyses are heavy and time-consuming, determining electric potentials (Sect. 2.2) and activity coefficients in cell compartments is difficult and an unrealistic modelling of the system may be responsible for erroneous conclusions.

4.2.2.4 Active Versus Passive Transports: Use of the Symmetry-Criterion

In order to avoid those difficulties, we have developed an alternative method, termed the "symmetry-criterion", for the determination of the active or passive character of the transport of S_j. To use the symmetry-criterion, the availability of an analogue, S_k, of S_j is requisite; S_k has to be sufficiently similar to S_j for being transported by the same carrying device, which implies that

$$L_k = L_j \tag{29}$$

and/or that L_j does not depend on the presence or absence of S_k in the external medium of the plants. The net flux of S_j, $J_j(c_k^e)$ [measured as a function of c_k^e and at a constant value of c_j^e] and that of S_k, $J_k(c_j^e)$ [measured as a function of c_j^e and at a constant value of c_k^e], may be written

$$J_j\left(c_k^e\right) = L_{jk} \cdot \ln\left(c_k^e / {}^{\circ}c_k^e\right) \tag{30}$$

and

$$J_k\left(c_j^e\right) = L_{kj} \cdot \ln\left(c_j^e / {}^{\circ}c_j^e\right) \tag{31}$$

in which the "cross-coefficients", L_{jk} and L_{kj}, are quasi-constant as long as c_j^e and c_k^e are not too different from the concentrations, ${}^{\circ}c_j^e$ and ${}^{\circ}c_k^e$, in the initial growth medium of the plants. Starting from Onsager's reciprocity relations (Onsager 1931), we have shown that verifying the relations

$$L_{jk} = L_{kj} \tag{32}$$

or

$$L_{jk} \neq L_{kj} \tag{33}$$

meant that S_j (and therefore also S_k) was passively (32) or actively (relation 33) transported [14].

4.2.3 Examples of Application of the Flow/Force Formulation

We have used compartment analysis [32], a morphometric study [38] and the flux-ratio equation (Sects. 4.1.2.1 and 4.2.2.3) to study sub-cellular exchanges of sulphate by *Lemna* fronds. The plants were pre-equilibrated in a nutrient solution containing 0.54 mM of ^{35}S-labelled sulphate. They were transferred into a solution of identical composition but non-radioactively labelled. The sulphate exit data was interpreted using a compartment model with the usual three main compartments (w, v and c) plus an extra compartment corresponding to sulphate metabolism. Under the experimental conditions used in these experiments, sulphate appeared to be passively distributed between cytoplasm and vacuole (equation (25) verified) but actively taken up from wall to cytoplasm (relation (23) verified) [38]. Moreover, when *Lemna* or *Riccia* plants were subjected to a transient depletion of sulphate, the conductance λ_j (index j = sulphate) was significantly enhanced [3]. This was consistent with the observation (Hawkesford et al. 1993) that sulphur transport is increased in sulphur-starved plants. With *Lemna* fronds studied in a range of temperatures 2–22°C, and using an Arrhenius plot (Sect. 4.2.2.2), the slopes of the linear approximations $\{1/T, \log \lambda_j\}$ were close to – 2,700, – 2,960 and – 3,080 (arbitrary units) for plants non-starved or sulphate-starved during 6 or 12 days, respectively [5]. Since those slopes differ by less than 8%, it may be concluded that the increase of sulphate-conductance induced by sulphate-starvation is of an essentially quantitative nature. It is likely that plants react to sulphate starvation by simply increasing the number of sulphate carriers per cell.

To apply the flux-ratio equation to the study of subcellular transports of boric acid by *Lemna* fronds, our experimental protocol [17] resembled that for sulphate studies. The system was modelled using compartments w, c and v, plus a fourth compartment corresponding to the binding of H_3BO_3 by cis-diol molecules in the cell walls. NCR (Sect. 2.1) and the stable isotopes ^{10}B and ^{11}B were used for the measurements. With an H_3BO_3-concentration equal to 0.16 mM in the growth and experimental solutions, boric acid appeared to be passively distributed between w, c and v, although the possible occurrence of small active effluxes could not be totally excluded [equations (22) and (25) not perfectly verified] [38].

The flux-ratio equation was applied again to study sub-cellular transports of lithium by *Lemna* fronds. The stable isotopes ^{6}Li and ^{7}Li and their detection by SIMS (Sect. 2.1) were used for compartment analysis. When the growth and experimental solutions contained 1 mM LiCl, lithium appeared to be pumped out of the cytoplasm, both towards the wall and the vacuole (relations (24) and (26) verified) [40–41]. One might be tempted to interpret the active character of Li-transport as a consequence of an interaction of lithium with the proton gradients involved in the co-transports of glucose and other solutes. The addition of glucose and that of fusicoccin are known to decrease or to strongly increase, respectively, the proton gradients (Marrè 1980). We have found that the addition of lithium decreased the absorption of glucose by *Lemna* fronds, but neither the addition of glucose nor that of fusicoccin had any effect on the unidirectional Li-fluxes [37].

Therefore, when lithium disturbs the intake of sugars or other solutes, this is rather due to an adverse effect on the cell devices catalysing the transport of these solutes than to a direct interaction of lithium with the couplings driving these transports.

We have used Rb^+ as an analogue of K^+ [7] to apply the symmetry-criterion (Sect. 4.2.2.4) to the determination of the active or passive character of the transport of potassium by *Lemna* fronds. With indices k and j referring to K^+ and Rb^+, and using the radioactive isotopes ^{40}K and ^{86}Rb as tracers, we have measured the fluxes, $J_k(c_j^e)$ and $J_j(c_k^e)$ [8]. Applying equations (30) and (31) to four similar experiments, we have found 6.71, 2.83, 5.41 and 1.14 for the value of the ratio of the cross-coefficients L_{kj}/L_{jk}. On average, L_{kj} was approximately four times as large as L_{jk} (relation (33) verified). Under the experimental conditions used in these experiments, potassium was thus actively transported. In another series of experiments, *Lemna* fronds were bathed in KCl solutions deprived of calcium and magnesium, or containing 1 mM of either calcium or magnesium. As could be expected (Elzam and Hodges 1967), at all external K^+-concentrations, c_k^e, the flux of K^+ transport, $J_k(c_k^e)$, was decreased in the presence of alkaline cations. When the data was analysed using the Flow/Force formulation (20), the decrease in the $J_k(c_k^e)$-values appeared to be mainly due to a decrease of the conductance, λ_k. Using an Arrhenius plot (Sect. 4.2.2.2) in the range of temperatures 5–20°C [10], the slopes of the linear approximations $\{1/T, \log \lambda_k\}$ were close to – 0.40, – 0.85 and – 0.87 (arbitrary units) for the plants in the absence of Ca^{2+} and Mg^{2+}, or in the presence of either Ca^{2+} or Mg^{2+}; that is the slopes were more than twice as large in the presence of calcium or magnesium than in the absence of any alkaline-earth cation. The decrease of λ_k induced by the presence of calcium or magnesium was thus of a qualitative nature. This could be due to alkaline-earth cations changing the specific activity of the membrane carriers of potassium. However, since divalent cations bind to the anionic sites of cell walls with a high selectivity vs. monovalent cations (last paragraph in Sect. 2.3), an alternative interpretation may be that the presence of Ca^{2+} and/or Mg^{2+} decreases the accessibility of K^+ to the cell wall and hence to the membrane K^+-carriers.

When plant samples are put in contact with an aqueous solution containing labelled bivalent cations, these cations passively bind to the anionic sites of the wall (Sect. 2.2) in a lapse of time so short that their transport to the cell interior remains negligible. A Flow/Force reinterpretation [4] of older data by Epstein and Leggett (1954) shows that Sr^{2+} can be used as an analogue of Ca^{2+}. With indices j and k referring to Ca^{2+} and Sr^{2+}, and using the radioactive tracers ^{45}Ca and ^{89}Sr, the symmetry-criterion is applicable and predicts that L_{jk} should be equal to L_{kj} (32). We have measured the fluxes, $J_j(c_k^e)$ and $J_k(c_j^e)$ [8] and, using equations (30) and (31) in three successive experiments, we have found 1.18, 1.25 and 1.20 for the value of the ratio L_{jk}/L_{kj}. This is written

$$L_{jk} \approx L_{kj} \tag{34}$$

which is consistent with the prediction by the symmetry-criterion.

In brief. *By imposing adequate structural constraints to enzymes, it is possible to create "artificial carriers" permitting the uphill transport of a solute. Again, there is no reason for the apparent parameters,* V_m^{app} *and* K_m^{app} *that can be determined by adjusting one or several rectangular hyperbolas to the kinetic data, to be equivalent to the real kinetic parameters,* V_m *and* K_m*; the number of hyperbolas adjusted to each kinetic curve is not representative of the number of enzymes involved; the existence of a sigmoid kinetic curve is not proof that an allosteric system is present. When a separated frog skin is mounted between two compartments filled with a classical Ringer solution, a stationary electric potential difference of a few tens mV is measured across the skin. When lithium was substituted for sodium in the solution on the external side of the skin, the electric potential difference became oscillatory. From the characteristics of this oscillation, and using an appropriate modelling of the skin epithelium, it was possible to determine the order of magnitude of the values taken by parameters that cannot be determined by a classical, reductionist approach. The conventional interpretation of the fluxes of solutes measured in cell transport is based on the adjustment of Michaelis–Menten hyperbolas to the experimental kinetic data. However, we have seen above that this approach leads to the determination of apparent parameters that are usually not representative of the real parameters of the transport systems. We have thus proposed an alternative approach based on using the linear Flow/Force relationships that exist when a system is close enough to equilibrium. In that approach, the main parameter is the overall conductance of the whole system for the transported solute. When there is a modification in the conductance, it is possible, using an Arrhenius plot, to check whether this modification is quantitative (e.g. corresponding to a modification of the number of carrier molecules per cell) or qualitative (e.g. corresponding to a change in the specific activity of the carriers). The active or passive character of the transport can be determined by use of the classical "flux-ratio equation". However, since this method, which is extremely convenient to study a transepithelium transport, is more cumbersome in its application to cell transport, we have introduced an alternative approach to the characterization of active transport, termed the "symmetry criterion". A few examples of application of these formulations to the study of the cell transport of solutes by small waterweeds are given.*

5 Plant Sensitivity to Stimuli

Plants are sensitive to environmental stimuli such as wind, rain, touching, wounding and infection by fungi, bacteria or viruses. They may react to stimulation by movement (*Mimosa pudica*, some carnivorous plants); however, the most frequent response is a reorientation of their metabolism and/or morphogenesis.

5.1 Immediate and Local Responses in Separated Tissues or Cells

5.1.1 Ageing of Foliar Discs

When a plant fragment is separated and incubated in water, it responds to this wound signal by a prompt increase of respiration and of metabolic activities that include the transport of solutes (Kahl 1974). This has been termed the "ageing" of the tissue.

With Georges Carlier, we have studied the uptake of radioactively labelled methylglucose (MeG) by freshly prepared foliar discs of *Pelargonium zonale* (L) Aiton [16]. In a few hours, the influx, J_j^{ei} (with j = MeG), increased by a factor of 4. Lithium at a concentration of 10 mM totally inhibited this J_j^{ei}-increase, while NaCl had no effect. The effect of Li^+ on the incorporation of radioactively labelled amino acids into the proteins of the foliar disc paralleled its effect on the increase of J_j^{ei}; however, the inhibition by Li^+ of amino acid incorporation was always partial, even at doses causing a total inhibition of the J_j^{ei}-increase. This is another argument (last sentence of the third paragraph of Sect. 4.2.3) supporting the idea that the increase of MeG influx corresponds to an increased biosynthesis of MeG-carriers and that lithium inhibits this biosynthesis of MeG-carriers without inhibiting the bulk of protein biosynthesis. Lithium also caused J_j^{ei} to decrease progressively in foliar discs previously aged in the absence of lithium. This is consistent with the existence of a rapid turn-over (degradation/biosynthesis) of the MeG-carriers (Carlier 1973).

5.1.2 Gas-Shock in Suspension Cultures of *Acer* Cells

Suspension cultures of *Acer* cells separated by filtration from their growth medium and re-suspended in a fresh nutrient solution (termed the "recovery medium") undergo a "gas shock" (Dorée et al. 1972). This shock causes an almost immediate collapse of various enzyme and transport activities, and then these activities are spontaneously restored within several hours (recovery from the shock).

The peroxidase activities increased progressively after the shock up to a factor of 5 after 1 day [62]. The characteristics (molecular weight, pH dependence, number and pI values of the different isoenzymes) of the peroxidases released in the recovery medium after the shock were different from those of the peroxidases released in the growth medium prior to the shock. Adding 1 mM Li^+ in the recovery medium immediately after the shock inhibited peroxidase release.

Concerning transport activities [18, 23, 27], the influx of sulphate, J_j (with here j = sulphate), fell to almost zero at the moment of the shock and then progressively came back to its initial value; LiCl had a strong inhibitory effect on the resumption of normal J_j-values, while NaCl had no effect. Using the Flow/Force formulation (Sect. 4.2.2), these changes in the J_j-values were found to correspond mainly to changes in the conductance λ_j. For instance, after full recovery in the absence of lithium, the λ_j-value was approximately ten times as large as that shortly after the shock; furthermore, in two comparable experiments carried out 12½ h after the

shock, the λ_j-values in the presence of 1 mM NaCl or LiCl were (arbitrary units) 13.0 and 3.4 (first experiment) and 8.8 and 1.4 (second experiment). Using Arrhenius plots (Sect. 4.2.2.2), the regression lines $\{1/T, \log \lambda_j\}$ obtained at the beginning of recovery and after full recovery were quasi parallel, which means that the change of λ_j was of an essentially quantitative nature. The transports of leucine, methionine, glucose, adenine and phosphate were as sensitive as that of sulphate to gas-shock, while that of K^+ was indifferent to the shock. The time-course of the recovery from gas-shock and the way in which the recovery was altered by the presence of lithium were approximately the same with the different sensitive substrates. A drop in temperature (e.g. down to 1°C), as well as a variety of inhibitors of protein biosynthesis, prevented the restoration of a normal activity of transport of the sensitive substrates after the shock. From all this data, it appears likely that Gas-shock is responsible for the degradation or the release of proteins involved in the transport of the sensitive substrates and that a lithium-inhibited neosynthesis of these transport proteins takes place during the recovery from the shock.

When 1 mM LiCl or various inhibitors of protein biosynthesis were added to the nutrient medium of *Acer* cells that had recovered from gas-shock, in a few hours these cells lost their ability to transport sensitive substrates such as sulphate, phosphate, leucine and glucose. This demonstrates turn-over of transport molecules as it implies that some peptidic component of the carrier devices of the sensitive substrates are subject to a spontaneous and relatively rapid degradation and that lithium inhibits the peptidic biosynthesis that normally compensates for this degradation.

5.2 Migration, Storage and Recall of Information in Entire Plants

5.2.1 Inhibition of Hypocotyl Elongation in *Bidens* Seedlings

Under natural conditions, the hypocotyl of *Bidens* seedlings elongates rapidly during the first 5–10 days following germination. In our experiments [51], the seedlings, which were initially grown in a classical nutrient solution, were either left in this nutrient solution or transferred on the fifth day into deionised water. As long as the seedlings were not subjected to a significant stimulus, the hypocotyl elongation was similar in the two media. However, if, on the sixth day, a few pricks were inflicted to the seedling cotyledons with a blunt needle, the hypocotyl elongation of the seedlings grown in water was inhibited whereas that of the seedlings grown in the nutrient solution was practically unchanged. An information of "hypocotyl-elongation inhibition" thus migrated from the pricked cotyledons to the hypocotyl.

When *Bidens* seedlings grown in the nutrient solution were subjected to an 8-prick stimulus on the fifth day, as could be expected no significant inhibition of hypocotyl elongation occurred. However, if the seedlings were transferred into deionised water on the sixth or seventh day, then hypocotyl elongation was

immediately inhibited. This means [101] that the information of hypocotyl-elongation inhibition, induced by the pricking stimulus, was stored (i.e. memorised) in the seedlings, but that this stored information could not be used and take effect as long as the seedlings were not enabled to recall it by transferring them into deionised water. Such behaviour implies that STO (for storage) and RCL (for recall) functions exist within the seedlings and that the inhibition of hypocotyl elongation can occur only when the STO and RCL functions have both been activated. In our present experiments, the memorisation time was 1 day (from fifth to sixth day) or 2 days (from fifth to seventh day). Since the same result (inhibition of hypocotyl elongation) was obtained whether the seedlings were subjected to the pricking stimulus before or after being transferred into deionised water, it may be concluded that the STO and RCL functions operate independently from each other.

With 8-day-old tomato seedlings, the perception of an abiotic stimulus also caused the storage of information of hypocotyl-elongation inhibition and transferring the seedlings into an extremely diluted nutrient medium also enabled them to recall the stored information and allow it to take effect [101].

When *Bidens* seedlings grown in deionised water were subjected to a pricking stimulus (STO and RCL functions both activated), the reduction in hypocotyl elongation was accompanied by an increase of the release of ethylene, an increase in the tissue content of the ethylene precursor ACC (1-aminocyclopropane-1-carboxylic acid) and of its malonyl derivative MAAC and an increase of the hypocotyl content of total peroxidases [51]. When 20–50 μM LiCl was added to the deionised water, this prevented the inhibition of hypocotyl elongation and the increase of hypocotyl peroxydases, whereas the addition of the same concentrations of NaCl had no effect [51]. Since it is the RCL function that is sensitive to the composition of the bathing medium of the seedlings, this means that a Li-sensitive neo-synthesis of peroxidases, and perhaps also of other enzymes and of transport proteins (Sect. 5.1), was involved in the processes enabling these seedlings to recall stored information of hypocotyl-elongation inhibition.

5.2.2 Breaking the Symmetry of Bud Growth in *Bidens* Seedlings

5.2.2.1 Asymmetry-Index, g, of a Set of *Bidens* Seedlings

The experiments were carried out with 2- to 3-week-old seedlings. At this age, the *Bidens* seedlings are bilaterally symmetrical. They consist of an axis [root, hypocotyl, actively growing terminal bud (also termed “apex”)] plus two opposite cotyledons and the buds at the axil of these cotyledons (also termed the “cotyledonary buds”). Normally, the growing apex inhibits the growth of the cotyledonary buds (“apical dominance”); but the cotyledonary buds start growing rapidly if the apex is removed (“seedling decapitation”). Under optimal conditions of light and mineral nutrition, the growth rate of the two buds is approximately the same and the seedlings remain symmetrical; under low light and starvation conditions, usually

one of the buds starts to grow before the other and the seedlings cease to be symmetrical.

Consider a set of n seedlings in which one cotyledon of each seedling is defined as being cotyledon A and the other cotyledon B and term a and b the corresponding cotyledonary buds. If n_a and n_b are the numbers of seedlings in which bud a or bud b is the first to start to grow, an asymmetry-index, g, can be defined by

$$g = (n_b - n_a)/n \tag{35}$$

According to whether $g \approx 0$, $0 << g \leq 1$ or $-1 \leq g << 0$, the set of seedlings is said to be symmetrical, asymmetrical in favour of bud b, or asymmetrical in favour of bud a [66].

5.2.2.2 STO and RCL Functions in the Control of Bud Growth

The experiments consisted in either pricking only one cotyledon or pricking equally the two cotyledons (asymmetrical or symmetrical stimulus) with a blunt needle. Cotyledon A was defined as being that subjected to the first asymmetrical stimulus. When x pricks were administered to cotyledon A or when y pricks were administered simultaneously to the two cotyledons, this was termed xa or yA-yB, respectively. When the seedlings were subjected to several successive stimuli, the time lapse between the stimuli was indicated in brackets. For example, xA(nh)yA-yB means that an asymmetrical stimulus xA was followed n hours later by a symmetrical stimulus yA-yB.

When non-decapitated seedlings were subjected to an asymmetrical stimulus, nothing externally visible occurred and the g-values were close to zero. When non-decapitated seedlings were subjected to an asymmetrical stimulus followed, a few days later, by seedling decapitation, these seedlings sometimes remained symmetrical ($g \approx 0$) and sometimes became asymmetrical ($g > 0$). This apparent inconsistency was cleared up by assuming that, again (second paragraph in Sect. 5.2.1), two functions, STO and RCL, were involved and that it is only when they were both activated that non-symmetrical sets of seedlings were observed. This is illustrated in Table 2. After an asymmetrical stimulus 4A, symmetry-breaking information relative to bud growth was stored in all cases (STO function activated), but the state of the RCL function depended on how seedling decapitation was carried out [101]. When the seedlings were decapitated using tweezers in the morning or using a razor blade at midday, they were unable to recall the stored information and the g-values remained close to zero. When decapitation took place using a razor blade in the morning or tweezers at midday, the seedlings became able to recall the stored information and the g-values were appreciably above zero. That the experimental condition "razor/midday" failed to activate the RCL function but did not deactivate the STO function is attested by the fact that the addition of a (non-asymmetrical) thermal treatment restored positive g-values.

Table 2 Asymmetry-index, *g*, under different experimental conditions

Seedling decapitation		Treatment after decapitation	*g*-Values	Interpretation	
				STO	RCL
Tweezers	Morning	–	0.09	Activated	Inactivated
	Midday	–	0.42	Activated	Activated
Razor blade	Morning	–	0.35	Activated	Activated
	Midday	–	0.08	Activated	Inactivated
	Midday	Thermal treatment	0.50	Activated	Activated

The seedlings were all subjected to an asymmetrical stimulus 4A. They were decapitated 2 days later, either by tearing off the apex with tweezers (a treatment that inflicts a big trauma to the seedlings) or by removing it neatly using a razor blade (minimal wounding) and either at the beginning or at the middle of the day ("Morning" and "Midday", respectively). In one case, the seedlings were also subjected to a thermal treatment (rapid temperature decrease down to 7°C, 24 h at 7°C and slow warming up to room temperature) begun immediately after seedling decapitation. The last two columns indicate the state in which the STO and RCL functions have to be for a coherent interpretation of the measured *g*-values

In the experiments in Table 2, the memorisation time (i.e. the time lapse between the pricking treatment and seedling decapitation) was equal to 2 days; but memorisation times up to 14 days occurred without any loss of information [39, 101]. Pricking one cotyledon of flax or tomato seedlings broke the symmetry of bud growth, but with smaller g-values than with *Bidens* seedlings [39]. Not only pricking but also rubbing a cotyledon, or deposing a droplet of an appropriate solution on a cotyledon stored symmetry-breaking information [39, 68, 101]. A wave of electric depolarization was associated with the signal migration from the pricked area to the buds [45]. The storage of symmetry-breaking information was an all-or-nothing and irreversible process [66]. By contrast, changing the decapitation conditions, adding a thermal treatment and/or asymmetrical or symmetrical pricking stimuli, modifying the time interval between two successive treatments or changing the ionic conditions made the seedlings reversibly able/unable to recall stored symmetry-breaking information [31, 34, 39, 53, 66, 68, 101]. Even apparently paradoxical data (Table 3) was easily interpreted by taking the state of the STO and RCL functions into consideration. Although it occurred that a given asymmetrical stimulus (e.g. a treatment 4A, see experiment 2 in Table 3) acted simultaneously on the STO and RCL functions, RCL appeared to be sensitive to a number of factors that had no effect on STO. This means probably that, again (second paragraph in Sect. 5.2.1), these two functions operate independently from each other.

When an asymmetrical stimulus, 2A, stored symmetry-breaking information and was followed by one or several symmetrical stimuli, 2A–2B, with appropriate values of the time lapses between the successive stimuli, the stored information was recalled, then non-recalled, then recalled again (*g*-values above zero, close to zero and again above zero in experiments 8–10 in Table 3). This means that the seedlings were able to recall stored symmetry-breaking information at least twice.

Table 3 Asymmetry-index, g, after various combinations of pricking treatments

Experimental series	Pricking treatment	g-Value	Interpretation	
			STO	RCL
1	1A	0.01	Activated	Inactivated
2	4A	0.56	Activated	Activated
3	10A	0.01	Activated	Inactivated
4	10A(24 h)1A	0.47	Activated	Activated
5	1A(1 h)4B	0.39	Activated	Activated
6	1A(3 h)4B	0.07	Activated	Inactivated
7	2A	0.06	Activated	Inactivated
8	2A(1 h)2A–2B	0.32	Activated	Activated
9	2A(1 h)2A–2B(3 h)2A–2B	0.05	Activated	Inactivated
10	2A(1 h)2A–2B(3 h)2A–2B(5 h)2A–2B	0.34	Activated	Activated

The table is a selection of published and still unpublished data originating from a considerable number of experiments (more than one million *Bidens* seedlings tested in total). An asymmetrical treatment of 1–10 pricks inflicted to cotyledon A stored symmetry-breaking information relative to bud growth (activation of the STO function) in all cases. Treatment 4A (experiment no. 2) activated not only the STO function but also the RCL function (causing the g-value to be >0), while weaker (experiments no. 1 and 7) or stronger (experiment no. 3) stimuli left RCL inactivated (with the consequence that $g \approx 0$); the addition of a stimulus 1A one day after 10A (experiment no. 4) activated the RCL function (g thus becoming >0). After a stimulus 1A, the addition of a stronger, opposing stimulus 4B (experiment no. 5 and 6) had no effect on the state of the STO function but either activated the RCL function (g-value > 0) or left RCL inactivated (g-value ≈ 0), according to whether this stimulus 4B was applied 1 or 3 h after the stimulus 1A. After a stimulus 2A, the repeated application of a symmetrical stimulus 2A–2B after appropriate intervals of time (experiments no. 8–10) activated the RCL function ($g > 0$) then inactivated it ($g \approx 0$) then activated it again ($g > 0$)

5.2.2.3 Mechanisms Involved in the Control of Bud Growth

The DNA content of the meristematic cells of the cotyledonary buds was studied in non-decapitated *Bidens* seedlings; after a stimulus 4A, almost all the cells that were in the G2 state of the cell cycle in bud a underwent division, while the number of cell divisions was much smaller in bud b [95]; molecules such as cyclins, which are involved in the control of the transition G2/mitosis (Francis 2009), may thus have a role in the storage of symmetry-breaking information. Lithium (at concentrations ranging from μM to mM values) altered the memorisation process [11, 64] and the effect (like other ionic effects) was mainly on the RCL function [53]. By analogy with the results obtained with separated tissues or cells (Sect. 5.1), this might mean that a lithium-sensitive biosynthesis of enzymes and/or of transport proteins is involved in the RCL functioning.

Jacques Demongeot, Yannick Kergosien, Donald Mikulecky and René Thomas have helped me modelling the system [22, 85, 110, 117]. This has permitted us (a) to obtain evidence for positive and negative reaction circuits and to determine the stationary states of the system [110], (b) to interpret the STO function as corresponding, in the phase space of the system, to the selection of one of the two limit cycles corresponding to the selection of the cotyledonary bud that will be the

first of the two to start to grow [117], (c) to interpret the RCL function as corresponding to the generation of a pathway towards the selected limit cycle [117] and (d) to make predictions that were subsequently verified by experimental analysis [85].

5.2.3 Induction of Meristems in the Hypocotyl of Flax Seedlings

We have induced the formation of epidermal meristems in the hypocotyl of flax seedlings, without any hormonal or wounding treatment, by subjecting these seedlings to an appropriate abiotic stimulus and to a transient depletion of calcium [93]. Meristems began to appear after 1 or 2 days, then their number increased and finally reached a plateau value after about 3 weeks. The numbers of meristems in seedlings subjected to only the abiotic stimulus or to only the transient depletion of calcium were always close to zero. If instead of the abiotic stimulus and the transient calcium-depletion being simultaneous, there was a lapse of time, δt, between stimulus and calcium-depletion, the start of meristem production was also delayed [93]. This means that meristem-production information was stored within the seedlings, without taking effect, during the time lapse δt. We did not observe any appreciable erosion of the stored information for memorisation-times, δt, up to 8 days [93, 124]. Again (Sects. 5.2.1 and 5.2.2.2), we have termed "STO" the seedling ability to store meristem-production information and "RCL" the seedling ability to recall the stored information and let it take effect by the actual production of meristems. Since the same response (meristem production) was obtained whether the transient depletion of calcium was applied after or before the abiotic stimulus [93, 124], it may be concluded again (Sects. 5.2.1 and 5.2.2.2) that STO and RCL function independently from each other.

Diverse abiotic stimuli (manipulation stimulus, wind, cold shock, slow cold treatment, drought stress and exposition to electromagnetic radiations in the approximate range 1–100 GHz) activated the STO function (i.e. stored meristem-production information) [93, 105, 111, 119]. Vian et al. (2006) have confirmed the plant sensitivity to electromagnetic radiations. A high concentration of external calcium was needed for the perception of the abiotic stimuli and the storage of meristem-production information [93, 124], which is consistent with data published by other authors (Takahashi et al. 1997; Plieth et al. 1999).

We have not found any treatment other than a transient depletion of calcium that could activate the RCL function (i.e. enable the seedlings to recall stored meristem-production information). Calcium-depletion periods ranging from 0.5 to 4 days were equally efficient to activate the RCL function, on the condition that the external calcium concentration was low enough (in the approximate range of 0–0.25 mM) during the period of depletion. When stimulated seedlings were subjected to a 2-day calcium depletion followed, 8 days later, by another 2-day calcium depletion, as could be expected the beginning of the curve was identical to that after a single 2-day calcium depletion; however, after the second transient calcium-depletion, the seedlings started to produce a new round of meristems,

which resulted in a final number of meristems per plant almost double that with a single 2-day calcium depletion. The seedlings were thus able to recall the stored meristem-production information twice [124].

In another series of experiments [124], stimulated seedlings were subjected to a 2-day calcium depletion followed by 4-day calcium excess (three times the normal calcium concentration). As could be expected, after calcium depletion, the seedlings began to produce meristems actively; but meristem production was blocked as a consequence of the application of the calcium-excess treatment. However, when a second 2-day calcium-depletion treatment was applied after the period of calcium excess, an active production of meristems was rapidly restored. Again, the seedlings recalled stored meristem-production information twice.

From the three preceding paragraphs, it appears that the storage of meristem-production information was favoured by a high calcium-concentration in the external medium, whereas rendering the seedlings able to recall the stored information was favoured by calcium-depletion and blocked by calcium excess. Complementary experiments [124] have shown that once the STO and RCL functions have both been activated, meristem-production is favoured by an intermediate external calcium-concentration. The role of calcium in plant sensitivity to stimuli thus is more complex than it is usually assumed.

We have used 2D electrophoresis to study the proteome modifications accompanying the activation of the STO and RCL functions [112]. Concerning the storage of meristem-production information, some proteome modifications were unspecific and others specific to each type of abiotic stimulus. For instance, a protein spot that we have termed "*Toucher-1*" was displaced (post-translational modification) during the first 10 min following inflicting a manipulation stimulus to non-calcium-depleted seedlings. Using the SIMS technique (Sect. 2.1), we were able to show that this displacement was corresponding to a transient phosphorylation of the protein. In other cases, new protein spots appeared or pre-existing spots disappeared (changes in gene expression). A few spots appearing when non-stimulated seedlings were subjected to calcium depletion are likely to be associated with the enabling of the seedlings to recall stored information.

The proteins involved in these changes in the proteome have still not been identified. It is not known whether some of them correspond to the carrier proteins, peroxidases or cyclins that were implicated in the inhibition of hypocotyl elongation or in the breaking of the symmetry of bud growth (Sects. 5.2.1 and 5.2.2.3). By contrast, our finding that the phosphorylation of *Toucher-1* occurs rapidly after inflicting a manipulation stimulus to the seedlings is consistent with the suggestion by previous authors that a transient phosphorylation of proteins (Takahashi et al. 1997) and the activation of a mitogen-activated protein kinase pathway (Jonak et al. 1996) are among the early events occurring after the perception of abiotic stimuli. This is again (see Sect. 5.2.2.3) an indication that molecules involved in the control of the cell cycle may have a role in the storage of information that is induced by abiotic stimuli.

In brief. *Plants are sensitive to a number of traumatic or non-traumatic stimuli, to which they usually respond by metabolic and/or morphogenetic modifications.*

The responses may be local and immediate, or they may occur at a distance from the stimulated area (thus involving a migration of information within the plant) or be stored and take effect only after being recalled at a later time (thus involving information memorization). Local and immediate responses (consisting of an increase of enzyme activities and of the cell transport of sugars and other solutes) have been observed with separated foliar discs and cell suspension cultures. The migration and the storage and recall of morphogenetic information following the perception of various abiotic stimuli has been studied with seedlings of different plant species in three different cases: "inhibition of hypocotyl growth", "breaking the symmetry of bud growth" and "induction of meristem production in the hypocotyl epiderm". In all three cases, the storage of the morphogenetic information (STO function) was a solid and apparently irreversible process, whereas the ability of the seedlings to recall the stored information (RCL function) was easily reversible and strongly dependent on the ionic conditions. The activation of both the STO and RCL functions was accompanied by specific modifications of some protein activities, modifications of the expression of a few genes and posttranslational modifications (including transient protein phosphorylation).

6 Reflection and Speculations

6.1 Methodological and Conceptual Implications

6.1.1 Use of Stable Versus Radioactive Isotopes

With NCR and SIMS (Sect. 2.1), we have contributed to the body of work showing that stable isotopes can be used instead of radioisotopes for labelling, imaging and quantitative analysis [103]. This avoids the administrative and operational constraints inherent in the use of radioactive substances and extends the use of isotopic methods to the study of elements that have no utilisable radioisotopes. The main drawbacks are that neither NCR nor SIMS is suited to in vivo imaging; moreover, NCR detects only one of the stable isotopes of the elements under study and SIMS is costly. That said, the sensitivity, the lateral resolution and the discrimination of NCR and SIMS have proved irreplaceable for many applications to fundamental and applied science (Sect. 2.3 and third paragraph in Sect. 4.2.3).

6.1.2 Molecular and Macroscopic Parameters of Cell Transport

Consider a copper wire in an electric circuit. The wire can be described at a microscopic level (using the atomic and crystalline properties of copper) or at a macroscopic level (by its resistance). The values of the microscopic and macroscopic parameters are determined using totally different types of measurements. Expressing

macroscopic phenomena in terms of microscopic parameters (or inversely) is possible, but it requires being able to model precisely the system.

The situation is similar in the case of transport systems in plant cells (Sect. 4.2). At the microscopic level, molecular biology and genetics are suitable for identifying carriers and assessing their molecular properties. At the macroscopic level, the measurement of the fluxes of solutes and of the forces driving these fluxes is needed to study the mineral nutrition of plants. Again, expressing microscopic phenomena in terms of macroscopic parameters (or inversely) requires being able to model precisely the system. However, doing this with cells (in which there are so many intricate structures) is fraught with difficulty. The conventional formulation of cell transports (Sect. 4.2.1), which infers the values of molecular parameters (carrier affinity and maximal rate of functioning) from flux measurements, thus risks being erroneous. The same conclusion is drawn from the study of artificial transport systems (Sect. 4.1.1). We have shown (Sects. 4.2.2 and 4.2.3) that the parameters appropriate for interpreting flux data are the conductance of the system for each transported solute, the intercept, ln ($^{\circ}c_j^{ei}$) or ln (c_j), of the straight line {ln (c_j^e), J_j} with the axis of abscissas and the cross-coefficients that permit to determine the passive or active character of a transport (Sect. 4.2.2.4).

6.1.3 Increasing Instead of Decreasing a System Complexity

In a reductionist approach, the system under consideration is split into simpler parts, the study of which is more easily accessible to experimental analysis. However, properties such as the permeability coefficients or the relative volumes of sub-cellular compartments can be lost as a result of the reduction of the system. An alternative approach is to increase the complexity of the whole system. In the case of frog skin, certain properties that could not be measured with a reductionist approach could, however, be estimated by constraining the system by lithium addition in such a way that the electric potential oscillated rather than staying constant (Sect. 4.1.2.2).

I suggest that, whenever possible, the reductionist approach to a living system, which reduces its complexity, should be complemented by an approach that increases its complexity. As with frog skin, this could be achieved by imposing extra constraints on the system under consideration and studying those modifications of the system's behaviour that occur only when one or more of the values of its characteristic properties lie in a restricted range.

6.2 Physiological Considerations

6.2.1 Biological Processes in Organised Media

Studies of enzyme kinetics under unusual conditions (Sect. 3.2) and of the fluxes of solutes in enzyme-constituted, artificial transport systems (Sect. 4.1.1) were the

bases for a patent on the extraction of specific compounds from complex solutions [54].

When enzyme systems were functioning within an organised or a partially aqueous/partially organic environment (conditions that are encountered in real cellular systems), (a) the apparent parameters were usually not representative of the real kinetic parameters of the proteins involved, (b) the existence of a sigmoidal kinetic curve did not necessarily mean that an allosteric enzyme was present and (c) the ability to explain a complex kinetic curve as the combination of two or more rectangular hyperbolas was not proof that two or more different proteins were involved. The apparent kinetic parameters, which can be determined by the adjustment of rectangular hyperbolas to experimental kinetic data of transport in vivo, do not therefore necessarily represent real molecular characteristics of the proteins in question. This is another reason (Sect. 6.1.2) to doubt the validity of the conventional, Michaelis–Menten interpretation of cell transports (Sect. 4.2.1).

In both types of the artificial transport systems described in Sect. 4.1.1, the transporting device had a structural symmetry, and it was a functional asymmetry (for instance, a pH gradient) that was responsible for the orientation of the transport from exterior to interior or inversely. Therefore, although all the real transport systems characterised so far correspond to proteins asymmetrically anchored in cell membranes, the past or present existence of structurally symmetrical systems of transport in vivo cannot be totally excluded.

6.2.2 Protein/Protein Interactions and Regulatory Properties

In the case of allosteric enzymes (Monod et al. 1965), the fact that there are subunit/subunit interactions in addition to the usual enzyme/substrate interactions causes the steady-state curves $\{c_s, v\}$ to be sigmoid. This step response enables such an enzyme to play a regulatory role in a pathway of sequential enzymes. When an allosteric enzyme was endowed with a feed-forward property (Sect. 3.2), we have shown [78] that the transient behaviour (before reaching steady-state) pre-adapted the pathway containing this enzyme to respond to variations in the concentration of the initial substrate, thus allowing it to act as an "anticipatory system" (Bali and Thomas 2001).

The transient association of enzymes that we have termed "functioning-dependent structure" or FDS (Sect. 3.3) may be considered as a generalisation of the concept of allostery. In the FDS case, (a) protein/protein interactions are added to the enzyme/substrate interactions and (b) the protein/protein interactions involve the different sequential enzymes that bind or release one another depending on whether the sequence starts or stops accomplishing a task. Bi-enzymatic functioning-dependent structures (FDSs) can generate steady-state kinetics exhibiting properties such as linearity, invariance, or the production of spike-, step- or inverse step-responses [115]. We have still to investigate transient behaviours to study real FDSs (Ovadi 1988) and not just abstract systems and to take into account that free peptides often undergo a degradation that is much more rapid than when they are in

complexes (Buchler et al. 2005). Nevertheless, data already exists consistent with a role for FDSs in the coordination between and within metabolic and signalling pathways (last paragraph in Sect. 3.3).

6.2.3 Lithium-Sensitive Responses to Wounds and Stresses

The way in which *Acer* cells recover from gas shock (Sect. 5.1.2) bears such resemblance to the way in which foliar discs of *Pelargonium* age (Sect. 5.1.1) that it is likely that the underlying mechanisms are the same. Both correspond to the neo-synthesis of enzymes and of proteins involved in the transport of substrates such as sulphate, phosphate, glucose (or glucose analogues), leucine, methionine and adenine. These transport proteins are degraded and resynthesized continuously during their functioning and lithium inhibits their biosynthesis without blocking protein synthesis in its entirety. This role of lithium in plant systems appears to be different from that in frog skin (Sect. 4.1.2.2), in which Li interfered with the functioning of the Na^+/K^+-pump without apparently altering protein synthesis. Unfortunately, the technique of analysis of the electrical noise (Sect. 2.2) has not proved very helpful here [47]. One reason is that this technique is especially suited to studying K^+-channels but K^+ transport is insensitive to gas-shock (third paragraph in Sect. 5.1.2). Moreover, to analyse the electrical noise of an *Acer* cell, one has to take the cell from its nutrient solution and impale it on a microelectrode, which may cause other shocks to interfere with the gas-shock under study.

6.2.4 Memorization of Information in Plants

As far as we are aware, we were the first to show that plants can memorise information [34]. Since then, many authors have reported examples of "plant memory". Generally, this has been by showing that the perception of the first signal modified the way in which a plant responded to subsequent signals (for citation, see e.g. [123]). We have preferred to keep to the definition of "memory" as it is given in dictionaries, e.g. "*Your memory is your ability to retain and recall information [. . .]*" (Sinclair 1988).

In the three systems that we have studied (Sects. 5.2.1–5.2.3), seedlings stored morphogenetic information as a consequence of perceiving an initial, abiotic stimulus; this information then remained latent, without any apparent effect on plant growth and development, until, at a later time, a second signal enabled the seedlings to recall the stored information and let it take effect. We have termed "STO function" and "RCL function" these abilities of the seedlings to store and recall information. We have observed that STO and RCL functions exist in several plant species (*Bidens*, tomato and flax) where they control different morphogenetic responses (inhibition of hypocotyl elongation, asymmetry of bud growth and meristem production). These functions depend on a variety of stimuli both for the initial storage of information and for the recall of the stored information. The reason

why evolution has selected the ability for plants to store and recall information may be that this permits them to generate, at an appropriate moment, an integrated response to the variety of environmental signals that they perceive in the course of time (for a discussion see [123]).

Memory lasted for days, and perhaps much longer, in all three systems (Sect. 5.2) and we have never been able to delete stored information. This means that the information storage that we are studying must involve components that are stable at some level. Candidates include chromatin silencing or the reactivation of silenced chromatin (Reyes et al. 2002) depending on histone modifications (Chinnusamy and Zhu 2009) and leading to changes in gene expression or in protein kinase activity (Trewavas 2003; Goh et al. 2003). We have shown that protein phosphorylation and substances controlling the cell cycle are implicated (Sects. 5.2.2.3 and 5.2.3). Størmer and Wielgolaski (2010) have suggested that a type of plant memory might be stored in magnetite in the core of ferritin.

It has always been easy to render the seedlings reversibly able/unable to recall stored morphogenetic information (Sect. 5.2). These RCL activations/deactivations are correlated with modifications to the proteome of the seedlings, which would be consistent with the involvement of Li-sensitive synthesis of peroxidases and/or carrier proteins. The RCL functioning is sensitive to the ionic status of the system and the ionic composition of the bathing medium of the seedlings controls the RCL activation/deactivation either directly (Sects. 5.2.1 and 5.2.3) or indirectly by modifying the way in which various sorts of signals act on the RCL function [53]. Calcium plays a particularly important role, although other inorganic ions are also implicated [53]. One possibility is that RCL functioning is an ion-based, delocalised process and physicochemical studies of the behaviour of inorganic ions in biological media are therefore required (Sect. 2.2). Changes in calcium levels via channels play a part (Trewavas 1999) whilst changes via ion condensation (Sect. 2.2) could play an integrative role [107]. In the breaking of the symmetry of bud growth (Sect. 5.2.2), when the seedlings were subjected to two successive stimuli with an interval of time, Δt, between them, the distribution of the measured g-values as a function of Δt exhibited a damped oscillation [39]; this may be an indication that the RCL functioning is linked with internal rhythms within the seedlings, e.g. to the oscillations of free calcium that occur in plants (Johnson et al. 1995).

Seedlings can recall what is effectively the same stored morphogenetic information at least twice and perhaps many times (Sects. 5.2.2 and 5.2.3). In one series of experiments (Sect. 5.2.2), a wave of electric depolarisation paralleled the signal migration from the stimulated to the reactive area. Although plants have no specialised cells resembling animal neurones, the functioning of the form of plant memory that we have studied thus bears a striking resemblance to the nervous conduction, information storage and repeated-evocation ability that exist in animal memory.

In brief. *With the physical techniques available, the use of stable isotopes may be substituted for that of radioactive isotopes in biological studies. Concerning cell transport, the molecular characteristics of the carriers can be determined by the*

techniques of molecular biology and genetics but usually not by flux measurements; flux measurements will rather give information about the macroscopic characteristics of the studied transport (e.g. the overall conductance of the whole system for the transported solute). The past or present existence of structurally symmetrical systems of transport cannot be excluded. Despite its efficiency in many domains of biology, the "reductionist method" fails to determine those parameters that are characteristic of constituents destroyed in the reduction process; in that case, it may be useful to increase (instead of decreasing) the system complexity so that an order of magnitude of these parameter-values can be determined via the modifications observed in the system behaviour. The protein/protein interactions that take place in functioning dependent structures are responsible for kinetic properties that do not exist with the free proteins; in particular, the spike and inverse-step responses might confer on these structures a regulatory function in metabolic pathways. In plants, lithium seems to inhibit those biosyntheses of enzymes and transport systems that occur in response to stimuli, without inhibiting the bulk of protein synthesis. Plants have no neurons; however, they can perceive stimuli, transfer the corresponding information from the sensitive to the responding areas and possibly store that information until the perception of another signal enables them to recall the stored information and let it take effect; a same stored information can be recalled several times (at least twice). Oscillations occur in the responses of plant to stimuli and the RCL function is extremely sensitive to the ionic conditions: taken together, these two observations might mean that, under natural conditions, RCL activation is controlled by the combination of rhythms modifying cyclically the tissue concentrations of inorganic ions in plants.

7 Back to the Initial Question About Life

This article began with a big question "*What is life?*" The preceding discussion may have helped persuade us that it is important to take the dynamics of living systems into consideration. This is not, however, sufficient because non-living, purely physical systems can also exhibit dynamic behaviour.

To go further, let us start from a simple example. Imagine a metal rod that is subjected to a thermal force by putting one end in contact with a hot source and the other with a cold source. A gradient of temperature develops along the rod as the rod conveys heat from the hot to the cold source (and thus is dynamic from the point of view of heat transfer). If the metal rod is then entirely wrapped with material impermeable to heat exchanges, the rod ceases to transfer heat and eventually the rod has the same temperature throughout, which corresponds to its internal equilibrium. If the insulating envelope is removed and the contact with the hot and cold sources thereby re-established, the system becomes dynamic again; if the rod is again wrapped in the envelope, it again goes to equilibrium; this can be continued for as long as the sources last.

Things are more complex with living beings. Under the effect of particular external or internal conditions, some living systems (spores, seeds, resurrection plants, etc.) have found a way to enter a state of "latent life", i.e. a well-ordered structural state, close to thermodynamic equilibrium (Minsky et al. 2002; Buitink and Leprince 2008; Rajjou and Debeaujon 2008), in which the dynamic processes of active life (assimilation, nutrition, growth, reproduction, mobility) are suspended; later, if conditions permit it, the system can return to the state of active life. To some extent, the systems with a capacity for latent life behave as the metal rod did (shifting from a dynamic state to equilibrium and inversely). However, isolating a living creature in order to interrupt all fluxes between its external and internal media usually causes it to become rapidly and irremediably dead (Spallanzani 1787; Lavoisier 1801); even the organisms with a capacity for latent life cannot reach the quasi-equilibrium state unless they follow an appropriate pathway to it; for instance, an unripe seed will reach the state of latent life only if it can go through the lengthy process of maturation, but it will die, instead of becoming a mature seed, if it is abruptly isolated from all possibilities of exchange with its mother plant. In sum, apart from those organisms that have reached a state of latent life, it is impossible for a living being to go to thermodynamic equilibrium and to remain able to return to a dynamic behaviour, as non-living, physical systems do. Why?

The concept of FDS might help us to get on the right track. We have seen (Sect. 3.3) that an FDS is a multimolecular structure that is created and maintained when it has to function but that disassembles when its task is over. It is thus a dynamic structure, in stark contrast with the permanent (or, at least, long lasting) cell structures such as walls, membranes, various organelles, etc. Moreover, FDSs are far from constituting an isolated case of dynamically maintained structure in biology. For instance, we have mentioned the transport systems that are inactivated by their own functioning and regenerated by novel protein biosyntheses (Sects. 5.1.1 and 5.1.2) and we have proposed the existence of delocalised integrative receptors that undergo constant re-arrangements following the perception of external or internal signals (fourth paragraph in Sect. 6.2.4). Other examples of dynamically maintained structures include (a) the microfilaments and microtubules of the cytoskeleton that are continuously losing and gaining molecules at their extremities (Alberts et al. 1983), (b) the Golgi apparatus that grows when it is secreting and shrinks when it is not (Kepes 2002), (c) the numerous cell proteins that are subjected to a permanent turnover in which synthesis compensates for degradation (Buchler et al. 2005; Belle et al. 2006).

In brief, in an actively living being, not only the processes (e.g. enzyme-catalysed reactions, transmembrane transports) but also many of the molecular and cellular structures are also dynamic, whereas spontaneous dynamic structures are not usually encountered in inanimate, physical systems. In this concept, death would correspond to the collapse of the dynamic structures and their ongoing functions, whereas the activities performed by permanent structures (e.g. reactions catalysed by stable enzymes) could continue for a while. When Xavier Bichat (1800) stated that "life is the whole set of the functions that resist death"

[*La vie est l'ensemble des fonctions qui résistent à la mort*], he had perhaps a premonition of the dynamic structures mentioned above. The organisms with a capacity for latent life would be able to lose progressively the dynamic components of their structure without dying and to re-establish them upon favourable conditions.

Although we are still not able to give a necessary and sufficient condition defining unambiguously what is life, at least we can state that life, as we know it on earth, implies the existence of non-equilibrium structural components in addition to the classical quasi-permanent components.

It is possible to give a quantitative expression to this assertion using the concept of "production of entropy" [73]. Entropy is a state function that has to temperature a relationship formally equivalent to that which volume has to pressure. According to the second law of thermodynamics, the functioning of any system entails a positive production of entropy, σ. If T is the absolute temperature, $T\sigma$ can be expressed as a sum of terms, each of which corresponds to each different process involved, j, and is written as the product of the flux, J_j, of this process by its driving force, X_j. For any type of system, $T\sigma$ is thus written

$$T\sigma = \Sigma(J_j \cdot X_j) = J_V \cdot X_P + J_{El} \cdot X_{El} + \Sigma(J_k \cdot X_k) + \Sigma(J_\rho \cdot X_\rho) + \cdots \qquad (36)$$

in which V and P hold for volume and pressure, El for the transport of electric charges, k for the transport of one or several chemical species, ρ for the progress of one or several chemical reactions, etc. Now, consider a pair of sequential enzymes, E_1 and E_2, catalysing the overall transformation of a solute S_1 into S_3 according to (4). Under steady-state conditions, the only molecules that undergo transformation are S_1 and S_3; therefore a term of the form $(J_\rho \cdot X_\rho)$ describes the contribution of this overall reaction to $T\sigma$ independently of whether the system is catalysed via free enzymes or an FDS. Away from steady-state conditions, however, the situation is different because E_1 and E_2 can act immediately on their substrates when they are free, whereas they have to assemble into an FDS before this FDS can act. Consequently, in a system that contains FDSs, terms of the form $(J_{FDS} \cdot X_{FDS})$, which correspond to the FDS assembly/disassembly, must be added to the expression of $T\sigma$ (36). A similar reasoning can be applied to the other occurrences of dynamic biological structures, DBS, with the result that a series of terms, $\Sigma(J_{DBS} \cdot X_{DBS})$, again have to be added to the expression of $T\sigma$.

We thus come to the conclusion that the same physico-chemical approach, that is the same way of expressing the production of entropy as a bilinear form of "Flux·Force" terms, is valid for all systems, but that living systems are nonetheless distinguishable from inanimate systems because the expression of $T\sigma$ for living systems contains complementary terms representing the entropic cost of maintaining those dynamic structures that characterise living systems.

Isn't that satisfactory!

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Part II
Genetics

To Divide and to Rule; Regulating Cell Division in Roots During Post-embryonic Growth

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Abstract Post-embryonic cell proliferation allows for the development of an extensive root system. Recent genetic analysis in *Arabidopsis thaliana* has revealed several mechanisms involved in cell proliferation control during root development, including hormone signaling and regulatory loops. Furthermore, cell division responds to changes in redox status induced by environmental stresses, and we explore putative connections to the pathways that regulate cell proliferation.

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1 Introduction

The regulation of cell division in roots is tightly linked to the development of the root system. Over the past years, intensive genetic analysis in *Arabidopsis* and other species has revealed key developmental regulatory pathways which mediate cell proliferation during development and growth. Here we review our knowledge of the pathways involved in the regulation of the cell cycle in the different tissues of the root apical meristem and during lateral root initiation and formation. We explore the connections to the mechanisms involved in cell cycle progression while focusing on observations in *Arabidopsis*.

2 The Cell Cycle in Roots and the Core Components

For the evolution of land plants, the development of vascularized roots was crucial and allowed for the progression toward larger plants such as ferns, horsetails, and seed plants. Post germination, root growth enables plants to explore their substrate and hence to extract nutrients and water from larger areas and from greater depths whilst also providing anchorage to the substrate. Furthermore, roots provide a niche for symbiotic organisms, and in several species roots have evolved so they can store nutrients, while in others they are a major vehicle of asexual propagation.

In seed plants, the root consists of concentric cylinders of different tissues in the radial dimension, a pattern which is set up during embryogenesis by formative cell divisions. The outside tissue layer or epidermis encloses the other root tissues and contains the trichoblast lineage, a cell lineage with a specific cell identity, which gives rises to root hairs by tip growth. These root hairs enhance the surface area for uptake and aid in anchoring the root. The cortical cell layers provide protection and mechanical support, whereas the endodermis, with its secondary thickening of the cell wall, forms a selective barrier for ions. Enclosed by the endodermis, the cells in the pericycle maintain meristematic properties that can give rise to lateral root primordia, and during secondary root growth the progeny of this cell layer can contribute to vascular tissues or diverge into cork cambium in some species. The vascular tissue originates from the procambium and can differentiate into the two types of conductive tissue in plants, phloem and xylem, and these tissues can be organized in different patterns in different taxa (Elo et al. 2009). Once again, these patterns are set up during embryogenesis in a series of asymmetric and formative divisions, resulting in well-defined cells layers with distinct cellular identities.

The forward growth of the root is achieved by a combination of cell wall biosynthesis and cell divisions perpendicular to the growth axis in a region at the tip of the root. In the root tip of seed plants (Fig. 1), the root cell files, apart from the epidermis and the lateral root cap tissues, originate around a region that in *Arabidopsis* consists of three to four cells which proliferate slowly. This quiescent center (QC) plays a major role in organizing the meristem and is required to maintain the stem cell identity

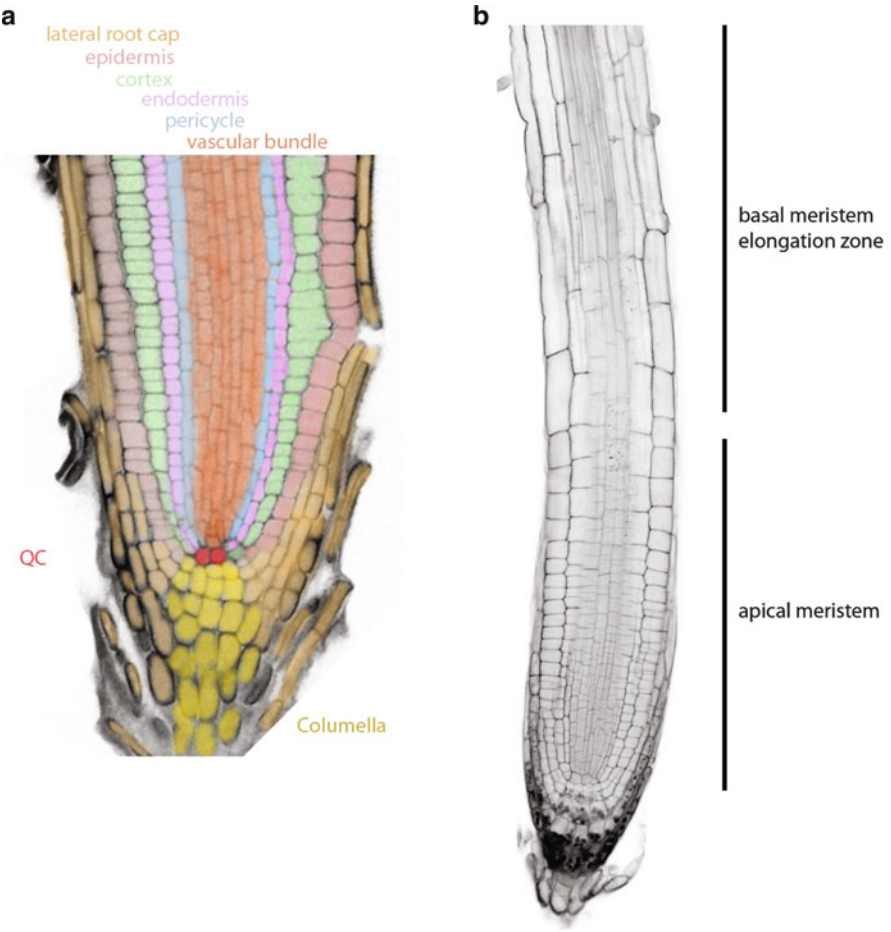

Fig. 1 Organization of the *Arabidopsis* root apex. (**a**) The different tissues of the root apical meristem. (**b**) Micrograph of a cleared root tip, revealing the apical meristem and the basal meristem with the onset of cellular elongation

of the neighbouring cells, the initials, which abut on the QC. Remarkably, this organization seems to have a high degree of autoregulation, as removal of the QC, either surgically (Feldman and Torrey 1977) or by laser ablation (Van den Berg et al. 1997), results in the de novo formation of new quiescent center with flanking initials. The stem cells flanking the cortex–endodermis stem cell on the outside give rise to the initials of the epidermis and the lateral root cap (Pernas et al. 2010). Cells above the QC form the proximal meristem, and cells below are referred to as the distal meristem. The progeny of the distal meristem will form the central root cap cells. Within the different tissues of the meristematic region, cells grow and divide at a relatively small yet constant size resulting in forward movement of the tip. Cells that reach a threshold distance from the actual tip gradually start to elongate, and it is still

unclear whether they divide at a large cell size, or whether division activity arrests before elongation rate increases (Fig. 1). Higher up the root, cell division arrests and cells rapidly elongate. In many tissues, cell elongation is associated with endoreduplication, a shunted cell cycle that allows for DNA replication without intervening mitosis, resulting in a logarithmic accumulation of genome copies in each nucleus. Unfortunately, at this point in time, we do not have a full map of nuclear ploidy in the different tissues and cell types at our disposal, and it would be interesting to establish the relationship between cell elongation and DNA ploidy in the different tissues. What we do know is that both pericycle and cambium cells away from the root tip maintain the potential to reinitiate division.

Pericycle cells at the xylem poles can be primed to become lateral root progenitors or founder cells in the transition zone of the meristem, and currently two mechanisms have been put forward. A local alternating pulse of auxin, or localized auxin response, has been proposed to be the major component of pericycle cell priming (De Smet et al. 2007; Dubrovsky et al. 2008). However, recently another mechanism based on endogenous and autonomous oscillations of gene expression was put forward (Moreno-Risueno et al. 2010). Primed cells have the potential to resume cell division and to create a de novo lateral root primordium with the same cellular and tissue organization as the primary root apex by a well-characterized sequence of transverse, anticlinal, and periclinal cell divisions (Casimiro et al. 2003). Intriguingly, while pericycle cells at the phloem pole remain in G_1, pericycle cells at the xylem poles have the capacity to progress to G_2 (Beeckman et al. 2001).

Periclinal divisions of the cambium cells give rise to additional cells, which differentiate into phloem or xylem and contribute to thickening of the root.

Hence, conceptually, cell division in the growing root is associated with three major processes: cell division in the apical root meristem provides cells during longitudinal growth, cell cycle reactivation activation of the pericycle results in the formation of lateral primordia for the ramification of the root system, and activation of the cell cycle in the cambium and the pericycle provides cells for secondary radial development.

A challenge lies in understanding how key growth and developmental pathways which mediate cell proliferation in the different root tissues interface and coordinate with the core cell cycle mechanisms. Cell cycle progression in eukaryotes is driven by the ordered consecutive action of different cyclin-dependent kinase (CDK) complexes (Inze and De Veylder 2006). Genome sequencing has revealed a significant number of core cell cycle regulators in higher plants (Renaudin et al. 1994; Vandepoele et al. 2002). The different cyclins and cyclin-dependent kinases are regulated post-translationally. This involves post-translational regulation by interactions with scaffolding proteins, inhibitors, and activating or inhibiting phosphorylation by regulatory kinases (Inze and De Veylder 2006). Hence, there is an extensive tool set for regulation of the cell cycle, and we are still exploring the composition (Van Leene et al. 2010) and function of the different complexes.

Apart from the CDKAs which are conserved in animals (CDKs) and yeast (CDK1/cdc2+/cdc28), plants have a unique family of CDKB cyclin-dependent kinases composed of two subfamilies. The CDKB1 and CDKB2 kinase activity is maximum during G2/M and mitosis, respectively (Inze and De Veylder 2006).

In *Arabidopsis*, the single CDKA is essential (Nowack et al. 2006), but in other species with more then one CDKA gene (e.g., rice) there might be a level of functional redundancy and specificity.

D-type cyclins were postulated to be important for the G_1/S transition by controlling the activity of retinoblastoma-related proteins (RBRs) by phosphorylation. In higher plants, the *CYCD* gene family has several members, which can be classified into seven conserved families (CYCD1–CYCD7) (Menges et al. 2007). Indeed, in plants a subset of D-type cyclin genes respond transcriptionally to mitogenic signaling (Riou-Khamlichi et al. 1999; Oakenfull et al. 2002) and CYCD/CDKA kinases are able to hyperphosphorylate the RBR protein (Nakagami et al. 2002). This in turn renders RBR unable to suppress transcription factors such as E2F/DP which promote S-phase progression. Furthermore, loss of function of the three members of the *CYCD3* gene family and *CYCD3;1* ectopic overexpression indicated that *CYCD3* genes are rate limiting for cell proliferation in aerial organs (Dewitte et al. 2003, 2007). Nevertheless, given the multiple *CYCD* genes (*Arabidopsis* has ten *CYCD* genes), it is feasible that some CYCDs interact with different CDKs, and other or additional functions can be anticipated for the different CYCD/CDK kinases.

A-type cyclins (CYCAs) are subdivided into three types (CYCA1–CYCA3) and the different members seem to have acquired quite different functions. The expression of a few members of the CYCA3 family peaks at the G_1/S transition, and those CYCAs are able to form active kinase complexes with CDKA which have activity toward RBR, making it not unlikely that they act in concert with CYCD complexes during the G_1/S transition (Menges et al. 2005; Takahashi et al. 2010). CYCA2;3 was found in a complex with CDKB1;1, and its prolonged expression inhibits the onset of endoreduplication, indicating that the CYCA2;3/CDKB plays an active role at the G_2/M boundary (Boudolf et al. 2009). Another member of the CYCA group, CYCA1;2, plays a crucial role in the two cell-cycle transitions of meiosis (Erfurth et al. 2010).

There are 11 CYCBs in *Arabidopsis* and they are divided into three different subgroups (CYCB1–CYCB3) (Menges et al. 2005). Transcript analysis in synchronized *Arabidopsis* cell cultures shows that transcripts of most B-type cyclins show a common regulation, with a distinct peak in G_2 and early mitosis. The main exception is *CYCB1;1*, which is activated from early S phase in synchronized cells and does not significantly increase further during G_2 phase (Menges et al. 2005). In *Arabidopsis* plants, the accumulation of the *CYCB1;1* transcript is correlated with meristematic tissues (Ferreira et al. 1994). Detailed analysis of the *CYCB1;1* promoter identified cis-acting elements and regulatory proteins involved in the regulation of *AtCycB1;1* gene transcription (Ito 2000; Planchais et al. 2002). B-type cyclins proteins are also a subject of cell cycle-dependent proteolysis, and their protein levels follow the transcriptional regulation. Interestingly, CYCB1;1 accumulates in several backgrounds that are defective in genome structure and chromatin maintenance (Wu et al. 2010) or in response to DNA damage (De Schutter et al. 2007). In all these backgrounds, the accumulation of CYCB1;1 is associated with root swelling, probably due to disorganization of cortical microtubules (Serralbo et al. 2006). Recently, Wu et al. (2010) hypothesize that plant separase is the link between microtubules

disorganization and the superabundance of CYCB1;1. Separase is a protease activated at the onset of anaphase cleaving the cohesin complex, which holds sister chromatids together (Oliveira and Nasmyth 2010), and its action seems to be involved in CYCB1;1 destruction, either directly or indirectly.

Modulation of CDK activity is also achieved by interaction with proteins of the interactor of CDK (ICK)/Kip-related protein (KRP) and SIAMESE (SIM) families (Dewitte and Murray 2003; Churchman et al. 2006; Inze and De Veylder 2006). The ICK/KRP family contains seven members (ICK/KRP1–7) (Vandepoele et al. 2002), whereas the SIM subfamily contains five members (Peres et al. 2007) in *Arabidopsis*. Global transcriptomic analysis in synchronized *Arabidopsis* cell cultures reveals that ICK/KRPs show different patterns of regulation during cell cycle reentry and cell cycle progression, with sequential peaking in cell cycle phases (Menges et al. 2005). Interestingly, *ICK/KRP2* is expressed at high levels in sucrose-starved cells, declines on cell cycle resumption, and does not show any later regulation during subsequent cell cycle progression, suggesting a specific role in cell cycle reentry (Menges and Murray 2002; Menges et al. 2005). Gene expression analysis of *ICK/KRPs* genes in *Arabidopsis* plants reveals that *ICK/KRPs* are generally expressed at low levels but showed preferential expression in specific tissue/developmental conditions (Torres Acosta et al. 2011). Furthermore, several *ICK/KRPs* and *SIM* members are transcriptionally regulated by hormones (Wang et al. 1998; Himanen et al. 2002) or in response to biotic and abiotic stresses (Peres et al. 2007). In addition to their transcriptional regulation, ICK/KRP proteins are post-translationally regulated by proteolysis (Verkest et al. 2005; Jakoby et al. 2006).

Overexpressing an *ICK/KRP* gene reduced plant size, suppressed lateral root formation, and conferred smaller serrated leaves with fewer yet larger cells (Wang et al. 2000; De Veylder et al. 2001; Jasinski et al. 2002, 2003; Zhou et al. 2002; Barroco et al. 2006; Bemis and Torii, 2007; Kang et al. 2007). In line with an interaction with the D-type cyclins, the effects of elevated KRP levels could be attenuated by co-expressing a D-type cyclin (Jasinski et al. 2002; Schnittger et al. 2002b; Zhou et al. 2003). Ploidy analysis reveals that high levels of ICK/KRP proteins inhibit the G_1-to-S phase transition of both mitotic and endocycles, whereas a moderate increase affects mainly mitotic cell cycles (Verkest et al. 2005). Furthermore, KRP proteins can act as scaffolds to promote the interaction between CYCDs and CDKs, as recently shown for KRP2 which catalyses an interaction between CYCD2 and CDKA (Sanz et al. 2011).

The orderly destruction of the kinase partners and regulators allows for the coordination of the cell cycle. The level of the several cell cycle factors is controlled by the action of ubiquitin ligases, as ubiquitinylation targets the factors for destruction by the proteasome. During the cell cycle, the anaphase promoting complex (APC), which plays an important role in exiting mitosis, and the SKP1/Cul1/F-Box protein complex (SCF complex) are the key E3 ubiquitin ligase complexes controlling the stability of cyclins and other regulatory factors. CDK-mediated phosphorylation can stimulate recognition by the F-box proteins of specific substrates, and target them for destruction, hence providing a mechanism

which gives direction to the cell cycle. Furthermore, the action of these cell cycle factors can link several cell cycle phases together.

G_1/S regulators, such as D-type cyclins, as they prime the cells for a mitotic cell cycle (Schnittger et al. 2002b), have been proposed to mediate the expression of mitotic CDKB kinases via the E2F/DP pathway (Boudolf et al. 2004).

Regulation of the cell cycle during root development under optimal conditions involves several aspects including the spatial control of cell proliferation in the different tissues and the coordination between cell division and cellular growth. During mitotic cell cycle progression, both cell growth and division seemed to be tightly linked, as revealed by loss of function of the Translationally Controlled Tumor Protein, a regulator of the TOR kinase pathway. Abolishing its function slows down both cell cycle progression and the growth of mitotic cycling cells (Brioudes et al. 2010).

In summary, cell division, driven by the action of CDKs, is restricted to specific tissues in roots.

3 Size Control of the Proximal Root Apical Meristem and the Mitotic Cell Cycle

In the root tip, the progeny of the stem cells undergo additional divisions in the proximal meristem, the so-called transit amplifying cell proliferation, before they elongate in the differentiation/elongation zone. While the elongating cell is often described as "cells leaving the meristem," the reality is that the stem cell niche at the root's tip (as defined by the QC and the initials) grows away from an elongating cell. Earlier elongation of the cells in the proximal meristem will deplete the meristem of dividing cells and will result in a shorter root apical meristem with fewer cells. Hence, the question "What controls the size of the root apical meristem?" can be reformulated toward "How does the distance from the stem cell niche influence the division/elongation probability of a cell?" when considering this problem from a cellular angle. We have now strong evidence that the decision between dividing or elongation is achieved by balancing auxin and cytokinin signaling in the transition and elongation zone, with auxin stimulating division and preventing elongation and cytokinin having an opposing function, which in combination with an auxin gradient of which the maximum migrates away together with the quiescent center, provides an elegant solution.

A model on the basis of available information on the density and orientation of auxin transporters, cell shape, and auxin transport parameters predicts a maximum auxin concentration in the QC and a steep auxin gradient in the proximal meristem, which drops according to the cell number from the quiescent center (Grieneisen et al. 2007; Laskowski et al. 2008). This is broadly in agreement with the analytical measurements of auxin levels on cell-sorted protoplasts derived from different apical tissues (Petersson et al. 2009), albeit they have a lower spatial resolution, as well as with the available expression patterns of auxin responsive genes, such as

members of the *PLETHORA (PLT)* family, in the different root tissues. Indeed, *PLT1* and *PLT2* proved to be crucial for interpreting this "instructive" auxin gradient in root growth and development (Aida et al. 2004). *PLT1* and *PLT2* encode for AP2-domain transcription factors, and losing the function of both results in the loss of stem cells, an arrest of transit-amplifying divisions, and reduction of cell expansion (Galinha et al. 2007).

Conceptually, meristem cells are characterized by their competence to divide and their undifferentiated status, and chromatin modifiers seem to play an important role in this process. Indeed, histone acetyltransferase are required to sustain *PLT* expression and maintain both transit-amplifying divisions and the root stem cell niche at the root's apex (Kornet and Scheres 2009). Furthermore, the action of SUMO E3 ligase is vital to repress endocycles in the root and shoot meristem, and in the root this SUMO E3 ligase acts in the PLT pathway (Ishida et al. 2009). In summary, the root tip is characterized by an auxin maximum, and auxin is required to sustain transit-amplifying divisions (Ishida et al. 2010).

A dynamic system for size control of the meristem during development was recently proposed by Moubayidin et al. (2010). In the transition zone at the border of the meristem, cytokinins stimulate the expression of *SHY2*, encoding for an auxin/indole acetic acid (AUX/IAA)-type protein that interacts with and suppresses the activity of auxin response factors, thereby inhibiting the expression of auxin responsive genes. Elevating SHY2 levels or reducing TIR1-AUX/IAA-ARF-dependent auxin signaling (Ishida et al. 2010) reduces the meristem size and promotes the onset of endoreduplication. Among the auxin responsive genes are the *PIN-FORMED* auxin efflux carriers, which are of major importance for polar auxin transport (Wisniewska et al. 2006; Feraru and Friml 2008; Geldner et al. 2009). Cytokinins reduce the meristem length by stimulating the expression of *SHY2* through ARR cytokinin responsive transcription factors, which are also stimulated by the RGA DELLA protein. The levels of *RGA* expression are suppressed by gibberellins. Gibberellin-mediated destruction of DELLA proteins also mediates the rate of cell proliferation in the root apical meristem, and this is proposed to be a consequence of reduced cell expansion and associated division of the endodermis layer in the root apical meristem (Achard and Genschik 2009; Ubeda-Tomas et al. 2009), suggesting a key role for the endodermis in controlling the growth rate in the root apical meristem. Auxin can also stimulate the biosynthesis or the activity of gibberellins (Fu and Harberd 2003), providing a positive feedback.

Apart from this elegant mechanism, brassinosteroids also influence meristem size (Gonzalez-Garcia et al. 2011; Hacham et al. 2011) by influencing both cell elongation and the proportion of dividing cells in the proximal meristem. Recent evidence suggests that the epidermis responds to brassinosteroids and that brassinosteroids can influence the activity of quiescent center cells and differentiation of cells in the distal meristem.

The potential to divide diminishes in cells above the proximal meristem and in terms of cell cycle control, one could argue that the transition from a mitotically cycling cell toward an endocycling cell can be achieved by removing M-phase factors. Certainly, manipulating levels of mitotic cyclins and plant-specific CDKBs

modulates the onset of endoreduplication (Schnittger et al. 2002a; Boudolf et al. 2004, 2009). CCS52A1, an activator of the anaphase complex, is able to target CYCA2;3, a mitotic cyclin, for destruction in the elongation zone of the root (Boudolf et al. 2009) and elevating CYCA2;3 levels delayed the onset of cell elongation (Ishida et al. 2010). These arguments indicate that mechanisms associated with the G_2–M transition are putative targets for elongation onset pathways to interfere with the cell cycle. Nevertheless, this does not exclude that factors involved in the G_1–S transition that prime cells for a mitotic division by linking the different cell cycle phases are important targets. Indeed G_1 cyclins, such as D-type cyclins, are able to stimulate the G_1–S transition and to prime cells for a mitotic cell cycle (Schnittger et al. 2002b; Dewitte et al. 2003, 2007; Qi and John 2007), thereby preventing endocycles and elongation. In this respect, one link to the core cell cycle machinery could be the stimulation of CYCD-interacting KRP2 and SIAMESE inhibitors by the DELLA proteins (Churchman et al. 2006; Peres et al. 2007; Achard and Genschik 2009). With increased levels of *CYCD2;1* throughout the plant, there is still a discernable transition zone above the meristem leading up to the final elongated zone (Qi and John 2007). Ectopic expression of CYCD2;1 conferred transit-amplifying cell divisions at a smaller cell size in the proximal meristem, resulting in shorter meristems with higher cell numbers. In addition, ectopic expression of *CYCD2;1* reduced endoreduplication and final cell expansion in tissues above the root apex. This indicates that D-type cyclins can promote transit-amplifying divisions in the RAM and overcome a cellular size threshold mechanism that integrates cell size with the decision to divide. However, our interpretation on the effectiveness of the SHY2 control mechanism for the size of apical meristem in these experiments is limited by our understanding of how modifying the threshold size for cell division and increasing the number of cell compartments in the proximal RAM influences the steepness of the auxin gradient, and it is therefore hard to judge whether elevating CYCD levels bypasses the SHY2-based control mechanism, or if the auxin gradient is affected as well. Nevertheless, loss of function of *CYCD4;1*, a D-type cyclin gene induced by sucrose, conferred premature elongation in the transition zone in the pericycle, indicating that D-type cyclins prevent, to some extent, the onset of elongation in some tissues (Nieuwland et al. 2009).

4 Maintaining the Stem Cell Population in the Distal Meristem and the Procambium; CLE Loops

In roots as in shoots, cells acquire different identities depending on their relative position, and their differentiation is governed by non cell autonomous regulatory loops, based on the diffusion of signaling ligands and recognition by membrane receptors, impinging on the action of *WUSCHEL*-related HOMEOBOX genes. Current models for the maintenance of the stem cell population in the distal and

vascular meristem comprise feedback loops related to the WUS/CLAVATA regulatory loop that controls the shoot stem cell population (Sarkar et al. 2007; Hirakawa et al. 2008, 2010a, b; Stahl et al. 2009). It is proposed that the duplication and diversification of the WUS/CLV module is part of the evolutionary origin of the root system of land plants (Dolan 2009). In the vasculature, the TDIF peptide (CLE41/44) is secreted by the phloem, and upon recognition by the TDR/PXY (TDIF receptor/phloem intercalated with xylem) membrane protein kinase in the cambial cells and probably in collaboration with other members of the CLE peptide family (Whitford et al. 2008), they regulate the proliferation of procambial /cambial stem cells and prevent their differentiation into xylem. Also, this interaction is required for correct orientation of the plane of cell division (Etchells and Turner 2010). In *Arabidopsis*, the *WUSCHEL*-related HOMEOBOX gene, *WOX4*, is a key target of the TDIF signaling pathway and is required for promoting cell division of the procambial/cambial population, but not for preventing differentiation of cambial cells into xylem (Hirakawa et al. 2010a). It is therefore feasible that the cytokinin signaling pathway, which prevents differentiation of cambial cells into xylem (Mahonen et al. 2006; Helariutta 2007), acts independently of *WOX4*. In agreement with this, related CLE peptides have the capacity to suppress protoxylem formation in a cytokinin-dependent manner (Kondo et al. 2010). Apart from controlling the fate and number of cells within the vasculature, cytokinins promote secondary radial growth in *Arabidopsis* roots and shoots, and the cambium cells are particularly responsive to cytokinins (Matsumoto-Kitano et al. 2008).

The differentiation of columella cells below the QC in the distal meristem is governed by the CLE40, a CLAVATA-like peptide, and the ARC4 receptor kinase signaling loop (Stahl et al. 2009). Unlike in the shoot apical meristem, where stem cells express the CLV3 ligand, the differentiated daughter cells of the columella stem cells express the CLE40 ligand, which is perceived by and stimulates expression of the ARC4 receptor-like kinase. In the root stem cell niche, *WOX5* is expressed in the quiescent center and it maintains cell fate in adjacent cells in the distal and proximal meristem (Sarkar et al. 2007). The action of ARC4 represses *WOX5* expression, thereby suppressing stem cell fate (De Smet et al. 2008; Stahl et al. 2009). This mechanism allows the CLE40 peptide produced in the tip of the columella to control the number of stem cells under the quiescent center, and the effect on differentiation is well documented; but it is still unclear how this connects to the temporal regulation of the cell cycle in the stem cells. In this context, it is interesting to note that the expression of *WOX5*, as of its shoot counterpart WUS, also depends on the action of two related phosphatases, POL1 and PPL1. These phosphatases appear to be of key importance for cellular polarization before cell division, asymmetric cell divisions, and stem cell maintenance (Gagne et al. 2008; Song et al. 2008). Losing the function of these phosphatases results in abnormalities of *PIN1* expression and distribution and ectopic expression of the *MP*/*ARF5* auxin response factor gene (Gagne et al. 2008). Moreover, since other members of the *WOX* family mediate *PIN* transcription (Breuninger et al. 2008), it is conceivable that WOX5-mediated auxin transport provides an additional regulatory mechanism.

The retinoblastoma-related (RBR) protein has emerged as a central player at the crossroads of cell differentiation and cell proliferation (Johnston and Gruissem 2009; Borghi et al. 2010; Johnston et al. 2010). RBR directly interacts with a cohort of factors including those involved in chromatin organization, regulates differentiation pathways via downstream targets of transcription factors, and regulates cell cycle progression via the E2/DP transcription factors (Shen 2002). Surprisingly, the stem cells of the distal meristem proved to be particularly sensitive to modulating RBR levels. Levels of RBR are inversely correlated with cell division frequency in those stem cells and directly with the cellular differentiation of stem cells into statocysts (Wildwater et al. 2005). Furthermore, enhancing levels of known suppressors of RBR activity, such as D-type cyclins and E2F/DP transcription factors, triggers cell division, while elevating KRP protein level stimulates differentiation and suppresses cell division, and one can speculate that auxin can mediate the CYCD/RBR/E2F module in these distal stem cells in several ways. For example, auxin can stabilize the E2F transcription factors (Magyar et al. 2005) that could stimulate CDKB levels (Boudolf et al. 2004), induce CYCD expression (Oakenfull et al. 2002), and stimulate KRP destruction (Himanen et al. 2002; Sanz et al. 2011).

5 Post-embryonic Formative Divisions in the RAM

The three principal root tissues are radially organized, the epidermal cell layer covers the ground tissues, cortex, and endodermis, which in turn surround the stele, and this organization is laid out during embryogenesis. During embryogenesis, the action of SCHIZORIZA, a heat shock transcription factor, is required for establishing the ground tissue stem cells (Pernas et al. 2010), and regulating the asymmetric cell divisions of the ground tissue stem cells and specification of the cortex cell fate (Ten Hove et al. 2010). The formative division of the ground tissue requires the action of the SCR/SHR transcription factor complex (Helariutta et al. 2000; Nakajima et al. 2001; Heidstra et al. 2004; Cui et al. 2007), which regulates genes related to cell cycle progression and CDK activity (Dhondt et al. 2010; Sozzani et al. 2010). Within the targets of SCR/SHR, the cyclin *CYCD6;1* gene was identified. In the root of embryos and seedlings, *CYCD6;1* is expressed in the cortex–endodermis initial. Further, in older seedlings, *CYCD6;1* expression marks the formative division of the endodermis cells which results in an additional cortex layer. CYCD6;1 proved to boost the division of the cortex endodermis initial during embryogenesis and growing seedlings (Sozzani et al. 2010). As CYCD6;1 lacks the canonical LXCXE motif in *Arabidopsis* and other plant species (Menges et al. 2007), it still remains to be established how this D-type cyclin acts in this process.

6 Environmental Signaling in the Root Apex via Redox Homeostasis

Redox homeostasis has emerged as an important player that mediates many developmental and physiological processes in plants, which include root growth, hypocotyl elongation in etiolated conditions, and responses to biotic and abiotic stresses. The cell's redox status is mainly determined by the net contribution of different redox couples and many short-lived reactive molecules, like reactive oxygen species (ROS) and reactive nitrogen species (RNS). In plants, the major redox systems are thioredoxin (NTR/TRX), glutathione (GSH/GSSG), and ascorbic acid (AA/DHA) (Banhegyi et al. 1997; May et al. 1998; Arrigo 1999; Schafer and Buettner 2001; Arrigoni and De Tullio 2002; Filomeni et al. 2002; Noctor et al. 2002; Potters et al. 2002, 2004; De Tullio and Arrigoni 2003; Bashandy et al. 2011a). GSH and AA are biochemically interconnected via the ascorbate/glutathione cycle. ROS and RNS have many diverse biological activities. The diversity comes from the enormous variety of chemical reactions and biological properties associated with them. Their concentrations within the cells seem to be determining their biological function. Initially, they were thought to be primarily harmful, increasing tissue damage after infection processes. However, their role was reevaluated after the discovery that these species are endogenously produced. We now know that these compounds not only are purposely synthesized, but also are fast and reliable direct indicators of "something is happening" in order to induce a proper adaptive response (De Tullio et al. 2010).

Redox balance is the basis for the activation of several enzymes and transcription factors, due to the presence of redox-sensitive residues, whose oxidation/reduction is responsible for changes in protein conformation and activity as well as for downstream consequences, such as cell cycle arrest. These enzymes include superoxide dismutases, peroxidases, dehydroascorbate reductases, glutathione reductases, and peroxiredoxins, which can shift their activity in response to redox conditions (Dietz 2008). Redox reactions can occur in membranes, such as thylakoids, plastid envelope, and plasma membrane, and in aqueous cell phases (Dietz and Pfannschmidt 2011). Upon such modifications, cellular redox status plays a critical role in regulating cell proliferation (Reichheld et al. 1999; Den Boer and Murray 2000; Shackelford et al. 2000). In plants, changes in the absolute amounts and/or ratios of reductants/oxidants affect cell proliferation, as shown by the *root meristemless 1* (*rml1*) mutant, in which less glutathione is synthesized because of a mutation in gamma-glutamylcysteine synthetase, the first enzyme in glutathione biosynthesis. In this mutant, the embryonic root meristem forms normally possibly due to maternal-derived glutathione, but cells within cease to produce new derivatives and the meristem disorganizes shortly after germination (Vernoux et al. 2000). Interestingly, the *rml1* mutant of *A. thaliana* is able to develop lateral roots (Cheng et al. 1995), which suggest that GSH is specifically required for cell divisions within the root apical meristem. Similarly, glutathione biosynthesis was required to sustain cell divisions in cell suspensions (Sanchez-Fernandez et al. 1997;

Potters et al. 2004). Interestingly, recent reports (Teotia et al. 2010) concluded that alteration of redox balance by knocking out two members (RCD1 and SRO1) of the poly(ADP-ribose) polymerase (PARP) superfamily led to compromised development. The double mutant *rcd1 sro1* accumulates both ROS and RNS and displays an abnormal root apical meristem. Furthermore, the zone of cell division is smaller than in wild-type roots and the identity of the QC is compromised. Additional studies with the AA/DHA redox couple also support previous results. High levels of AA increase the rates of cell proliferation in plant cells (Kerk and Feldman 1995; Liso et al. 2004), whereas addition of the oxidized form of AA, DHA, delays cell cycle progression (Potters et al. 2002, 2004).

In conclusion, several lines of evidence suggest that the accumulation of reduced components (AA and GSH) increases the rates of cell proliferation in plant cells, whereas the accumulation of the oxidized forms (DHA and GSSG) delays cell cycle progression. Furthermore, the in vivo localization of these redox couples reinforces their connection with cell proliferation, whereas GSH is mainly associated with the actively dividing proximal meristem cells, characterized by a short cell cycle, GSH is almost not detectable in QC cells, characterized by a long cell cycle (Sanchez-Fernandez et al. 1997). Similarly, ASC is absent in QC cells and ASC oxidase, an enzyme involved in the oxidation of AA to DHA, is accumulated in QC cells (Kerk and Feldman 1995; Liso et al. 2004), suggesting a linkage between the accumulation of oxidized forms and the arrest of QC cells in cell cycle progression (Jiang et al. 2003).

A possible mechanism underlying direct redox control of the cell cycle involves A-type cyclins. Several A-type cyclins are involved in S-phase progression (Dewitte and Murray 2003). Previous reports pointed out the differential expression of two A-type cyclins in BY-2 tobacco cells under oxidative stress, which result in cell cycle arrest (Reichheld et al. 1999). Interestingly, these A-type cyclins are not detected in the QC of the *Arabidopsis* primary root (Burssens et al. 2000), characterized by a more oxidized status and the arrest of cells in G_1 phase (Kerk and Feldman 1995; Sanchez-Fernandez et al. 1997; Kerk et al. 2000; Jiang et al. 2003; Liso et al. 2004).

Many lines of evidence link auxin signaling to changes in redox status (Takahama 1996; Jiang and Feldman 2003). High levels of auxin induce in plants a more oxidizing environment (Joo et al. 2001; Pfeiffer and Hoftberger 2001; Schopfer 2001; Schopfer et al. 2002; Tyburski et al. 2008, 2009). This change in redox status is mainly due to the generation of several ROS, such as hydrogen peroxide (Brightman et al. 1988; Joo et al. 2001) and superoxide ions (Schopfer 2001). These ROSs could be generated by oxidation of IAA (Kawano 2003) or, indirectly, as a consequence of auxin affecting the activities of redox-associated systems (Takahama 1996; Kisu et al. 1997; Jiang et al. 2003; Pignocchi et al. 2003; Pignocchi and Foyer 2003). In agreement with this concept, the redox status of the QC, where auxin is strongly accumulated, is different from that in adjacent rapidly dividing cells. The QC has a more oxidizing environment (Kerk and Feldman 1995; Sanchez-Fernandez et al. 1997; Kerk et al. 2000; Jiang et al. 2003; Liso et al. 2004) and a large group of transcripts associated with regulating redox status is found (Jiang et al. 2010). Similarly, perturbing basipetal auxin flux by transport inhibitors

induces an increase in the ASC/DHA ratio in QC cells and a decrease in the other tissues of the root apical meristem (Jiang et al. 2003). These data collectively show that auxin could affect gene expression by both stimulating the degradation of the AUX/IAA proteins (Tan et al. 2007) and via redox regulation. Interestingly, recent reports also conclude that alteration in redox status influence auxin signaling. Auxin transport and levels are perturbed in mutants affected in TRX and GSH redox systems (Bashandy et al. 2011a, b; Cheng et al. 2011). Treatments with redox components (DHA and the AA precursor GalL) or the use of inhibitors of GSH synthesis (BSO) confer remarkably similar effects on the expression pattern of QC identity markers and auxin housekeeping in roots. Taken together, these results suggest a regulatory loop between redox status and auxin levels, with profound implications for QC specification and maintenance.

Changes in environmental and nutrient conditions affect root apical meristem organization (Lopez-Bucio et al. 2003). ROS and RNS have been reported to be induced rapidly by several different types of environmental stresses in a variety of plant species and to regulate the plant response to the biotic and abiotic stresses. Novel tools, such as redox-sensitive promoters driving reporter genes, have enabled us to detect changes in redox conditions in vivo in response to changing conditions (Jiang et al. 2006; Heiber et al. 2007). It is well-known that low temperatures (Lee et al. 2004; Aroca et al. 2005), metals (Sharma and Dubey 2007), pathogens (Torres 2010), and nutrient deficiency (Tyburski et al. 2009) induce production of ROS and RNS in specific tissues. Although these diverse forms of stress affect root morphology in an analogous way by reducing primary root growth and promoting branching (Potters et al. 2007), the mechanisms of redox generation and sensing in response to those specific conditions are still poorly understood. The characteristic response of the *Arabidopsis* root system to low phosphorous (P) availability is an interesting example to illustrate the complexity of these processes. In an effort to understand how P deficiency is perceived in plants and influences root development, Tyburski et al. (2010) showed that ROS are involved in the developmental adaptation of the root system to low P availability. Interestingly, rapidly growing roots of plants grown on P-sufficient medium synthesize ROS in the elongation zone and QC of the root. When seedlings are grown in conditions of P deficiency, the primary root growth slows down and concomitantly, ROS in the QC relocate to cortical and epidermal tissues. Previously, Sanchez-Calderon et al. (2005) indicated that when *Arabidopsis* plants are grown in conditions of P deficiency, the number of cells in the root apical meristem decreases until it is depleted. In these roots, all root apical meristem cells differentiate and the QC is almost indistinguishable. One could argue that these changes in root architecture in response to phosphate starvation could be directly due to changes in ROS location and/or abundance. However, this scenario is even more complicated since these responses are also modulated by the auxin (Lopez-Bucio et al. 2003) and gibberellin–DELLA signaling pathways (Jiang et al. 2007). Intriguingly, plant DELLAs promote survival during adversity by reducing the levels of ROS (Achard et al. 2008), suggesting a putative linkage between ROS and gibberellin signaling pathways in the developmental adaptation of the root system to low P availability. In conclusion, many lines of evidence allow us to conclude that redox status in response

to stress is a major regulatory element in order to induce a proper adaptive response of the root system, which is dependent on the establishment and maintenance of the primary root meristem. Identifying direct ROS and RNS target genes from secondary affect genes now requires further study.

7 Cell Proliferation Outside the Root Apex: Reactivation of the Pericycle

Lateral root founder cells are primed in the pericycle at the xylem poles in the basal meristem. In line with this, reducing the density of cells in the basal meristem, as a consequence of losing *CYCD4;1* function, reduces the final density of lateral roots (Nieuwland et al. 2009). Transcript profiling revealed oscillating gene expression in the basal meristem correlated with the gain of competence for lateral root formation, as marked with the synthetic auxin response reporter DR5. This oscillating gene expression, which can be influenced by auxin, was proposed to be the underlying mechanism for establishing the spatiotemporal distribution of lateral root primordial (Moreno-Risueno et al. 2010). Another model for founder cell specification relies on localized auxin fluxes acting as instructive signals in the basal meristem (De Smet et al. 2007; Dubrovsky et al. 2008) and in agreement with this, the localized destruction of the AUX/IAA28 triggers expression of *GATA23*, encoding for a transcription factor required for founder cell specification (De Rybel et al. 2010). Future research is expected to reveal how both models can be united.

Cell division is reactivated once the distance between the root tip and founder cell reached a threshold to form a lateral root primordium by a series of divisions according to a well-defined sequence of transversal, periclinal, and anticlinal divisions (Casimiro et al. 2003; Peret et al. 2009). Auxin treatment is sufficient to induce the first asymmetric division, and lateral root initiation requires the destruction of the SLR/IAA14 AUX/IAA repressor, which is catalyzed by auxin, resulting in activation of the auxin response factors ARF7 and ARF19. The successive activation of the Bodenlos/IAA12-MONOPTEROS/ARF5 module is required for the organization and patterning of the lateral root (De Smet 2010). The number of cells at the pericycle cell pole that undergo periclinal divisions and thus participate in the formation of the lateral root primordium is restricted by the action of the ARC4 receptor kinase, which is expressed in the smallest daughters of the first two asymmetric transversal divisions, resulting in the definition of a core in the primordium, of which the two cells undergo the first periclinal divisions (De Smet et al. 2008).

Although cell proliferation is required for the formation of lateral roots, stimulation of the cell cycle by enhancing the levels of G1/S regulators, such as CYCDs or E2F/DP transcription factors, is not sufficient for lateral root induction, but these factors mediate the sensitivity of the pericycle to auxin (Vanneste et al. 2005; De Smet 2010; Sanz et al. 2011). Indeed CYCD2;1 is rate limiting, to a certain extent, for auxin-induced lateral root formation (Sanz et al. 2011). Likewise, enhancing the

level of KRP proteins, which can inhibit CYCD/CDKA activity, suppresses lateral root formation and conversely, mutations in KRP2 stimulate lateral root formation (Himanen et al. 2002; Sanz et al. 2011). While CYCD2;1 is induced by sucrose, as is the related CYCD4;1, KRP2 levels are controlled by auxin, enabling auxin to control CYCD2;1 activity post-translationally (Himanen et al. 2002; Sanz et al. 2011). It is still an open question whether these CYCD-related pathways are targets of the SLR/IAA14-ARF7-ARF19 pathway or if they control the basal sensitivity toward auxin by intervening with the initial cell cycle progression at the pericycle xylem pole (Casimiro et al. 2003).

8 Conclusion and Perspective

Analysis of the genetic model plant *Arabidopsis* has recently revealed key regulatory pathways that mediate cell proliferation in the various processes in the apex and mature tissues of the root and has highlighted the role of cell cycle regulators in specific processes. The challenge now is to unravel how developmental and environmental signaling pathways are connected to the core cell cycle machinery and to establish how these mechanisms influence the architecture of the root system under various growth conditions.

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Metabolic Engineering of Cyanobacteria for Direct Conversion of CO_2 to Hydrocarbon Biofuels

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Abstract Cyanobacteria are oxygenic photosynthesizers like plant and algae and hence can capture CO_2 via the Calvin cycle and convert it to a suite of organic compounds. They are Gram-negative bacteria and are well suited for synthetic biology and metabolic engineering approaches for the phototrophic production of various desirable biomolecules, including ethanol, butanol, biodiesel, and hydrocarbon biofuels. Phototrophic biosynthesis of high-density liquid biofuels in cyanobacteria would serve as a good complement to the microbial production of biodiesel and hydrocarbons in heterotrophic bacteria such as *Escherichia coli*. Two groups of hydrocarbon biofuels that are being considered in microbial production systems are alkanes and isoprenoids. Alkanes of defined chain lengths can be used as drop-in fuel similar to gasoline and jet fuel. Many cyanobacteria synthesize alkanes, albeit in minute quantities. Optimizing the expression of the alkane biosynthesis genes and enhancing the carbon flux through the fatty acid and alkane biosynthesis pathways should lead to the accumulation and/or secretion of notable amounts of alkanes. It also becomes important to understand how to control the chain lengths of the produced alkane molecules. Isoprenoids, e.g., the monoterpene pinene and the sesquiterpene farnesene, are considered precursors for future

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biodiesel or next-generation jet fuel. Cyanobacteria produce carotenoids and extending the carotenoid biosynthetic pathways by the introduction of constructs for appropriate terpene synthases should allow the biosynthesis of selected mono- and sesquiterpenes.

1 Introduction

Photosynthetic organisms offer the potential to convert sunlight and CO_2 directly to transportation fuels, bypassing the need for biomass deconstruction. This is a contrast to approaches for biofuel production that require either conversion of resistant feedstock (lignocellulose) to sugars for fermentation, or lipid extraction, isolation, and transesterification. Cyanobacteria and eukaryotic microalgae (often collectively referred to as "algae" or "microalgae") hold particular interest in the biofuel sector since they can tolerate high CO_2 levels such as flue gas streams (Ono and Cuello 2007; Li et al. 2008; Jansson and Northen 2010), and may be superior to higher plants in energy efficiency, biomass and oil productivity, and land and water usage (Chisti 2007, 2008; Gressel 2008; Hu et al. 2008; Packer 2009; Sheehan 2009; Costa and de Morais 2011; Lu 2010; Radakovits et al. 2010; Scott et al. 2010; Ono and Cuello 2007; Li et al. 2008; Clarens et al. 2010). Since a large number of cyanobacterial and microalgal species are halophilic, they can be grown in seawater, saline drainage water, or brine from the petroleum production and refining industry or CO_2 injection sites, thereby sparing freshwater supplies. The use of potable water for cultivation can also be avoided by utilizing municipal wastewater as a nutrient source. Additionally, cyanobacteria occupy a wide range of extreme environments such as hot springs, deserts, bare rocks, and permafrost zones, and they are exposed to the highest rates of UV irradiance known on the Earth. The thermophilic nature of many cyanobacteria, as well as microalgae, allows them to tolerate high temperatures characteristic of flue gas.

Cyanobacteria are photosynthetic Gram-negative bacteria that perform oxygenic photosynthesis similar to plants and algae. As opposed to microalgae that can accumulate large amounts of triacylglycerols (TAGs) as storage lipids, the cyanobacteria studied to date produce little or no TAGs, but their fatty acids (FAs) are directly shuttled to membrane lipid synthesis (see further below). Conversely, cyanobacteria are well suited for approaches aimed at redirecting carbon flux in lipid metabolism to specific biofuel molecules. First, whereas in plants and algae, including microalgae, lipid metabolism involves several different cellular compartments, in cyanobacteria, all metabolism occurs via soluble or membrane-bound enzymes in the cytoplasm. Second, being bacteria, cyanobacteria are amenable to homologous recombination, which allows rapid site-directed mutagenesis, gene insertions, replacements, and deletions in a precise, targeted and predictable manner.

Two major groups of hydrocarbon biofuels being considered in microbial production systems are FA-based products like alkanes and alkenes, and isoprenoids

such as monoterpenes and sesquiterpenes(Fortman et al. 2008; Keasling and Chou 2008; Kirby and Keasling 2008; Lennen et al. 2010; Lu 2010; Steen et al. 2010). In the following, we will focus on pathway engineering in cyanobacteria for the biosynthesis of alkanes from free FAs and of selected terpenes.

2 Fatty Acid Metabolism in Cyanobacteria

Fatty acid (FA) synthesis in cyanobacteria and other bacteria is accomplished by a type II FAS (FASII), a multienzyme system, utilizing a freely dissociable acyl carrier protein ACP (Campbell and Cronan 2001) (Fig. 1). The FASII enzymes comprise acetyl-CoA carboxylase (ACC; EC 6.4.1.2); malonyl-CoA:ACP transacylase (FabD; 2.3.1.39); three condensing enzymes, ß-ketoacyl-ACP synthase I (FabB; EC 2.3.1.41), ß-ketoacyl-ACP synthase II (FabF; EC 2.3.1.41), and ß-ketoacyl-ACP synthase III (FabH; EC 2.3.1.180) required for initiation of FA synthesis; ß-ketoacyl-ACP reductase (FaBG; EC 1.1.1.100); ß-hydroxyacyl-ACP dehydratase/isomerase (FabA and FabZ); and enoyl-ACP reductase I (FabI; EC 1.3.1.9). The products of FASII are released as acyl-ACPs and may be directly incorporated into membrane lipids by two acyltransferases, glycerol-3-phosphate *O*-acyltransferase (GPAT or PlsB; EC 2.3.1.15) and 1-acylglycerol-3-phosphate *O*-acyltransferase, also called

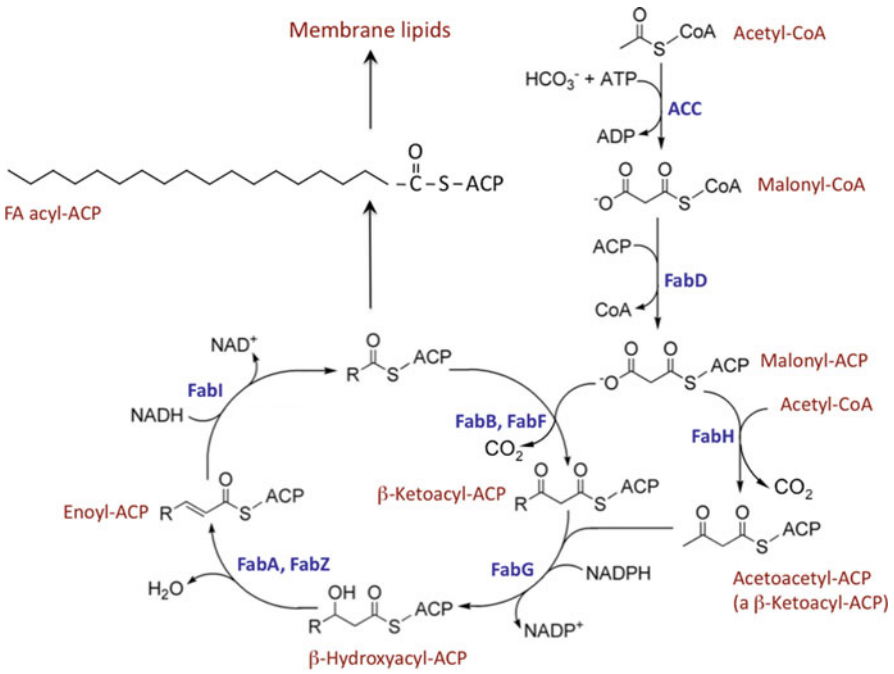

Fig. 1 Fatty acid synthesis in cyanobacteria. See text for abbreviations

lysophosphatidic acid acyltransferase (AGPAT, LPAT, or PlsC; EC 2.3.1.51) that each attaches an FA to the glycerol 3-phosphate (G3P) backbone to form the key intermediate, phosphatidic acid (PA) (Voelker and Kinney 2001; Cronan 2003).

In plants and algae, de novo FA synthesis occurs in the plastids, which also exhibit the FASII machinery (Jones et al. 1995; Voelker and Kinney 2001; Thelen and Ohlrogge 2002). In the plastids, acyl-ACPs are hydrolyzed by acyl-ACP thioesterases (TE; EC 3.1.2.14, e.g., FatB in *Arabidopsis*) to yield free FAs for transport across the plastid envelope (Jones et al. 1995; Bonaventure et al. 2003). Upon arrival at the outer plastid surface, the free FAs are re-activated by acyl-CoA synthetase or long-chain-FA-CoA ligase (FadD; EC 6.2.1.3) to form acyl-CoA. Acyl-CoA is the starting substrate for synthesis of TAGs but can also be used for ß-oxidation and for synthesis of membrane lipids (Bonaventure et al. 2003). Most bacteria lack intracellular TEs that act on FA-ACPs, and formation of free FAs mainly occurs during recycling of membrane lipids or degradation of acylated proteins. *Escherichia coli* and other bacteria that can take up and metabolize exogenous FAs possess periplasmic TEs, e.g., TesA in *E. coli* (Cho and Cronan 1994) that liberate FAs for import. Heterologous expression of plant TEs in bacteria can yield high production of free FAs (Voelker and Davies 1994; Jones et al. 1995; Yuan et al. 1995; Jha et al. 2006; Lu et al. 2008). The concomitant decrease in acyl-ACP levels also relieves the rigorous feedback inhibition of ACC exerted by this end product. Thus, expression of plant TEs in bacteria has the dual effect of producing free FA and enhancing FA synthesis [greater than fivefold; Khosla 2008].

Cyanobacteria seem to lack TE enzymes and the generation of free FAs occurs during recycling of degraded membrane lipids (Figs. 1 and 2). In *E. coli*, which can

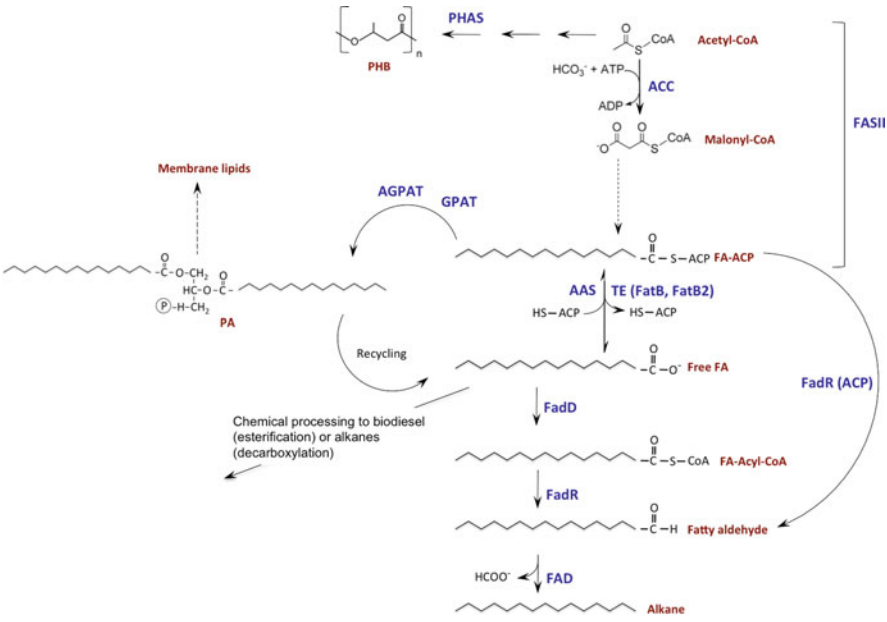

Fig. 2 Proposed pathway(s) for alkane synthesis in cyanobacteria. FadR (ACP), FadR accepting FA-ACP rather than FA-Acyl-CoA as a substrate. See text for other abbreviations

take up and metabolize exogenous FAs, the incoming FAs are activated to acyl-CoA by the soluble but plasma membrane-associated FadD for subsequent utilization in ß-oxidation (Yoo et al. 2001; Kaczmarzyk and Fulda 2010). Uptake of FAs is not a normal *modus operandi* for cyanobacteria but they are able to import both free FAs and complex lipids and incorporate the exogenous FAs in their membranes (Hagio et al. 2000; Kaczmarzyk and Fulda 2010). Together with their capacity for alkane biosynthesis, this indicates the presence of an activating enzyme like FadD in cyanobacteria. Cyanobacteria most likely lack FadD but the FA-activating process is instead provided by an Acyl-ACP synthetase or long-chain-FA-ACP ligase (AAS; EC 6.2.1.20) (Kaczmarzyk and Fulda 2010; Lu 2010; Schirmer et al. 2010).

Biosynthesis of free FAs is of interest since they can be used for downstream chemical processing to biofuels (Lennen et al. 2010) and also because cyanobacterial biosynthesis of alkanes with specified chain lengths may require free FAs as an intermediate metabolite (Fig. 2).

3 Biosynthesis of Hydrocarbons

3.1 Alkanes

The pathway for alkane synthesis in cyanobacteria seems to proceed via decarbonylation of fatty aldehydes (Schirmer et al. 2010), the major route for alkane synthesis in most organisms (Ladygina et al. 2006). The decarbonylation pathway implies the involvement of the fatty acyl-CoA or fatty acyl-ACP reductase (FadR; EC 1.2.1.50) and fatty aldehyde decarbonylase (FAD; EC 4.1.99.5) (Walsh et al. 1998; Ladygina et al. 2006) (Fig. 2). Gene sequences for *FadR* and *FAD* have recently been identified from several cyanobacteria (Schirmer et al. 2010). Interestingly, the decarbonylation step in cyanobacterial alkane biosynthesis may involve the release of formate ($HCOO^-$) rather than CO (Warui et al. 2011).

To generate FAs of desired chain lengths (e.g., C_8, C_{10}, and C_{12} saturated species) for jet fuel or gasoline, cyanobacteria can be endowed with genes encoding TEs (Fig. 3) with different substrate specificities in the 16:0–8:0 FA-ACP range: *FatB* from *Arabidopsis* (Accession NP_172327), *FatB2* from *Cuphea hookeriana* (GenBank: U39834.1), *FatB1* (pCGN3822) from *Umbellularia californica* (GenBank: M94159.1), and *FatB1* from *C. hookeriana* (GenBank: Q39513.1). Another potential TE is the mature TES enzyme from *E. coli* (Cho and Cronan 1994). In addition to inserting an appropriate TE, high-yield production of free FAs in cyanobacteria most likely will also require additional optimization by increasing the carbon flux toward FA synthesis. Such efforts may entail the insertion of extra copies of the gene for ACC, which catalyzes the rate-limiting step in FA-ACP synthesis. ACC is a heterotetramer consisting of AccA, AccB, AccC, and AccD. The genes for the different subunits are distributed in most, if not all, cyanobacterial genomes. For the sake of increasing ACC activity, an *ACC* operon can be

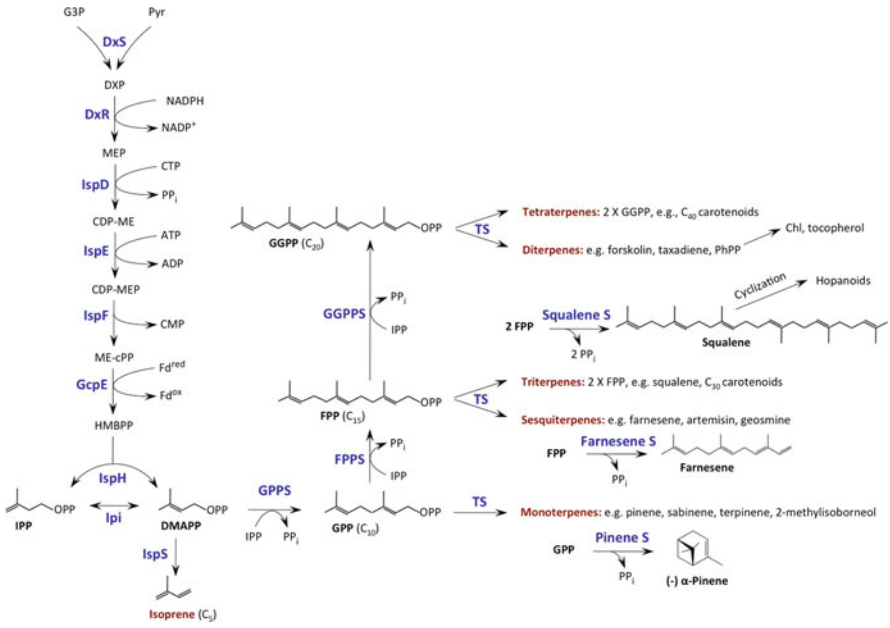

Fig. 3 Generic pathways for synthesis of isoprenoids in bacteria

constructed behind a strong promoter. For example, in our own work in *Synechocystis* PCC6803 (*S*. 6803), the *sll0728*, *slr0435*, *sll0053*, and *sll0336* loci, encoding AccA, AccB, AccC, and AccD, respectively, were assembled by utilizing the promoter and SD sequences from the *S*. 6803 *cpc* operon and the terminator from the *S*. 6803 *atp1* operon. Additionally, an ATG start codon was substituted for the original GTG codon in *AccB*. The operon was inserted into the *S*. 6803 *slr1830* locus that harbors the *phaC* gene, which encodes polyhydroxyalkanoate synthase (PHAS). This insertion has the additional effect of preventing diversion of carbon flux from acetyl-CoA to polyhydroxybutyrate (PHB) synthesis (Fig. 2). Intuitively, another optimizing step would be to inactivate the *AAS* gene to prevent re-thioesterification of free FAs. However, since AAS rather than FadD may serve as the sole FA-activating enzyme in cyanobacteria, the yield of metabolites downstream of acyl-ACP, like alkanes, might benefit from increasing the copy number of *AAS* genes so as to speed up the activation of recycled FAs from the degradation of membrane lipids (Figs. 1 and 2).With few exceptions, *AAS* exists as a single-copy gene in cyanobacteria, encoding an enzyme with broad substrate specificity (Kaczmarzyk and Fulda 2010). For the single purpose of free FA production, a simultaneous increase in ACC activity and inactivation of the gene for AAS is likely to improve yield.

Microbial biosynthesis of alkanes was recently demonstrated in *E. coli* by utilizing genes for a FadR with high specificity for acyl-ACP, and an FAD, both identified by comparing the genomes of alkane-producing and non-alkane-producing cyanobacteria (Schirmer et al. 2010). Whether or not the substrate specificities of the AAS, FadR, and

FAD enzymes are broad enough to efficiently utilize C_8–C_{14} FAs produced from the various TEs is an open question. Attempts to alter the substrate specificity of selected enzymes by protein engineering may be an option. The applicability of such an approach has been demonstrated by the successful modification of the substrate specificity of FatB1 from *U. californica* from 12:0 FA-ACP to 14:0 FA-ACP (Yuan et al. 1995).

Cyanobacteria synthesize a wide array of linear, branched, and cyclic alkanes (Table 1), some of which, like branched methyl- and ethylalkanes, are only found in cyanobacteria. For example, the cyanobacterium *Microcoleus vaginatus* produces four *n*-alkanes and more than 60 different branched alkanes (Dembitsky et al. 2001). Another strain that merits investigation is *Anabaena cylindrica*, which forms C_9–C_{16} *n*-alkanes under high NaCl conditions (Bhadauriya et al. 2008), presumably due to an increase in short-chain FA during salt stress. Notably, this range of carbon lengths is particularly well suited for development of alkane-based jet fuels. Exploring the enormous diversity of cyanobacteria is likely to reveal alkanes with a rich variety of lengths and structures.

3.2 Isoprenoids

Branched hydrocarbons, which have higher octane rating than *n*-alkanes, can be produced by engineering the carotenoid pathway in cyanobacteria. While it is possible to use carotenoids themselves to make gasoline, e.g., via hydrocracking (Hillen et al. 1982), many carotenoids are solid at room temperature, complicating refining approaches. Cyanobacteria contain genes for carotenoid synthesis and thus synthesize geranyl pyrophosphate (GPP), farnesyl pyrophosphate (FPP), and geranylgeranyl pyrophosphate (GGPP), which are precursors for monoterpenes, sesqui- and triterpenes, and di- and tetraterpenes, respectively (Fig. 3). Most, if not all, cyanobacteria produce sesquiterpenes such as geosmine, and monoterpenes such as 2-methylisoborneol (Agger et al. 2008), but synthesis of isoprene in naturally occurring cyanobacteria has not been reported. By introduction of an isoprene synthase (IspS) gene based on the mature enzyme from the Kudzu plant (*Pueraria montana*; GenBank: AY316691), Lindberg et al. (2010) demonstrated the production of volatile isoprene hydrocarbons in the cyanobacterium *S.* 6803 (Lindberg et al. 2010). The rationale for engineering cyanobacteria for isoprene, monoterpene, or sesquiterpene synthesis is straightforward as it involves the addition of a single gene, IspS, or different terpene synthases (TS). A desirable objective will be to extend the carotenoid pathway for synthesis of pinene (a monoterpene) and farnesene (a sesquiterpene). Pinene is being considered for next-generation jet fuel, and farnesene is being developed as precursors to diesel fuels (Rude and Schirmer 2009). For example, synthetic gene constructs could be based on the mature proteins of (−)-α-pinene synthase from *Pinus taeda* (GenBank: AF543527.1) and α-farnesene synthase from *Pyrus communis* (GenBank: AY566286.1).

Table 1. Biosynthesis of alkanes in cyanobacteria

Alkanes	Cyanobacterium	Comment	Refs.
Very long *n*-alkanes (C_{23}-C_{31})	*Anabaena cylindrica* *Schizothrix* sp. *Scytonema* sp.		(Jones and Young 1970; Thiel et al. 1997)
Nonadecane	*Calothrix* sp.		(Dembitsky and Srebnik 2002)
Octadecane	*Anabaena cylindrica* *Anacystis cyanea* Calothrix sp. *Microcystis aeruginosa* *Synechocystis* UTEX 2470		(Jones and Young 1970; Dembitsky and Srebnik 2002)
Heptadecane	Most cyanobacteria	Predominant alkane in most cyanobacteria	(Han et al. 1968; Jones 1969; Jones and Young 1970; Thiel et al. 1997; Dembitsky and Srebnik 2002; Ozdemir et al. 2004; Ladygina et al. 2006; Bhadauriya et al. 2008)
Hexadecane	*Anacystis* sp. *Lyngbya* sp. *Microcoleus* sp. *Microcystis aeruginosa* *Plectonema terebrans* *Spirulina platensis*		(Dembitsky and Srebnik 2002)
Pentadecane	*Anacystis* sp. *Microcoleus lyngbyaceus* *Microcystis aeruginosa* *Oscillatoria* sp. *Plectonema terebrans* *Spirulina platensis*		(Han et al. 1968; Winters et al. 1969; Dembitsky and Srebnik 2002)
Tetradecane	*Spirulina platensis*		(Ozdemir et al. 2004)

Short-medium n-alkanes (C_7-)	*Anabaena cylindrica* *Nostoc* sp.	*A. cylindrica* (C_9-C_{16} during salt stress) *Nostoc* sp. (C_7-C_{10})	(Dembitsky et al. 1999; Bhadauriya et al. 2008)
Branched alkanes (methylalkanes, dimethylalkanes, trimethylalkanes, ethylalkanes, polybranched alkanes)	*Chlorogloea fritschii* *Calothrix scopulorum* *Mastigocladus laminosus* *Microcoleus vaginatus* *Nostoc* sp. *Phormidium* sp. *Schizothrix* sp. *Scytonema* sp.	Some that are unique to cyanobacteria	(Han et al. 1968; Han and Calvin 1969; Kenig et al. 1995; Thiel et al. 1997; Köster et al. 1999; Kenig 2000; Dembitsky et al. 2001; Jahnke et al. 2001; Ladygina et al. 2006)
Cyclic alkanes (alkylated or hydroxylated cyclopentanes, cyclohexanes, cyclooctanes)	*Nostoc* sp.		(Dembitsky et al. 1999; Dembitsky and Rezanka 2005)

4 Conclusions and Perspectives

The employment of cyanobacteria for biofuel production is in its infancy. Most of the attention in the algal biofuel space is devoted to eukaryotic microalgae, mainly because of their capacity to store large amounts of TAGs. However, the successful biosynthesis of FA ethylesters (FAEE; a biodiesel) and hydrocarbon fuels in *E. coli* (Kalscheuer et al. 2006; Beller et al. 2010; Schirmer et al. 2010; Steen et al. 2010) suggests that similar strategies in pathway engineering should prove achievable also in cyanobacteria, where sunshine and CO_2, rather than organic feedstocks, will serve as energy and carbon source, respectively. Furthermore, cyanobacteria have previously been engineered to produce alcohol-based fuels such as ethanol and isobutanol (Deng and Coleman 1999; Atsumi et al. 2009).

The capacity of cyanobacteria to thrive in high CO_2 concentrations makes them an attractive system for beneficial recycling of CO_2 from point sources such as coal-fired power plants via biofuel synthesis. Since many cyanobacteria are halophilic, raceway ponds can be sited away from agricultural land, making use of seawater or various sources of saline wastewater. A conceivable future scenario where CO_2 recycling is combined with utilization of brine produced from CO_2 injections during geological carbon capture and storage (CCS) is shown in Fig. 4.

Although the photoautotrophic cyanobacteria and microalgae offer obvious advantages over heterotrophic microorganisms and plants in biofuel synthesis, they also present several challenges that need to be addressed. (1) For example,

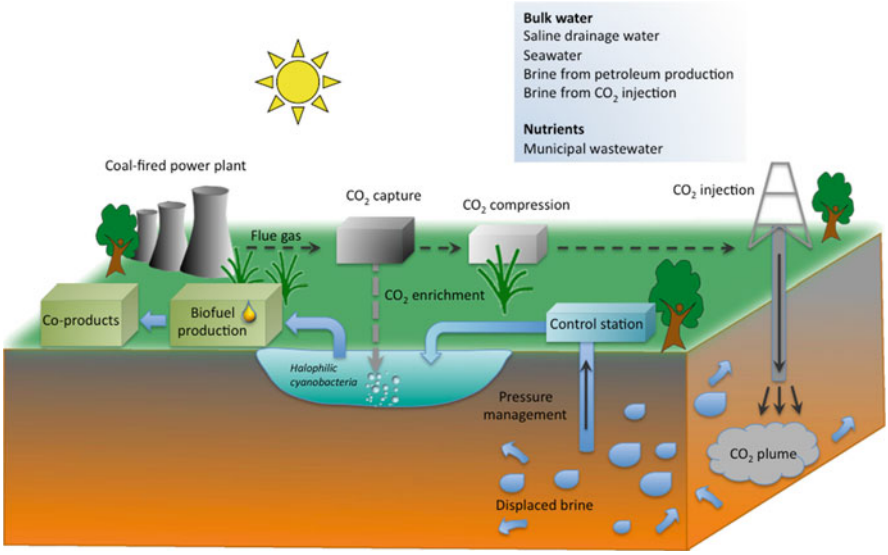

Fig. 4 Pictorial simulation of industrial-scale implementation of cyanobacteria for biofuel synthesis via CO_2 recycling from flue gas. The use of saline water is illustrated with brine from geological carbon capture and storage (CCS) operations

photosynthesis occurs only in light, which begs the question of how day–night cycles will affect biofuel production and accumulation; (2) A related issue is the efficiency of light harvesting and how to avoid light limitation and photoinhibition; (3) Whereas cultivation in raceway ponds requires measures for crop protection, growing cells in more controlled environments such as photobioreactors is currently all but cost prohibitive. Thus, how to prevent or mitigate contamination and grazing in open pond systems becomes an important question; (4) A major cost in the algal biofuel industry is associated with harvesting and extraction, and strategies that facilitate, or obviate the need for, these steps need to be further developed. One solution is to use filamentous or self-flocculating strains to expedite harvesting. Another approach is to achieve release of the biofuel molecules to the medium, either through cell lysis or by secretion. An example of the former is a nickel-inducible lysis system reported for *S*. 6803 (Liu and Curtiss 2009). The feasibility of secretion was illustrated by the release of free FAs from *S*. 6803 and *Synechococcus elongatus* PCC 7942 cells to the medium after inactivation of the *AAS* gene (Kaczmarzyk and Fulda 2010).

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Part III
Physiology

Interaction Between Salinity and Elevated CO_2: A Physiological Approach

Usue Pérez-López, Amaia Mena-Petite, and Alberto Muñoz-Rueda

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Abstract This review presents a detailed analysis of the papers published over the past 10 years addressing the response of several physiological parameters to the interaction between salinity and elevated CO_2, which are both anticipated to affect more severely future environmental conditions. This review demonstrates that the response to this interaction is species and even cultivar specific and that the predicted higher biomass production at elevated CO_2 is not a general trend. Most strategies to cope with salt stress involve active regulation of water potential and stomatal conductance, active regulation of photosynthetic metabolism as a whole, and active regulation of other salt-tolerance mechanisms. However, these salt-tolerance mechanisms are energetically expensive, and even if under elevated CO_2 there is generally higher energy supply than under ambient CO_2, biomass production will depend on the trade-off between the anabolic (photosynthesis) and catabolic (chloro-, photo-, and basal-respiration) rates, as well as the ability to control the stomatal conductance.

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1 A Brief Introduction

Plants need for their development both water and mineral salts, which are absorbed from the soil through plant roots, and CO_2, which is taken from the atmosphere through the stomata. These elements permit cells to continue to divide and expand, thus generating plant growth and development. However, the availability of minerals, water, and CO_2 is not always at optimal levels, and restriction of these factors limits plant growth throughout the different stages of development.

Soil salinity is a major environmental constraint limiting plant productivity and distribution. According to recent statistics (FAO 2000), it already affects 19.5% of the world's irrigated area and also occurs in nonirrigated croplands and rangelands. Moreover, salinity-affected areas are rapidly increasing because of faulty irrigation systems and poor-quality water. In addition, the increase in temperature associated with climate change will elevate evaporation rates, extending saline land areas still further. Meanwhile, the pressures of increasing populations and demand for plant production will make it imperative to bring even more saline lands into agricultural production.

Together with this rise in the saline surface, it is expected that by the end of the century, the atmospheric concentration of CO_2 will double (IPCC 2007). Because CO_2 is the basic substrate for photosynthesis, many scientists have argued that in CO_2-enriched atmospheres, plant growth will be more rapid and abundant. In addition, high CO_2 mitigates damage and the growth reduction that various stressors such as salinity provoke, provided the plant growth had diminished initially because of some factor directly or indirectly dependent on CO_2 availability. However, this relationship is complex and not well understood because higher CO_2 simultaneously affects both plant growth and salinity tolerance by, between others, increasing the carbon supply and reducing the transpiration rate (Maggio et al. 2002).

2 Background Up to 1999

As previously noted, the results of some laboratory experiments (Idso and Idso 1994; Munns et al. 1999 and literature therein) have indicated that elevated CO_2 will enhance plant growth in saline soils. However, this enhancement depends on many factors, such as the degree of salinity, whether there are stomatal limitations to growth, and the effect on growth of other edaphic factors such as waterlogging and nutrient deficiencies (Munns et al. 1999). Based on these studies, the authors suggested that under salinity, the elevated CO_2 appeared to improve the growth of the plants mainly by the increase in net photosynthetic rate and, to a lesser extent, by the improved water relations and/or the lower internal salt concentrations of the leaves. However, how this mechanism could apply when salt-affected plants already have apparently ample reserves of carbohydrates remained unclear.

Since 1999, several papers have been published in an effort to clarify this enigma. Species such as *Solanum muricatum* (Chen et al. 1999a, b), *Cucumis melo* (Mavrogianopoulos et al. 1999), *Lycopersicon esculentum* (Li et al. 1999a, b; Maggio et al. 2002; Takagi et al. 2009), *Hordeum vulgare* (Pérez-López et al. 2008, 2009a, b, 2010a, b), *Aster tripolium* (Geissler et al. 2009a, b, 2010), *Spartina densiflora* (Mateos-Naranjo et al. 2010), *Cleopatra mandarin* (*Citrus reticulate*), *Carrizo citrange* (*Citrus sinensis* × *Poncirus trifoliate*) (García-Sánchez and Syvertsen 2006), and *Olea europaea* (Melgar et al. 2008) have been studied. All of the studied plants were subjected to different salinities (from 25 through 500 mM NaCl) and CO_2 concentrations (from 350 through 1,200 μmol CO_2 mol^{-1} air) in different experiments; nevertheless, we have formulated from these data a review of advances regarding issues such as water and ionic relations, photosynthetic metabolism, and growth and salt-tolerance mechanisms, all parameters that are altered under saline soils. As we already know, the water in saline soils may not be available for the plant because ions such as Na^+ and Cl^- retain it. This characteristic provokes many changes in plant physiology, including altering water and nutrient uptake, reducing photosynthetic assimilation, inducing oxidative stress, and increasing respiration rate, consequently provoking a decrease in the absolute growth of the plant (Munns 2002, 2005; Parida and Das 2005). We will start the next section by analyzing water and ionic relations.

3 Water and Ionic Relations

Regarding the four major constraints of salinity on plant growth, the osmotic effect, resulting from ionic withholding of water from the soil, is the primary factor influencing plant growth (Rodríguez et al. 1997; Parida and Das 2005; Navarro et al. 2007). Thus, as a result of lower water availability, the plant has to ensure a continued uptake of water by lowering its water potential while at the same time diminishing water losses by lowering transpiration. As Table 1 shows, some plants become relatively more responsive to atmospheric CO_2 enrichment as soil salinity rises. Both in *A. tripolium* (Geissler et al. 2009b) and *H. vulgare* (Pérez-López et al. 2009a), as long as salt stress was more severe, the decrease of water potential under elevated CO_2 was lower than under ambient CO_2. Other species are relatively less responsive, and some exhibit no change in water potential between two CO_2 concentrations, such as *S. muricatum* (Chen et al. 1999b), *L. esculentum* (Takagi et al. 2009), *C. mandarin* (García-Sánchez and Syvertsen 2006), and *O. europaea* (Melgar et al. 2008). Thus, there appears to be significant interaction between CO_2 and soil salinity overall, depending on the species and the salt concentration used.

In *A. tripolium* (Geissler et al. 2009b) and *H. vulgare* (Pérez-López et al. 2009a), species in which the interaction occurs, the plant reduces the water potential of its cells by decreasing the osmotic potential via passive dehydration (DH) and/or active osmotic adjustment (OA), making it more negative than in the soil and thus ensuring water uptake. In plants, OA is achieved by means of accumulation

Table 1 Compilation of published data on the effects of CO_2 enrichment on water relations and ionic parameters

Variable measured	Species	CO_2 (μmol CO_2 mol^{-1} air); time scale (weeks)	Salinity (mM NaCl); time scale (weeks)	Effect	Reference(s)
Water potential	*Solanum muricatum* Ait.	350, 700; 4	0, 25; 8	0 (−17) 25 (−17)	Chen et al. (1999b)
	Solanum muricatum Ait.	350, 1,050; 4	0, 25; 8	0 (−25) 25 (−23)	Chen et al. (1999b)
	Lycopersicon esculentum L.	370, 1,000; 1	0, 100; 1	0 (n.s.)[a] 100 (n.s.)	Takagi et al. (2009)
	Lycopersicon esculentum L.	370, 1,000; 2	0, 100; 2	0 (n.s.)[a] 100 (n.s.)	Takagi et al. (2009)
	Aster tripolium L.	370, 520; 5	0, 125, 250, 375, 500; 4	0 (n.s.)[a] 125(n.s.) 250 (n.s.) 375 (−31) 500 (−18)	Geissler et al. (2009b)
	Cleopatra mandarin	360, 700; 8	0, 50; 8	0 (n.s.) 50 (n.s.)	García-Sánchez and Syvertsen (2006)
	Carrizo citrange	360, 700; 8	0, 50; 8	0 (+21) 50 (+32)	García-Sánchez and Syvertsen (2006)
	Olea europaea L. cv. Koroneiki	360, 700; 12	0, 100; 12	0 (n.s.) 100 (+34)	Melgar et al. (2008)
	Olea europaea L. cv. Picual	360, 700; 12	0, 100; 12	0 (n.s.) 100 (+73)	Melgar et al. (2008)
	Hordeum vulgare L. cv. Alpha	350, 700; 4	0, 80, 160, 240; 2	0 (n.s.) 80 (n.s.) 160 (−22) 240 (−23)	Pérez-López et al. (2009a)
	Hordeum vulgare L. cv. Iranis	350, 700; 4	0, 80, 160, 240; 2	0 (n.s.) 80 (n.s.) 160 (−23) 240 (n.s.)	Pérez-López et al. (2009a)

Osmotic potential	*Cleopatra mandarin*	360, 700; 8	0, 50; 8	0 (n.s.) 50 (n.s.)	García-Sánchez and Syvertsen (2006)
	Carrizo citrange	360, 700; 8	0, 50; 8	0 (−13) 50 (n.s.)	García-Sánchez and Syvertsen (2006)
	Olea europaea L. cv. Koroneiki	360, 700; 12	0, 100; 12	0 (n.s.) 100 (n.s.)	Melgar et al. (2008)
	Olea europaea L. cv. Picual	360, 700; 12	0, 100; 12	0 (n.s.) 100 (+11)	Melgar et al. (2008)
	Hordeum vulgare L. cv. Alpha	350, 700; 4	0, 80, 160, 240; 2	0 (n.s.) 80 (n.s.) 160 (n.s.) 240 (n.s.)	Pérez-López et al. (2009a)
	Hordeum vulgare L. cv. Iranis	350, 700; 4	0, 80, 160, 240; 2	0 (n.s.) 80 (n.s.) 160 (n.s.) 240 (n.s.)	Pérez-López et al. (2009a)
Turgor potential	*Cleopatra mandarin*	360, 700; 8	0, 50; 8	0 (n.s.) 50 (n.s.)	García-Sánchez and Syvertsen (2006)
	Carrizo citrange	360, 700; 8	0, 50; 8	0 (−20) 50 (−7)	García-Sánchez and Syvertsen (2006)
	Olea europaea L. cv. Koroneiki	360, 700; 12	0, 100; 12	0 (n.s.) 100 (n.s.)	Melgar et al. (2008)
	Olea europaea L. cv. Picual	360, 700; 12	0, 100; 12	0 (n.s.) 100 (n.s.)	Melgar et al. (2008)
	Hordeum vulgare L. cv. Alpha	350, 700; 4	0, 80, 160, 240; 2	0 (n.s.) 80 (n.s.) 160 (+34) 240 (+70)	Pérez-López et al. (2009a)
	Hordeum vulgare L. cv. Iranis	350, 700; 4	0, 80, 160, 240; 2	0 (n.s.) 80 (n.s.) 160 (+47) 240 (+52)	Pérez-López et al. (2009a)

(continued)

Table 1 (continued)

Variable measured	Species	CO_2 (μmol CO_2 mol^{-1} air); time scale (weeks)	Salinity (mM NaCl); time scale (weeks)	Effect	Reference(s)
Dehydration	*Hordeum vulgare* L. cv. Alpha	350, 700; 4	0, 80, 160, 240; 2	0 (n.s.) 80 (−45) 160 (−35) 240 (−8)	Pérez-López et al. (2010a)
	Hordeum vulgare L. cv. Iranis	350, 700; 4	0, 80, 160, 240; 2	0 (n.s.) 80 (−27) 160 (−33) 240 (−19)	Pérez-López et al. (2010a)
Osmotic adjustment	*Hordeum vulgare* L. cv. Alpha	350, 700; 4	0, 80, 160, 240; 2	80 (+100) 160 (+45) 240 (+50)	Pérez-López et al. (2010a)
	Hordeum vulgare L. cv. Iranis	350, 700; 4	0, 80, 160, 240; 2	80 (+63) 160 (+62) 240 (+83)	Pérez-López et al. (2010a)
Sugar content	*Lycopersicon esculentum* L.	360, 1,200; 4	0, 53; 4	0 (+325)[a,b] 53 (+233)	Li et al. (1999b)
	Lycopersicon esculentum L.	360, 1,200; 4	0, 53; 13	0 (n.s.)[a,b] 53 (n.s.)	Li et al. (1999b)
	Aster tripolium L.	370, 520; 5	0, 250, 375, 500; 4	0 (+96)[a] 250 (+60) 375 (+140) 500 (+77) In adult leaf	Geissler et al. (2009b)
	Aster tripolium L.	370, 520; 5	0, 250, 375, 500; 4	0 (n.s.)[a] 250 (n.s.) 375 (n.s.) 500 (n.s.) In main root	Geissler et al. (2009b)

	Hordeum vulgare L. cv. Alpha	350, 700; 4	0, 80, 160, 240; 2	0 (+37) 80 (+22) 160 (+30) 240 (+40) In adult leaf	Pérez-López et al. (2010a)
	Hordeum vulgare L. cv. Iranis	350, 700; 4	0, 80, 160, 240; 2	0 (+28) 80 (+24) 160 (+33) 240 (+32) In adult leaf	Pérez-López et al. (2010a)
Na^+ content	*Lycopersicon esculentum* L.	370, 1,000; 2	0, 100; 2	0 (n.s.)[a] 100 (n.s.) in the leaf	Takagi et al. (2009)
	Lycopersicon esculentum L.	370, 1,000; 2	0, 100; 2	0 (−26)[a] 100 (n.s.) In the root	Takagi et al. (2009)
	Cleopatra mandarin	360, 700; 8	0, 50; 8	0 (n.s.)[a] 50 (n.s.) In adult leaf	García-Sánchez and Syvertsen (2006)
	Cleopatra mandarin	360, 700; 8	0, 50; 8	0 (n.s.)[a] 50 (−33) In main root	García-Sánchez and Syvertsen (2006)
	Carrizo citrange	360, 700; 8	0, 50; 8	0 (n.s.)[a] 50 (−57) In adult leaf	García-Sánchez and Syvertsen (2006)
	Carrizo citrange	360, 700; 8	0, 50; 8	0 (n.s.)[a] 50 (n.s.) In main root	García-Sánchez and Syvertsen (2006)
	Olea europaea L. cv. Koroneiki	360, 700; 12	0, 100; 12	0 (n.s.)[a] 100 (−28) In the leaf	Melgar et al. (2008)

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Table 1 (continued)

Variable measured	Species	CO_2 (μmol CO_2 mol^{-1} air); time scale (weeks)	Salinity (mM NaCl); time scale (weeks)	Effect	Reference(s)
	Olea europaea L. cv. Koroneiki	360, 700; 12	0, 100; 12	0 (n.s.)[a] 100 (−18) In the root	Melgar et al. (2008)
	Olea europaea L. cv. Picual	360, 700; 12	0, 100; 12	0 (n.s.)[a] 100 (n.s.) In the leaf	Melgar et al. (2008)
	Olea europaea L. cv. Picual	360, 700; 12	0, 100; 12	0 (n.s.)[a] 100 (n.s.) In the root	Melgar et al. (2008)
	Hordeum vulgare L. cv. Alpha	350, 700; 4	0, 80, 160, 240; 2	0 (n.s.) 80 (n.s.) 160 (n.s.) 240 (n.s.) In the leaf	Pérez-López et al. (2010a)
	Hordeum vulgare L. cv. Alpha	350, 700; 4	0, 80, 160, 240; 2	0 (n.s.) 80 (n.s.) 160 (n.s.) 240 (n.s.) In the root	Pérez-López et al. (2010a)
	Hordeum vulgare L. cv. Iranis	350, 700; 4	0, 80, 160, 240; 2	0 (n.s.) 80 (n.s.) 160 (n.s.) 240 (n.s.) in the leaf	Pérez-López et al. (2010a)
	Hordeum vulgare L. cv. Iranis	350, 700; 4	0, 80, 160, 240; 2	0 (n.s.) 80 (n.s.) 160 (n.s.) 240 (n.s.) in the root	Pérez-López et al. (2010a)

Cl^- content	*Lycopersicon esculentum* L.	400, 900; 4	14 levels of salinity; 4	0 (n.s.)[a] 40 (−38) 80 (−29) 120 (−27) In the leaf	Maggio et al. (2002)
	Cleopatra mandarin	360, 700; 8	0, 50; 8	0 (n.s.)[a] 50 (n.s.) In adult leaf	García-Sánchez and Syvertsen (2006)
	Cleopatra mandarin	360, 700; 8	0, 50; 8	0 (n.s.)[a] 50 (−23) in main root	García-Sánchez and Syvertsen (2006)
	Carrizo citrange	360, 700; 8	0, 50; 8	0 (n.s.)[a] 50 (−28) In adult leaf	García-Sánchez and Syvertsen (2006)
	Carrizo citrange	360, 700; 8	0, 50; 8	0 (n.s.)[a] 50 (n.s.) In main root	García-Sánchez and Syvertsen (2006)
	Olea europaea L. cv. Koroneiki	360, 700; 12	0, 100; 12	0 (n.s.)[a] 100 (n.s.) In the leaf	Melgar et al. (2008)
	Olea europaea L. cv. Koroneiki	360, 700; 12	0, 100; 12	0 (n.s.)[a] 100 (−22) In the root	Melgar et al. (2008)
	Olea europaea L. cv. Picual	360, 700; 12	0, 100; 12	0 (n.s.)[a] 100 (n.s.) in the leaf	Melgar et al. (2008)
	Olea europaea L. cv. Picual	360, 700; 12	0, 100; 12	0 (n.s.)[a] 100 (n.s.) in the root	Melgar et al. (2008)
	Hordeum vulgare L. cv. Alpha	350, 700; 4	0, 80, 160, 240; 2	0 (n.s.) 80 (n.s.) 160 (n.s.) 240 (n.s.) in the leaf	Pérez-López et al. (2010a)

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Table 1 (continued)

Variable measured	Species	CO_2 (μmol CO_2 mol^{-1} air); time scale (weeks)	Salinity (mM NaCl); time scale (weeks)	Effect	Reference(s)
	Hordeum vulgare L. cv. Alpha	350, 700; 4	0, 80, 160, 240; 2	0 (n.s.) 80 (n.s.) 160 (−20) 240 (−21) in the root	Pérez-López et al. (2010a)
	Hordeum vulgare L. cv. Iranis	350, 700; 4	0, 80, 160, 240; 2	0 (−24) 80 (−24) 160 (−6) 240 (−19) in the leaf	Pérez-López et al. (2010a)
	Hordeum vulgare L. cv. Iranis	350, 700; 4	0, 80, 160, 240; 2	0 (+50) 80 (−14) 160 (−25) 240 (−23) in the root	Pérez-López et al. (2010a)
p-ATPase[c]	*Aster tripolium* L.	370, 520; 5	0, 375; 4	0 (n.s.)[a] 375 (+13)	Geissler et al. (2010)
f-ATPase[d]	*Aster tripolium* L.	370, 520; 5	0, 375; 4	0 (n.s.)[a] 375 (+14)	Geissler et al. (2010)
v-ATPase[e]	*Aster tripolium* L.	370, 520; 5	0, 375; 4	0 (n.s.)[a] 375 (+14)	Geissler et al. (2010)
Dark respiration rate	*Solanum muricatum* Ait.	350, 700; 4	0, 25; 8	0 (−15) 25 (−19)	Chen et al. (1999b)
	Solanum muricatum Ait.	350, 1,050; 4	0, 25; 8	0 (−40) 25 (−58)	Chen et al. (1999b)
	Aster tripolium L.	370, 520; 5	0, 250, 500; 4	0 (−13) 250 (+137) 500 (+31)	Geissler et al. (2009b)
	Hordeum vulgare L. cv. Alpha	350, 700; 4	0, 80, 160, 240; 2	0 (n.s.) 80 (n.s.) 160 (n.s.) 240 (n.s.)	Pérez-López et al. (unpublished data)

	Hordeum vulgare L. cv. Iranis	350, 700; 4	0, 80, 160, 240; 2	0 (n.s.) 80 (n.s.) 160 (n.s.) 240 (+44)	Pérez-López et al. (unpublished data)
Light respiration rate	*Hordeum vulgare* L. cv. Alpha	350, 700; 4	0, 80, 160, 240; 2	0 (+91) 80 (+76) 160 (+23) 240 (n.s.)	Pérez-López et al. (unpublished data)
	Hordeum vulgare L. cv. Iranis	350, 700; 4	0, 80, 160, 240; 2	0 (+29) 80 (+35) 160 (+57) 240 (+21)	Pérez-López et al. (unpublished data)
Transpiration rate	*Solanum muricatum* Ait.	350, 700; 4	0, 25; 8	0 (−6) 25 (−7)	Chen et al. (1999b)
	Solanum muricatum Ait.	350, 1,050; 4	0, 25; 8	0 (−23) 25 (−22)	Chen et al. (1999b)
	Lycopersicon esculentum L.	400, 900; 4	14 levels of salinity; 4	0 (−13)[a] 40 (−29) 80 (−38) 120 (−49)	Maggio et al. (2002)
	Aster tripolium L.	370, 520; 5	0, 250, 500; 4	0 (+51) 250 (+60) 500 (+39)	Geissler et al. (2009a)
	Cleopatra mandarin	360, 700; 8	0, 50; 8	0 (−32)[a] 50 (−35)	García-Sánchez and Syvertsen (2006)
	Carrizo citrange	360, 700; 8	0, 50; 8	0 (−40)[a] 50 (−30)	García-Sánchez and Syvertsen (2006)
	Olea europaea L. cv. Koroneiki	360, 700; 12	0, 100; 12	0 (−21)[a] 100 (−42)	Melgar et al. (2008)
	Olea europaea L. cv. Picual	360, 700; 12	0, 100; 12	0 (−27)[a] 100 (−33)	Melgar et al. (2008)

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Table 1 (continued)

Variable measured	Species	CO_2 (μmol CO_2 mol^{-1} air); time scale (weeks)	Salinity (mM NaCl); time scale (weeks)	Effect	Reference(s)
	Hordeum vulgare L. cv. Alpha	350, 700; 4	0, 80, 160, 240; 2	0 (−51) 80 (−38) 160 (−33) 240 (−51)	Pérez-López et al. (2009a)
	Hordeum vulgare L. cv. Iranis	350, 700; 4	0, 80, 160, 240; 2	0 (−32) 80 (−30) 160 (−25) 240 (−36)	Pérez-López et al. (2009a)
Total transpiration	*Lycopersicon esculentum* L.	400, 900; 4	14 levels of salinity; 4	0 (−39)[a] 40 (−43) 80 (−46) 120 (−63)	Maggio et al. (2002)
	Hordeum vulgare L. cv. Alpha	350, 700; 4	0, 80, 160, 240; 2	0 (−14) 80 (n.s.) 160 (−14) 240 (−11)	Pérez-López et al. (2009a)
	Hordeum vulgare L. cv. Iranis	350, 700; 4	0, 80, 160, 240; 2	0 (−11) 80 (n.s.) 160 (−11) 240 (n.s.)	Pérez-López et al. (2009a)
Stomatal conductance	*Solanum muricatum* Ait.	350, 700; 4	0, 25; 8	0 (−15) 25 (−19)	Chen et al. (1999b)
	Solanum muricatum Ait.	350, 1,050; 4	0, 25; 8	0 (−28) 25 (−29)	Chen et al. (1999b)
	Cucumis melo L.	400, 800 for the first 5 h of the day; 20	0, 25, 50; 20	0 (−12) 25 (−15) 50 (n.s.)	Mavrogianopoulos et al. (1999)
	Cucumis melo L.	400, 1,200 for the first 5 h of the day; 20	0, 25, 50; 20	0 (−10) 25 (n.s.) 50 (+26)	Mavrogianopoulos et al. (1999)

Lycopersicon esculentum L.	370, 1,000; 1	0, 100; 1	0 (−28)[a] 100 (+56)	Takagi et al. (2009)
Lycopersicon esculentum L.	370, 1,000; 2	0, 100; 2	0 (n.s.)[a] 100 (n.s.)	Takagi et al. (2009)
Aster tripolium L.	370, 520; 5	0, 250, 500; 4	0 (+43) 250 (+45) 500 (+40)	Geissler et al. (2009a)
Spartina densiflora	380, 700; 12	0, 171, 510; 12	0 (−30)[a] 171 (−30) 510 (n.s.)	Mateos-Naranjo et al. (2010)
Cleopatra mandarin	360, 700; 8	0, 50; 8	0 (−20) 50 (+28)	García-Sánchez and Syvertsen (2006)
Carrizo citrange	360, 700; 8	0, 50; 8	0 (−20) 50 (+26)	García-Sánchez and Syvertsen (2006)
Olea europaea L. cv. Koroneiki	360, 700; 12	0, 100; 12	0 (n.s.) 100 (−28)	Melgar et al. (2008)
Olea europaea L. cv. Picual	360, 700; 12	0, 100; 12	0 (−33) 100 (−30)	Melgar et al. (2008)
Hordeum vulgare L. cv. Alpha	350, 700; 4	0, 80, 160, 240; 2	0 (−53) 80 (−44) 160 (−46) 240 (−50)	Pérez-López et al. (2008)
Hordeum vulgare L. cv. Iranis	350, 700; 4	0, 80, 160, 240; 2	0 (−34) 80 (−32) 160 (−22) 240 (−30)	Pérez-López et al. (2008)

Effects are expressed as percentage increase (+) or decrease (−) resulting from increases in atmospheric CO_2 concentration at various soil salinity values
n.s. refers to nonsignificant
[a]Results have been obtained from the figures given in the original cited articles, so some of them could be not accurately determined
[b]In the paper of Li et al. (1999b) the data of dSm^{-1} were transformed to mM using the following equation, $dSm^{-1} = 0.097\ mM + 1.87$.
[c]Plasma-membrane ATPase
[d]Mitochondrial ATPase
[e]Vacuolar ATPase

of inorganic and organic solutes (Hasegawa et al. 2000). Despite the fact that using inorganic solutes requires less energy, both processes require extra ATP (the cost of salt tolerance; Munns 2002) and work together; the accumulation of compatible organic solutes in the cytoplasm balances the differential potential between vacuoles and the rest of the cell. In *A. tripolium*, the contribution made by DH and OA cannot be calculated, but when the results of the two CO_2 concentrations in both cultivars of *H. vulgare* are compared, under elevated CO_2, osmotic potential is predominantly dependent on a greater OA while the increase of osmotic potential caused by dehydration is inferior. This higher OA probably occurs because under elevated CO_2 conditions, the availability of sugars and ATP required for the synthesis of organic osmolite is higher. In fact, in both *A. tripolium* (Geissler et al. 2009b) and *H. vulgare* (Pérez-López et al. 2010a), a significant interaction between CO_2 and salinity on soluble sugar content has been identified, with higher soluble sugars under elevated CO_2 and salt stress than under ambient CO_2 (Table 1).

Results from other groups have also supported the concept that a higher content of ions such as K^+, Na^+, and Cl^- could lead to a better OA. In *O. europaea* cv. Koroneiki (Melgar et al. 2008) and citrus rootstocks (García-Sánchez and Syvertsen 2006), differences in mineral content have been detected under different CO_2 concentrations studied; however, in other reviewed species, such as *H. vulgare* (Pérez-López et al. 2010a), *A. tripolium* (Geissler et al. 2009b), *S. densiflora* (Mateos-Naranjo et al. 2010), and *O. europaea* cv. Picual (Melgar et al. 2008), no interaction has been found between salt levels and CO_2 enrichment relative to either Na^+ or Cl^- absolute levels (Table 1). Despite this lack of interaction, under elevated CO_2 conditions, the availability of carbon skeletons for respiration, and thus for ATP production, is higher. This higher availability could make possible a more efficient compartmentalization of both Na^+ and Cl^- in the vacuole, favoring a greater OA and better ionic homeostasis in the cytoplasm at elevated CO_2 than at ambient CO_2 with the same levels of ions. In fact, Geissler et al. (2010) demonstrated that under the same salt treatment, *A. tripolium* plants grown under elevated CO_2 concentrations showed significantly higher ATPase activity than plants grown under ambient CO_2 (Table 1), reinforcing this hypothesis. In barley (Pérez-López et al. unpublished data), light respiration rate was also higher under elevated CO_2 than under ambient CO_2 (Table 1). Mechanisms such as ion compartmentalization in the vacuole, osmolite synthesis, and/or protein recycling are more active under saline conditions. These processes require high levels of ATP, and higher rates of light respiration registered under saline conditions would indicate that these mechanisms are more active. At elevated CO_2, a higher availability of C skeletons (Pérez-López et al. 2010a) would permit their use for respiration, with a subsequent increase in ATP production, which would allow more effective functioning of these mechanisms and a greater tolerance for salinity than under ambient CO_2.

Regarding dehydration, in barley (Pérez-López et al. 2010a), the lesser dehydration at elevated CO_2 could result from a more developed root system, which would enhance the absorption surface. However, this tendency has been detected only in barley but not in other species, so it cannot be taken as a general trend. A lower transpiration rate also could underlie the lower DH; the stomata remain more closed

at elevated CO_2 than at ambient CO_2 (Robredo et al. 2007, 2010). Except for *A. tripolium* (Geissler et al. 2009b) and citrus rootstocks (García-Sánchez and Syvertsen 2006), the other species exhibit the tendency to lower transpiration rate under combined conditions of salt stress and elevated CO_2 (Table 1). In fact, tomato (Maggio et al. 2002), barley (Pérez-López et al. 2009a), and olive (Melgar et al. 2008) show lower transpiration rates under salt stress and elevated CO_2 than under ambient CO_2, indicating an interaction between both variables; their lower transpiration rates are the result of more closed stomata. In fact, except for *A. tripolium*, all the other studied species are responsive to atmospheric CO_2 enrichment as the salinity of the soil solution rises, exhibiting lower stomatal conductance relative to ambient CO_2 conditions (Mavrogianopoulos et al. 1999; Maggio et al. 2002; Melgar et al. 2008; Pérez-López et al. 2009a; Table 1). We analyze the consequences of reduced stomatal conductance and its effect in photosynthetic metabolism in the next section.

4 Photosynthetic Metabolism and Growth Parameters

Under environmental CO_2 conditions, the stomatal closure provoked by salinity implies a lower diffusion of CO_2 to the inside of the leaf, which results in a decreased assimilation rate. On the contrary, when the environmental CO_2 concentrations are increased, although stomata tend to close, there is a higher driving force for the entrance of the CO_2 because the gradient of CO_2 between the outside and the inside of the leaf increases. In this case, the diffusion is usually higher and, for the same stomatal closure, the values of photosynthetic rate are generally higher. Because many physiological studies have related inhibition of plant growth by salinity to a reduction in photosynthesis (Munns 1993; Tattini et al. 2002; Muscolo et al. 2003), the study of the interaction between elevated CO_2 and salt stress on photosynthetic rate deserves attention. In all species reviewed, except for *S. densiflora* (Mateos-Naranjo et al. 2010), under elevated CO_2, the photosynthetic rate was higher than under ambient CO_2, despite a higher stomatal closure (Chen et al. 1999b; Mavrogianopoulos et al. 1999; García-Sánchez and Syvertsen 2006; Melgar et al. 2008; Pérez-López et al. 2008; Geissler et al. 2009b; Takagi et al. 2009; Table 2).

These photosynthetic rates, higher at elevated CO_2 than under ambient CO_2, could be the result of higher Ci in the former, provoked by the higher diffusion of CO_2 to the interior of the leaf. As Table 2 shows, the majority of species studied are responsive to atmospheric CO_2 enrichment as the salinity of the soil solution rises, showing higher Ci at elevated CO_2 than the Ci shown at ambient CO_2. This fact reinforces the hypothesis that the higher photosynthetic rates are in part caused by a higher gradient of CO_2 between the outside and the inside of the leaf, in spite of the stomatal closure provoked by elevated CO_2 concentration per se.

However, the higher photosynthetic rates of the leaves do not always develop in a higher biomass production. The higher photosynthetic rate at elevated vs. ambient CO_2 under salinity conditions has resulted in more biomass production in barley,

Table 2 Compilation of published data on the effects of CO_2 enrichment on photosynthetic metabolism and growth parameters

Variable measured	Species	CO_2 (μmol CO_2 mol^{-1} air); time scale (weeks)	Salinity (mM NaCl); time scale (weeks)	Effect	Reference(s)
Maximum photosynthetic rate	*Aster tripolium* L.	370, 520; 5	0, 250, 500; 4	0 (+56) 250 (+82) 500 (+71)	Geissler et al. (2009a)
Photosynthetic rate	*Solanum muricatum* Ait.	350, 700; 4	0, 25; 8	0 (+74) 25 (+98)	Chen et al. (1999b)
	Solanum muricatum Ait.	350, 1,050; 4	0, 25; 8	0 (+72) 25 (+90)	Chen et al. (1999b)
	Cucumis melo L.	400, 800 for the first 5 h of the day; 20	0, 25, 50; 20	0 (+75) 25 (+74) 50 (+77)	Mavrogianopoulos et al. (1999)
	Cucumis melo L.	400, 1,200 for the first 5 h of the day; 20	0, 25, 50; 20	0 (+112) 25 (+114) 50 (+134)	Mavrogianopoulos et al. (1999)
	Lycopersicon esculentum L.	370, 1,000; 1	0, 100; 1	0 (+15)[a] 100 (+29)	Takagi et al. (2009)
	Lycopersicon esculentum L.	370, 1,000; 2	0, 100; 2	0 (n.s.)[a] 100 (+25)	Takagi et al. (2009)
	Spartina densiflora	380, 700; 12	0, 171, 510; 12	0 (n.s.)[a] 171 (n.s.) 510 (n.s.)	Mateos-Naranjo et al. (2010)
	Cleopatra mandarin	360, 700; 8	0, 50; 8	0 (+66) 50 (+38)	García-Sánchez and Syvertsen (2006)
	Carrizo citrange	360, 700; 8	0, 50; 8	0 (+71) 50 (+108)	García-Sánchez and Syvertsen (2006)
	Olea europaea L. cv. Koroneiki	360, 700; 12	0, 100; 12	0 (+51) 100 (+19)	Melgar et al. (2008)
	Olea europaea L. cv. Picual	360, 700; 12	0, 100; 12	0 (+24) 100 (+54)	Melgar et al. (2008)

	Hordeum vulgare L. cv. Alpha	350, 700; 4	0, 80, 160, 240; 2	0 (+15) 80 (+33) 160 (+38) 240 (+43)	Pérez-López et al. (2008)
	Hordeum vulgare L. cv. Iranis	350, 700; 4	0, 80, 160, 240; 2	0 (+27) 80 (+47) 160 (+56) 240 (+40)	Pérez-López et al. (2008)
CO_2 concentration in the sub-stomatal cavity (Ci)	*Solanum muricatum* Ait.	350, 700; 4	0, 25; 8	0 (+102) 25 (+76)	Chen et al. (1999b)
	Solanum muricatum Ait.	350, 1,050; 4	0, 25; 8	0 (+276) 25 (+246)	Chen et al. (1999b)
	Cucumis melo L.	400, 800 for the first 5 h of the day; 20	0, 25, 50; 20	0 (+98) 25 (+99) 50 (+102)	Mavrogianopoulos et al. (1999)
	Cucumis melo L.	400, 1,200 for the first 5 h of the day; 20	0, 25, 50; 20	0 (+210) 25 (+213) 50 (+212)	Mavrogianopoulos et al. (1999)
	Lycopersicon esculentum L.	370, 1,000; 1	0, 100; 1	0 (+275)[a] 100 (+366)	Takagi et al. (2009)
	Lycopersicon esculentum L.	370, 1,000; 2	0, 100; 2	0 (+295)[a] 100 (+230)	Takagi et al. (2009)
	Aster tripolium L.	370, 520; 5	0, 250, 500; 4	0 (+51) 250 (+60) 500 (+39)	Geissler et al. (2009a)
	Spartina densiflora	380, 700; 12	0, 171, 510; 12	0 (+65)[a] 171 (+150) 510 (+130)	Mateos-Naranjo et al. (2010)
	Hordeum vulgare L. cv. Alpha	350, 700; 4	0, 80, 160, 240; 2	0 (+90) 80 (+97) 160 (+85) 240 (+71)	Pérez-López et al. (2008)

(continued)

Table 2 (continued)

Variable measured	Species	CO_2 (μmol CO_2 mol^{-1} air); time scale (weeks)	Salinity (mM NaCl); time scale (weeks)	Effect	Reference(s)
	Hordeum vulgare L. cv. Iranis	350, 700; 4	0, 80, 160, 240; 2	0 (+100) 80 (+98) 160 (+104) 240 (+92)	Pérez-López et al. (2008)
Total dry biomass	*Solanum muricatum* Ait.	350, 700; 4	0, 25; 8	0 (+48) 25 (+50)	Chen et al. (1999a)
	Solanum muricatum Ait.	350, 1,050; 4	0, 25; 8	0 (+80) 25 (+89)	Chen et al. (1999a)
	Lycopersicon esculentum L.	360, 1,200; 4	0, 16, 34, 53; 19	0 (n.s.)[a,b] 16 (+7) 34 (+41) 53 (+95)	Li et al. (1999a)
	Lycopersicon esculentum L.	370, 1,000; 1	0, 100; 1	0 (+15)[a] 100 (+30)	Takagi et al. (2009)
	Lycopersicon esculentum L.	370, 1,000; 2	0, 100; 2	0 (+20)[a] 100 (+68)	Takagi et al. (2009)
	Aster tripolium L.	370, 520; 5	0, 125, 250, 375, 500; 4	0 (n.s.)[a] 125 (n.s.) 250 (n.s.) 375 (n.s.) 500 (n.s.)	Geissler et al. (2009b)
	Spartina densiflora	380, 700; 12	0, 171, 510; 12	0 (+46)[a] 171 (+46) 510 (n.s.)	Mateos-Naranjo et al. (2010)
	Cleopatra mandarin	360, 700; 8	0, 50; 8	0 (+39) 50 (+27)	García-Sánchez and Syvertsen (2006)
	Carrizo citrange	360, 700; 8	0, 50; 8	0 (+43) 50 (+49)	García-Sánchez and Syvertsen (2006)
	Olea europaea L. cv. Koroneiki	360, 700; 12	0, 100; 12	0 (+37) 100 (n.s.)	Melgar et al. (2008)

	Olea europaea L. cv. Picual	360, 700; 12	0, 100; 12	0 (+37) 100 (n.s.)	Melgar et al. (2008)
	Hordeum vulgare L. cv. Alpha	350, 700; 4	0, 80, 160, 240; 2	0 (+17) 80 (+35) 160 (+35) 240 (+44)	Pérez-López et al. (unpublished data)
	Hordeum vulgare L. cv. Iranis	350, 700; 4	0, 80, 160, 240; 2	0 (+19) 80 (+24) 160 (+25) 240 (+34)	Pérez-López et al. (unpublished data)
Shoot dry biomass	*Cleopatra mandarin*	360, 700; 8	0, 50; 8	0 (+45) 50 (+27)	García-Sánchez and Syvertsen (2006)
	Carrizo citrange	360, 700; 8	0, 50; 8	0 (+43) 50 (+80)	García-Sánchez and Syvertsen (2006)
	Olea europaea L. cv. Koroneiki	360, 700; 12	0, 100; 12	0 (+45) 100 (n.s.)	Melgar et al. (2008)
	Olea europaea L. cv. Picual	360, 700; 12	0, 100; 12	0 (+37) 100 (n.s.)	Melgar et al. (2008)
	Hordeum vulgare L. cv. Alpha	350, 700; 4	0, 80, 160, 240; 2	0 (+17) 80 (+35) 160 (+35) 240 (+44)	Pérez-López et al. (unpublished data)
	Hordeum vulgare L. cv. Iranis	350, 700; 4	0, 80, 160, 240; 2	0 (+19) 80 (+24) 160 (+25) 240 (+34)	Pérez-López et al. (unpublished data)
Root dry biomass	*Cleopatra mandarin*	360, 700; 8	0, 50; 8	0 (+26) 50 (+31)	García-Sánchez and Syvertsen (2006)
	Carrizo citrange	360, 700; 8	0, 50; 8	0 (+18) 50 (n.s.)	García-Sánchez and Syvertsen (2006)
	Olea europaea L. cv. Koroneiki	360, 700; 12	0, 100; 12	0 (n.s.) 100 (n.s.)	Melgar et al. (2008)
	Olea europaea L. cv. Picual	360, 700; 12	0, 100; 12	0 (n.s.) 100 (n.s.)	Melgar et al. (2008)

(continued)

Table 2 (continued)

Variable measured	Species	CO_2 (μmol CO_2 mol^{-1} air); time scale (weeks)	Salinity (mM NaCl); time scale (weeks)	Effect	Reference(s)
	Hordeum vulgare L. cv. Alpha	350, 700; 4	0, 80, 160, 240; 2	0 (+13) 80 (+23) 160 (+31) 240 (+42)	Pérez-López et al. (unpublished data)
	Hordeum vulgare L. cv. Iranis	350, 700; 4	0, 80, 160, 240; 2	0 (+9) 80 (+8) 160 (+9) 240 (+25)	Pérez-López et al. (unpublished data)
Total leaf area	*Lycopersicon esculentum* L.	360, 1,200; 4	0, 16, 34, 53; 19	0 (−35)[a, b] 16 (−25) 34 (−15) 53 (+56)	Li et al. (1999a)
	Spartina densiflora	380, 700; 12	0, 171, 510; 12	0 (+50)[a] 171 (+154) 510 (+50)	Mateos-Naranjo et al. (2010)
	Olea europaea L. cv. Koroneiki	360, 700; 12	0, 100; 12	0 (+34) 100 (n.s.)	Melgar et al. (2008)
	Olea europaea L. cv. Picual	360, 700; 12	0, 100; 12	0 (+21) 100 (n.s.)	Melgar et al. (2008)
	Hordeum vulgare L. cv. Alpha	350, 700; 4	0, 80, 160, 240; 2	0 (+26) 80 (+41) 160 (+37) 240 (+38)	Pérez-López et al. (2009a)
	Hordeum vulgare L. cv. Iranis	350, 700; 4	0, 80, 160, 240; 2	0 (+20) 80 (+30) 160 (+31) 240 (+38)	Pérez-López et al. (2009a)
Water use efficiency	*Solanum muricatum* Ait.	350, 700; 4	0, 25; 8	0 (+84) 25 (+112)	Chen et al. (1999b)

Solanum muricatum Ait.	350, 1,050; 4	0, 25; 8	0 (+123) 25 (+147)	Chen et al. (1999b)
Lycopersicon esculentum L.	400, 900; 4	14 levels of salinity; 4	0 (+39)[a] 40 (+41) 80 (+119) 120 (+157)	Maggio et al. (2002)
Aster tripolium L.	370, 520; 5	0, 250, 500; 4	0 (+13) 250 (+26) 500 (+60)	Geissler et al. (2009b)
Spartina densiflora	380, 700; 12	0, 171, 510; 12	0 (+44)[a] 171 (+19) 510 (+21)	Mateos-Naranjo et al. (2010)
Cleopatra mandarin	360, 700; 8	0, 50; 8	0 (+70) 50 (+38)	García-Sánchez and Syvertsen (2006)
Carrizo citrange	360, 700; 8	0, 50; 8	0 (+67) 50 (+99)	García-Sánchez and Syvertsen (2006)
Olea europaea L. cv. Koroneiki	360, 700; 12	0, 100; 12	0 (+39) 100 (+41)	Melgar et al. (2008)
Olea europaea L. cv. Picual	360, 700; 12	0, 100; 12	0 (+38) 100 (+78)	Melgar et al. (2008)
Hordeum vulgare L. cv. Alpha	350, 700; 4	0, 80, 160, 240; 2	0 (+35) 80 (+48) 160 (+56) 240 (+61)	Pérez-López et al. (2009a)
Hordeum vulgare L. cv. Iranis	350, 700; 4	0, 80, 160, 240; 2	0 (+34) 80 (+26) 160 (+41) 240 (+43)	Pérez-López et al. (2009a)

Effects are expressed as percentage increase (+) or decrease (−) resulting from increases in atmospheric CO_2 concentration at various specific values of soil salinity

n.s. refers to nonsignificant

[a]Results have been obtained from the figures given in the original cited articles, so some of them could be not accurately determined

[b]In the paper of Li et al. (1999a) the data of dSm^{-1} were transformed to mM using the following equation, $dSm^{-1} = 0.097\ mM + 1.87$

tomato, pepino, and citrus rootstocks but not in *O. europaea* (Melgar et al. 2008) and *A. tripolium* (Geissler et al. 2009b). These last two species showed higher photosynthetic rates under salt stress and elevated CO_2 than at ambient CO_2, but they did not exhibit higher biomass production. What is happening in between these two processes? First of all, we should take a deeper look at what is going with the photosynthetic metabolism. The lower photosynthetic rate in plants subjected to salinity, more under ambient CO_2 than under elevated CO_2, results in generation of more ATP and NADPH than the plant can consume in the reduction of CO_2 due to the fact that salinity causes a lower activity of the Calvin cycle, owing to the lack of CO_2 and biochemical limitations, provoking a diminution in the rate of turnover between NADPH and $NADP^+$. This imbalance leads to an overreduction of the electron transport chain (ETC), diverting the electrons to O_2 with the subsequent production of radical oxygen species (ROS). Although the ROS produced in the presence of NaCl and other stressors play a role as signal molecules and in cross-tolerance mechanisms, they could also cause oxidative stress (Sgherri et al. 1996; Dionisio-Sese and Tobita 1998; Meneguzzo et al. 1999; Pérez-López et al. 2009b). Consequently, it is necessary a close relationship between the generation of NADPH and ATP -through the thylakoid ETC- and its use by Calvin cycle and other metabolic pathways to reduce the damage. Plants have different mechanisms to achieve this, including increased photorespiratory rate, nonphotochemical dissipation, or Mehler reaction, and/or a decreased thylakoid electron transport rate (ETR) (Noctor and Foyer 1998; Hernández et al. 2000). However, these mechanisms are not necessarily energetically free. Thus, the imbalance between the enhancement of photosynthetic rate and the constancy in biomass production (Table 2) could result from the increased energy consumption (traduced in a higher respiration rate) for these salt-tolerance mechanisms.

5 Salt-Tolerance Mechanisms

Salt-tolerance mechanisms are quite broad in nature; so, we focus here on those that have been studied under the interaction of elevated CO_2 and salt stress. Geissler et al. (2009a) found that under salt stress, the overreduction in the photosynthetic ETC due to the energy surplus of the photosystem was counteracted by thicker outer epidermal cells and cuticle and wider cuticle ridges. They also found that these structural changes were more frequent under elevated CO_2 than under ambient CO_2. On the other hand, the reduced chlorophyll content of the leaves can fulfill a similar function (less energy conduction by the light-harvesting complex). In *O. europaea* (Melgar et al. 2008) and *S. densiflora* (Mateos-Naranjo et al. 2010), CO_2 enrichment and salt stress interact, yielding lower chlorophyll content under elevated CO_2 than under ambient CO_2 (Table 3), reinforcing the hypothesis that under elevated CO_2 the overreduction of the ETC is lessened compared to the conditions under ambient CO_2.

Regarding antioxidant metabolism, among different species, there is an induction of the antioxidant system under saline conditions and in response to the

Table 3 Compilation of published data on the effects of CO_2 enrichment on other salt-tolerance mechanisms

Variable measured	Species	CO_2 (μmol CO_2 mol^{-1} air); time scale (weeks)	Salinity (mM NaCl); time scale (weeks)	Effect	Reference(s)
Total chlorophyll content	*Solanum muricatum* Ait.	350, 700; 4	0, 25; 8	0 (−12) 25 (−16)	Chen et al. (1999b)
	Solanum muricatum Ait.	350, 1,050; 4	0, 25; 8	0 (−25) 25 (−28)	Chen et al. (1999b)
	Cucumis melo L.	400, 800 for the first 5 h of the day; 20	0, 25, 50; 20	0 (+28) 25 (+19) 50 (+27)	Mavrogianopoulos et al. (1999)
	Cucumis melo L.	400, 1,200 for the first 5 h of the day; 20	0, 25, 50; 20	0 (+27) 25 (+25) 50 (+33)	Mavrogianopoulos et al. (1999)
	Aster tripolium L.	370, 520; 5	0, 250, 500; 4	0 (n.s.)[a] 250 (n.s.) 500 (n.s.)	Geissler et al. (2009a)
	Cleopatra mandarin	360, 700; 8	0, 50; 8	0 (+38) 50 (n.s.)	García-Sánchez and Syvertsen (2006)
	Carrizo citrange	360, 700; 8	0, 50; 8	0 (+14) 50 (n.s.)	García-Sánchez and Syvertsen (2006)
	Olea europaea L. cv. Koroneiki	360, 700; 12	0, 100; 12	0 (−13) 100 (−26)	Melgar et al. (2008)
	Olea europaea L. cv. Picual	360, 700; 12	0, 100; 12	0 (n.s.) 100 (−24)	Melgar et al. (2008)
Chlorophyll-*a*	*Spartina densiflora*	380, 700; 12	0, 171, 510; 12	0 (n.s.)[a] 171 (−45) 510 (−33)	Mateos-Naranjo et al. (2010)
	Hordeum vulgare L. cv. Alpha	350, 700; 4	0, 80, 160, 240; 2	0 (−17) 80 (−12) 160 (n.s.) 240 (n.s.)	Pérez-López et al. (2008)

(continued)

Table 3 (continued)

Variable measured	Species	CO_2 (μmol CO_2 mol^{-1} air); time scale (weeks)	Salinity (mM NaCl); time scale (weeks)	Effect	Reference(s)
	Hordeum vulgare L. cv. Iranis	350, 700; 4	0, 80, 160, 240; 2	0 (−17) 80 (−13) 160 (n.s.) 240 (n.s.)	Pérez-López et al. (2008)
Superoxide dismutase (SOD) activity	*Aster tripolium* L.	370, 520; 5	0, 375; 4	0 (−10)[a] 375 (+56)	Geissler et al. (2009a; 2010)
	Hordeum vulgare L. cv. Alpha	350, 700; 4	0, 80, 160, 240; 2	0 (+20) 80 (n.s.) 160 (n.s.) 240 (n.s.)	Pérez-López et al. (2009b)
	Hordeum vulgare L. cv. Iranis	350, 700; 4	0, 80, 160, 240; 2	0 (n.s.) 80 (−10) 160 (−16) 240 (n.s.)	Pérez-López et al. (2009b)
Ascorbate peroxidase (APX) activity	*Lycopersicon esculentum* L.	370, 1,000; 1	0, 100; 1	0 (+15)[a] 100 (+30)	Takagi et al. (2009)
	Lycopersicon esculentum L.	370, 1,000; 2	0, 100; 2	0 (+20)[a] 100 (+68)	Takagi et al. (2009)
	Aster tripolium L.	370, 520; 5	0, 375; 4	0 (n.s.)[a] 375 (+57)	Geissler et al. (2010)
	Hordeum vulgare L. cv. Alpha	350, 700; 4	0, 80, 160, 240; 2	0 (n.s.) 80 (n.s.) 160 (−25) 240 (−15)	Pérez-López et al. (2009b)
	Hordeum vulgare L. cv. Iranis	350, 700; 4	0, 80, 160, 240; 2	0 (n.s.) 80 (n.s.) 160 (n.s.) 240 (n.s.)	Pérez-López et al. (2009b)

Catalase (CAT) activity	*Lycopersicon esculentum* L.	370, 1,000; 1	0, 100; 1	0 (n.s.)[a] 100 (−73)	Takagi et al. (2009)
	Lycopersicon esculentum L.	370, 1,000; 2	0, 100; 2	0 (−51)[a] 100 (−59)	Takagi et al. (2009)
	Hordeum vulgare L. cv. Alpha	350, 700; 4	0, 80, 160, 240; 2	0 (+12) 80 (+7) 160 (−15) 240 (−10)	Pérez-López et al. (2009b)
	Hordeum vulgare L. cv. Iranis	350, 700; 4	0, 80, 160, 240; 2	0 (n.s.) 80 (n.s.) 160 (n.s.) 240 (n.s.)	Pérez-López et al. (2009b)
Ascorbate content	*Hordeum vulgare* L. cv. Alpha	350, 700; 4	0, 80, 160, 240; 2	0 (−10) 80 (−28) 160 (−25) 240 (n.s.)	Pérez-López et al. (2010b)
	Hordeum vulgare L. cv. Iranis	350, 700; 4	0, 80, 160, 240; 2	0 (−14) 80 (−27) 160 (−26) 240 (−28)	Pérez-López et al. (2010b)
Solute leakage	*Hordeum vulgare* L. cv. Alpha	350, 700; 4	0, 80, 160, 240; 2	0 (n.s.) 80 (n.s.) 160 (−16) 240 (−25)	Pérez-López et al. (2009b)
	Hordeum vulgare L. cv. Iranis	350, 700; 4	0, 80, 160, 240; 2	0 (n.s.) 80 (−11) 160 (−23) 240 (−23)	Pérez-López et al. (2009b)
Nonphotochemical quenching	*Solanum muricatum* Ait.	350, 700; 4	0, 25; 8	0 (−25) 25 (−41)	Chen et al. (1999b)
	Solanum muricatum Ait.	350, 1,050; 4	0, 25; 8	0 (−18) 25 (−25)	Chen et al. (1999b)

(continued)

Table 3 (continued)

Variable measured	Species	CO_2 (μmol CO_2 mol^{-1} air); time scale (weeks)	Salinity (mM NaCl); time scale (weeks)	Effect	Reference(s)
	Olea europaea L. cv. Koroneiki	360, 700; 12	0, 100; 12	0 (+25) 100 (+37)	Melgar et al. (2008)
	Olea europaea L. cv. Picual	360, 700; 12	0, 100; 12	0 (+13) 100 (+24)	Melgar et al. (2008)
	Hordeum vulgare L. cv. Alpha	350, 700; 4	0, 80, 160, 240; 2	0 (n.s.) 80 (−26) 160 (−45) 240 (−41)	Pérez-López et al. (unpublished data)
	Hordeum vulgare L. cv. Iranis	350, 700; 4	0, 80, 160, 240; 2	0 (n.s.) 80 (−24) 160 (−20) 240 (n.s.)	Pérez-López et al. (unpublished data)

Effects are expressed as percentage increase (+) or decrease (−) resulting from increases in atmospheric CO_2 concentration at various specific values of soil salinity

n.s. refers to nonsignificant

[a]Results have been obtained from the figures given in the original cited articles, so some of them could be not accurately determined

imbalance between the supply and demand of NADPH and the possible generation of ROS. In the case of *A. tripolium* (Geissler et al. 2009a, 2010), the plants seemed to use more energy supply under elevated CO_2 than under ambient CO_2 concentrations for increasing the investment in antioxidant metabolism. In this case, the SOD and APX activities resulting from salt treatment were significantly enhanced under elevated CO_2 as compared with their counterparts under ambient CO_2 (Table 3), so that ROS could be scavenged more effectively. However, in *H. vulgare* (Pérez-López et al. 2009b, 2010b), although salinity caused lessened oxidative damage, measured as solute leakage (Table 3), a reduced upregulation of the antioxidant system at elevated CO_2 compared to ambient CO_2 was observed. This trend was also detected in tomato for CAT activity (Takagi et al. 2009). These findings suggest that in both barley and tomato under these combined conditions, the lower oxidative stress generated by salinity is a consequence of a reduced production of ROS, more probably because of superior photosynthetic rates than because of effective scavenging of them.

The nonphotochemical dissipation of energy also showed different trends depending on the species analyzed. In *O. europaea* (Melgar et al. 2008), nonphotochemical quenching coefficient values were higher under elevated CO_2 and salt stress than under ambient CO_2, indicating a greater dissipation of energy under elevated CO_2, while in *H. vulgare* (Pérez-López et al. unpublished data), the nonphotochemical quenching coefficient values were lower under elevated CO_2 than under ambient CO_2 (Table 3), as occurred with the antioxidant system.

6 Final Conclusions

From these results and trends, we can conclude that under elevated CO_2 concentrations in the surrounding atmosphere, each plant species follows a species-specific strategy to cope with salt stress. However, most of these strategies rely on mechanisms related to active regulation of (1) water potential and stomatal conductance, (2) photosynthetic metabolism as a whole, and (3) other salt-tolerance mechanisms. In fact, we have seen that under salt stress, some plant species (1) perform active OA by accumulating sugars and/or by compartmentalizing ions in the vacuoles, (2) regulate their dehydration status by decreasing stomatal conductance, or (3) regulate the balance between photosynthetic rate and oxidative stress by different mechanisms such as avoiding overreduction of the ETC or scavenging ROS.

The key point is that under elevated CO_2, all of these mechanisms seem to be favored because the higher CO_2/O_2 ratio around the leaf favors CO_2 diffusion with lower stomatal conductance, which increases Ci and consequently photosynthetic rate. The higher photosynthetic rate and lower stomatal conductance imply (1) a higher availability of sugars and ATP for an active OA, (2) a lower transpiration rate, (3) a lower risk for oxidative stress, and (4) a greater availability of proteins and other constituents to create more effective antioxidant networks. However, the other key point is that most of these mechanisms that elevated CO_2 could exacerbate are

energetically expensive. For this reason, in some species, a higher photosynthetic rate or better water potential do not always correlate with a higher biomass production.

In fact, we see in *A. tripolium* that the biomass production is similar under both CO_2 concentrations, even though this species shows a less negative water potential and higher photosynthetic rates under salt stress and elevated CO_2 than under ambient CO_2. The lack of change in biomass probably results from its high investment in antioxidant metabolism and other tolerance mechanisms, increasing respiration rate, and the inability to control stomatal conductance. On the other hand, both tomato and barley, for example, show less negative water potential and higher photosynthetic rates under salt stress and elevated CO_2 than under ambient CO_2, which results in a greater biomass production under elevated CO_2; this increase probably relates to the fact that these species use less expensive tolerance mechanisms and can better control their transpiration rate, achieving higher water use efficiency values.

Thus, the interaction between salt stress and elevated CO_2 is based on the intrinsic characteristics of each species and seems to result from (1) a compromise between the photosynthetic and respiration rates (cost of salt tolerance) and (2) the ability to control stomatal conductance. However, further studies will be needed to analyze other important aspects of plant physiology, such as the response of the photosynthetic enzymes, nitrogen metabolism, and other salt-tolerance mechanisms, and the cost of salt tolerance.

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Mechanisms of Cd Hyperaccumulation and Detoxification in Heavy Metal Hyperaccumulators: How Plants Cope with Cd

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Abstract Cadmium (Cd) is classified as a toxic heavy metal and is of major concern in environmental heavy metals contamination. Some special plants, referring to Cd hyperaccumulators, could accumulate $\geq$100 mg kg^{-1} Cd in their shoots without showing phytotoxicity. The mechanisms of Cd accumulation and tolerance in Cd hyperaccumulators have provoked the interest of scientists. In the latest decade, many efforts have been put in investigating the biomolecular basis of Cd hyperaccumulation and associated Cd hypertolerance, including some important heavy metal transporters correlated to Cd accumulation and ion homeostasis in plants. This review provides an overview of the main aspects involving Cd uptake, translocation, distribution in hyperaccumulators, and the evolution of Cd hyperaccumulation. Besides, the hypothesis about the physiological role of Cd may play in hyperaccumulators is also highlighted.

Abbreviations

ADP	Adenosine diphosphate
ATP	Adenosine-5′-triphosphate
ATPase	Adenosine triphosphatase
ASA	Ascorbate
APX	Ascorbate peroxidase
ABC	ATP-binding cassette
BSO	Buthionine sulfoximine
Cd	Cadmium
CAT	Catalase
CA	Carbonic anhydrase
CDF	Cation diffusion facilitator
CAXs	Cation/H^+ exchangers
DW	Dry weight
EDX	Energy-dispersive X-ray spectrometer
FW	Fresh weight
FBPase	Fructose 1,6-bisphosphatase
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GPX	Guaiacol peroxidase
GSH	Glutathione
GR	Glutathione reductase
HMA4	Heavy metal-transporting ATPase4
HMW	High molecular weight
HE	Hyperaccumulating ecotype
Pb	Lead
LFDI	Leaf feeding damage index
LMW	Low molecular weight
MDA	Malondialdehyde
MTP	Microsomal triglyceride transfer protein

NRAMP	Natural resistance-associated macrophage protein
NADH	Nicotinamide adenine dinucleotide
NADPH	Nicotinamide adenine dinucleotide phosphate
Ni	Nickel
NHE	Non-hyperaccumulating ecotype
POD	Peroxidase
PCs	Phytochelatins
Put	Putrescine
QTL	Quantitative trait locus
ROS	Reactive oxygen species
RuBP	Ribulose-1,5-bisphosphate
Rubisco	Ribulose-1,5-bisphosphate carboxylase-oxygenase
Se	Selenium
Spd	Spermidine
Spm	Spermine
SOD	Superoxide dismutase
ZIP	ZRT1/IRT1-like protein
Zn	Zinc

1 Introduction

Cadmium (Cd) contamination is of considerable concern worldwide due to its potential toxicity to various organisms. In spite of its non-essentiality, Cd is readily taken up by many plants and thus poses adverse effects on plant growth and development mainly by disturbing enzyme activities (Prasad 1995), generating oxidative stress (Schutzendubel et al. 2001), and interfering with water balance and nutrient uptake (Das et al. 1997; Prasad 1995).

Higher plants have developed two principal strategies to resist the toxicity of Cd: avoidance and accumulation (Baker 1987). Most tolerant species are often excluders, limiting the entry and root-to-shoot translocation of Cd, while few others accumulate Cd to an extraordinarily high level without visual toxicity symptoms. Brooks et al. (1977) first coined the term "hyperaccumulators" to refer to the plants that contain at least 1,000 mg kg^{-1} nickel (Ni) in their dry leaves. The term was then extended to describe plants that can accumulate 100 mg kg^{-1} Cd; 1,000 mg kg^{-1} arsenic, cobalt, copper, lead (Pb), and selenium (Se); and 10,000 mg kg^{-1} manganese and zinc (Zn) in their aerial parts (Baker and Brooks 1989). Although the arbitrary threshold for defining metal hyperaccumulation has been criticized for years, the listed values have received wide acceptance.

Metal hyperaccumulation is not a common feature in terrestrial higher plants. So far, a total of approximately 500 plant taxa (~ 0.2% of all angiosperms) worldwide have been identified as heavy metal hyperaccumulators, the majority of them being Ni hyperaccumulators (nearly 75%) (Krämer 2010). In comparison, the list of Cd hyperaccumulators is much shorter, but in recent years there is an increasing trend

in the discovery of new species using various screening methods, including field surveys in Pb/Zn mining and smelting areas or naturally mineralized soils (in particular, calamine), pot experiments with spiked Cd in soils, and hydroponic culture. According to the recent studies, we summarize the Cd hyperaccumulators discovered to date, half of which were identified in the past five years (Table 1).

Cadmium hyperaccumulators are currently found in 11 different families, with the highest occurrence among the Brassicaceae (43%), followed by Asteraceae (14%), while the remaining species distribute almost equally in various families. Among them, *Thlaspi caerulescens*, the best known Cd hyperaccumulator, has been considered an interesting model plant for investigating the underlying mechanisms of metal hyperaccumulation and homeostasis (Assunção et al. 2003). *Arabidopsis halleri*, another recommended model metal hyperaccumulator (Roosens et al. 2008), also attracts many studies because of its close relatedness to the model plant *Arabidopsis thaliana* (Weber et al. 2004). Apart from the Brassicaceae family, *Sedum alfredii*, a Crassulaceae, has received a lot of attention in recent years as this species may provide additional basic knowledge of Cd hyperaccumulation. Among the Cd hyperaccumulators identified, more than half of them are also shown to have the ability to hyperaccumulate another heavy metal simultaneously, such as Zn, Pb, or Ni (Table 1). In particular, *Arabis paniculata*, *T. caerulescens*, and *Thlaspi praecox* are able to hyperaccumulate triple metals.

During the past decades, Cd hyperaccumulation has attracted great attention because this property may be exploited in the remediation of Cd-contaminated soils (Chaney et al. 1997; Mcnair 2003). However, a prerequisite for the feasible application using Cd hyperaccumulators is to gain a better understanding of the mechanisms involved in Cd accumulation and tolerance in these exceptional species. In this review, we aim to summarize our current knowledge of the key processes of Cd uptake, translocation, storage, and detoxification in Cd hyperaccumulators. Meanwhile, hypotheses associated with the adaptive and ecological significance of Cd hyperaccumulation are also discussed to explore how this trait has evolved.

2 Cd Accumulation in Hyperaccumulators

2.1 Rhizosphere Mobilization and Root Uptake

There are three mechanisms by which hyperaccumulator plants most likely obtain Cd from the soil: (1) rhizospheric and microbial mobilization of Cd; (2) active foraging for Cd, by means of enhanced root growth and root branching in regions of Cd enrichment; and (3) effective transporters facilitating Cd uptake from soil to roots in hyperaccumulating plants (Whiting et al. 2000).

Table 1 Cd hyperaccumulators discovered worldwide

Family	Species	Shoot Cd concentration (mg kg^{-1}, DW)	Growth condition	Hyperaccumulation of other metals	Locality discovered	References
Asteraceae	*Bidens pilosa* L.	303(leaf)	100 mg kg^{-1} Cd in soil	–	China	Sun et al. (2009)
	Corydalis pterygopetala	102	0.5 mg L^{-1} Cd in solution	–	Thailand	Tanhan et al. (2007)
	Picris divaricata	246 (mean)	Pb/Zn mine	Zn	China	Tang et al. (2009a)
Athyriaceae	*Athyrium yokoscense*	164.8 (leaf)	mineralized soil	Pb	Japan	Nishizono et al. (1987) and Kamachi et al. (2005)
Brassicaceae	*Arabis gemmifera*	1,810	Cd-polluted sites	Zn	Japan	Kubota and Takenaka (2003)
	Arabis paniculata	457 (mean)	Pb/Zn mine	Zn/Pb	China	Tang et al. (2009b)
	Arabidopsis halleri	281	metallophyte grassland	Zn	France	Dahmani-Muller et al. (2000)
	Biscutella laevigata	198 (leaf)	4 mg L^{-1} Cd in solution	Pb	Poland	Pielichowska and Wierzbicka (2004) and Wenzel and Jockwer (1999)
	Rorippa globosa	104.5	25 mg kg^{-1} Cd in soil	–	China	Wei and Zhou (2006)
	Thlaspi caerulescens	14,000	500 μM Cd in solution	Zn/Ni	France	Lombi et al. (2000)
		2,800	500 mg kg^{-1} Cd + 100 mg kg^{-1} Zn in soil	–	France	Lombi et al. (2000)
		164	Field survey	–	UK	Baker et al. (1994)
	Thlaspi goesingense	830		Zn/Ni	Austria	Lombi et al. (2000)

(continued)

Table 1 (continued)

Family	Species	Shoot Cd concentration (mg kg^{-1}, DW)	Growth condition	Hyperaccumulation of other metals	Locality discovered	References
			500 mg kg^{-1} Cd + 100 mg kg^{-1} Zn in soil			
	Thlaspi praecox	5,960 (maximum)	Pb mine	Zn/Pb	Slovenia	Vogel-Mikuš et al. (2005)
	Thlaspi rotundifolium	108	Mineralized soil	Pb	Austria	Wenzel and Jockwer (1999)
Caprifoliaceae	*Lonicera japonica*	403	50 mg L^{-1} Cd in solution	–	China	Liu et al. (2009)
Chenopodiaceae	*Beta vulgaris*	300	100 mg kg^{-1} Cd in soil	–	China	Li et al. (2007)
Crassulaceae	*Sedum alfredii*	7,800 (leaf)	200 μM Cd in solution	Zn	China	Yang et al. (2002, 2004)
Iridaceae	*Iris iactea*	529	80 mg L^{-1} Cd in solution	–	China	Han et al. (2007)
Poaceae	*Echinochloa polystachya*	233(leaf)	100 mg L^{-1} Cd in solution	–	Mexico	Solís-Domínguez et al. (2007)
Rosaceae	*Potentilla griffithii*	1,670	20 mg L^{-1} Cd in solution	Zn	China	Hu et al. (2009) and Qiu et al. (2006)
Solanaceae	*Solanum nigrum*	167 (leaf)	100 mg kg^{-1} Cd in soil	–	China	Wei et al. (2005)
Violaceae	*Viola baoshanensis*	1,168 (mean)	Pb/Zn mine	–	China	Liu et al. (2004)

2.1.1 Rhizospheric and Microbial Mobilization

The rhizospheres of hyperaccumulator plants may have some influence on the availability and solubility of metals by both acidification of the rhizosphere and exudation of carboxylates (Whiting et al. 2000). However, several studies have indicated that rhizosphere acidification and root exudates are not responsible for improvement of metal capture (Whiting et al. 2001a, b; Zhao et al. 2001).

On the contrary, the release of root exudates by some hyperaccumulators can bind and sequester heavy metals in soil (Schwab et al. 2005). This mechanism could protect roots from the toxic effects of heavy metals (Liao and Xie 2004) and may also decrease Cd uptake by plants (Sarwar et al. 2010). Microbes are ubiquitous even in soils with high heavy metal concentrations. Rhizosphere microorganisms have also been shown to influence metal availability and mobility by reducing soil pH or by producing chelators and siderophores (Abou-Shanab et al. 2003; Wenzel et al. 2003; Whiting et al. 2001a, b). The existence of active rhizospheric and microbial metal-mobilizing mechanisms has not conclusively been demonstrated.

2.1.2 Root Foraging

High metal uptake has also been associated with enhanced root proliferation in artificially metal-enriched soil patches under experimental conditions (Whiting et al. 2000). Thus, root foraging (Fig. 1a) plays a significant role in scavenging of metals from soil (Haines 2002; Liu et al. 2010a). Hyperaccumulators have greater requirements for the metals of interest, which may be the reason why they allocate more roots into metal-rich soil patches (Liu et al. 2010a). For example, zincophilic root foraging responses have been demonstrated in *T. caerulescens* (Haines 2002).

Unlike Zn, Cd is a nonessential element which has no known essential biological function in plants, except in some marine diatoms (Lane and Morel 2000; Lane et al. 2005). However, some hyperaccumulator species also allocate root proliferation in Cd-enriched patches (Schwartz et al. 2003; Whiting et al. 2000). For example, Liu et al. (2010a) observed that the Zn/Cd hyperaccumulator plant *S. alfredii* consistently allocated approximately 90% of root biomass to Cd-enriched patches when grown on a heterogeneous substrate. The principal explanation for the phenomenon is that Cd may have a beneficial effect on growth by positively replacing Zn in some enzymes, proteins, or other Zn compounds (Liu et al. 2008) or by increasing the cellular physiological availability of Zn, such as by interfering with the constitutive Zn sequestration machinery (Liu et al. 2010a).

2.1.3 Cd Uptake from Soil to Roots

Root uptake of Cd^{2+} typically exhibits two phases: apoplastic binding and symplastic uptake (Hart et al. 1998; Zhao et al. 2002). Cadmium transport across

the plasma membrane of root cells has been shown to occur via a concentration-dependent process exhibiting saturable kinetics in many species, suggesting that Cd is taken up via a carrier-mediated system (Verbruggen et al. 2009a; Zhao et al. 2006). For such a non-essential element, it is likely that Cd enters cells through Ca channels or broad-range metal (e.g., Zn or Fe) transporters (Fig. 1b). Ca transport pathway might be involved in the uptake of Cd. Adding La^{3+} (a potent Ca channel inhibitor) or increasing the concentration of Ca appeared to suppress the metabolically dependent Cd uptake substantially in the Prayon ecotype of the hyperaccumulator *T. caerulescens* (Zhao et al. 2002). Except for Ca channel-mediated uptake, abundant evidence suggests that Fe may also lead to the upregulation of genes encoding Fe transporter proteins such as IRT1, which also mediate Cd uptake (Lombi et al. 2002; Zhao et al. 2006). However, Plaza et al. (2007) have reported that although TcIRT1-G may be involved in Cd hyperaccumulation in the Ganges ecotype of *T. caerulescens*, the transporter expressed in yeast seems to be incapable of Cd transport in contrast to AtIRT1. Therefore, the unique Cd accumulating ability of the *T. caerulescens* Ganges ecotype may be correlated with the expression of other transporter proteins or interacting proteins. Cadmium and Zn uptake are genetically correlated and competitive as they may be transported, at least partly, by the same transporter(s) or are controlled by common regulators because of their similar electronic structure (Baker et al. 1994; Benavides et al. 2005). In some cases, Zn was found to depress Cd uptake in *T. caerulescens* (Lombi et al. 2000, 2001). ZNT1 which is a Zn transporter cloned from the Prayon ecotype of *T. caerulescens* can also mediate Cd transportation with low affinity (Pence et al. 2000).

Most studies have shown that the main site of Cd accumulation in roots is the apoplast, particularly cell walls. Using energy-dispersive X-ray microanalysis, Cd was determined in cortex parenchyma cells, endodermis, parenchyma cells of the central cylinder, and xylem vessels in *T. caerulescens* (Wójcik et al. 2005). After treatment with 20 ppm Cd, about 13% of the total Cd in *T. caerulescens* roots was associated with organic acids (Boominathan and Doran 2003). Nedelkoska and Doran (2000) have found retention of almost the whole pool of Cd taken up in the fraction of cell wall of *Thlaspi* roots, and then diffused into the symplast after 7–10 days of exposure to 200 μM Cd. The authors also suggested that such a delay in Cd transport through membranes inside cells is an important defense mechanism against Cd toxicity, enabling simultaneous activation of intracellular detoxification mechanisms of Cd such as chelation and antioxidative defense.

2.2 Cd Translocation from Roots to Shoots in Hyperaccumulators

An efficient Cd translocation from roots to shoots is generally considered to be one of the most important characters of metal hyperaccumulators, although this process is not well understood (Ueno et al. 2008). It has been suggested that

hyperaccumulation may be a result of the evolution of a particularly effective metal translocation pathway from roots to shoots (Verbruggen et al. 2009a).

2.2.1 Xylem Loading of Cd Translocation

Several studies have suggested that root-to-shoot Cd translocation via the xylem, presumably mainly as free Cd, is the major and common physiological process in hyperaccumulating plants (Lu et al. 2009; Ueno et al. 2008; Uraguchi et al. 2009).

Metals could reach the xylem through either the symplast or the apoplast (White et al. 2002) (Fig. 1c). Symplastic bypass is considered as an energy-consuming (positive) pathway involving metal transporter proteins. Apoplastic bypass is known to be a passive pathway which is usually correlated to transpiration. Transpiration as a controller of Cd accumulation in shoots has been investigated either by manipulating transpiration or by observing unperturbed transpiration rates (Salt et al. 1995; Van der Vliet et al. 2007). However, results have led to equivocal conclusions. Liu et al. (2010b) reported that transpiration has an important role in Cd accumulation in shoots of the metal accumulator *Phytolacca americana*. Their argument is based mainly on a comparison of literature data on Zn influx in roots and accumulation in shoots. Meanwhile, as admitted by White et al. (2002), their calculation excluded the possible contribution by low-affinity transporters, which would be more prominent in the range of high external Zn concentrations. In *T. caerulescens*, EDX microanalysis revealed the presence of Cd in leaf along the path of water migration from the vascular cylinder to epidermal cells, which is in line with passive Cd transport by the transpiration stream (Wójcik et al. 2005). However, some current hypotheses suggest a dominant role of the symplastic pathway in the transport of Cd to xylem in several plant species. Xing et al. (2008) have reported that the Cd translocation efficiency did not correlate with the apoplastic bypass flow in 11 *Thlaspi* accessions, indicating that Cd and Zn translocation is not controlled by the apoplastic pathway. In the study by Ueno et al. (2008), xylem loading of Cd by *A. halleri* is an energy-dependent and rapid process, which appears to be partly shared with the Zn and Fe pathway. In addition, the metabolic inhibitor significantly reduced the Cd concentration in xylem sap, while the transpiration rate had no effect on Cd accumulation in shoots of the hyperaccumulating ecotype (HE) of *S. alfredii*. This suggests that Cd translocation to the xylem is an energy-driven process, and apoplastic bypass makes a very small contribution to Cd hyperaccumulation in shoots of HE of *S. alfredii* (Lu et al. 2009).

2.2.2 Metal Transporters in Hyperaccumulators

The transport of metals across plasma membranes by means of primary and secondary active transporters is of central importance in the metal homeostasis network in plants (Kotrba et al. 2009). With the use of genetic and molecular

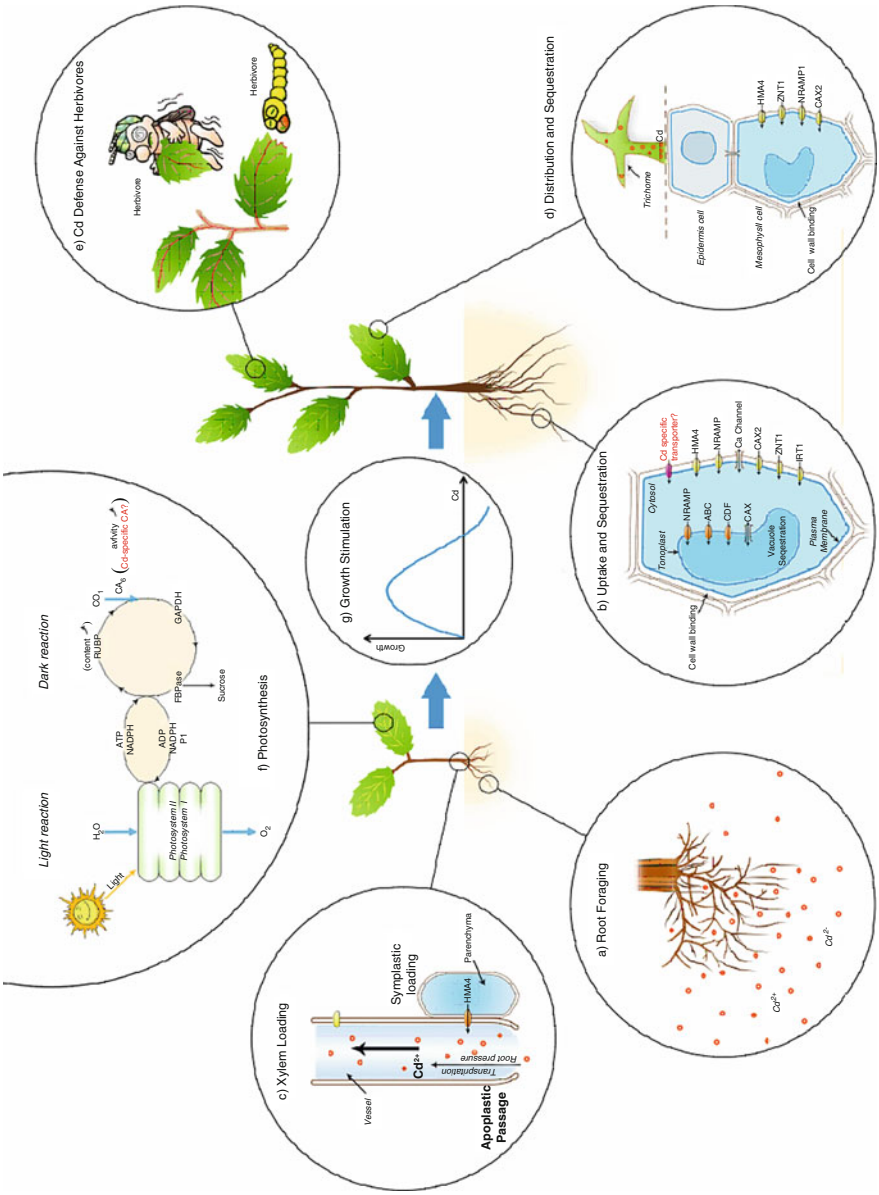

Fig. 1 Molecular and physiological mechanisms involved in Cd hyperaccumulation and detoxification (**a**–**d**), and their evolutionary significance for Cd hyperaccumulators (**e**–**g**). (**a**) Root foraging for Cd is a strategy that hyperaccumulator species employ to acquire high levels of Cd in Cd-enriched patches in a heterogeneous substrate. (**b**) In the root, uptake of Cd ion is mediated by various metal transporters residing in the plasma membrane. (**c**) Cd is transported to

techniques such as sequence comparison to identify transporters, functional complementation of yeast mutants, and plant transformation to regulate gene activities, a wide range of gene families have now been identified in plants that are likely to be involved in transition metal transport (Hall and Williams 2003). Although a picture of Zn hyperaccumulation is beginning to emerge, which attributes a central role to metal transport proteins and improves our mechanistic understanding of Zn hyperaccumulation and homeostasis, the molecular basis of Cd uptake into plant cells has not yet been elucidated clearly (Fig. 1b, d).

2.2.2.1 P_{1B}-ATPase Family

The heavy metal-transporting P_{1B}-ATPases belong to the superfamily of P-type ATPases which use energy from ATP hydrolysis to translocate cations across biological membranes (Williams and Mills 2005). The first identified transporter involved in Cd transportation from roots to shoots is the P_{1B}-type ATPase AtHMA4 (heavy metal-transporting ATPase4), which could mediate efflux of Zn and Cd from the xylem parenchyma cells to the xylem vessels, and therefore plays a key role in their transport from roots to shoots in *A. thaliana* (Mills et al. 2003; Verret et al. 2004). It has been reported that *T. caerulescens* and *A. halleri* have a much higher level of expression of the P_{1B}-type ATPase genes, especially *TcHMA4* or *AhHMA4* (Hanikenne et al. 2008; Papoyan and Kochian 2004). The authors have reported that TcHMA4 expression in roots of the Prayon accession of *T. caerulescens* is significantly induced by Zn deficiency and exposure to high Zn or Cd levels. The characterization of TcHMA4 protein has shown that its C-terminus contains numerous His and Cys repeated residues, which may participate in heavy metal binding (Papoyan and Kochian 2004). Additionally, HMA4 has been shown to be a strong candidate in determining the Cd hyperaccumulator phenotype: results from a cross between *A. halleri* and *A. lyrata* show how a major Cd-tolerance QTL colocalized with AtHMA4 (Courbot et al. 2007). By contrast, difference between Ganges and Prayon accessions in the root-to-shoot translocation of Cd and Zn was not attributable to different expression levels of *TcHMA4* (Xing et al. 2008).

Fig. 1 (continued) the shoot via the xylem through either the symplastic or the apoplastic way. HMA4 is responsible for Cd transfer from the xylem parenchyma to the vessel. (**d**) In the shoot, Cd is preferentially sequestered in the trichomes, epidermis, and mesophyll mediated by various metal transporters located at the plasma membrane and tonoplast. Cell wall binding and vacuole sequestration are suggested to be the main pathways of Cd detoxification. (**e**) According to the metal defense hypothesis, elevated Cd concentrations in plant tissues protect hyperaccumulators from herbivores or pathogens. (**f**) The enhanced photosynthesis by addition of suitable Cd dosage results from the stimulation of the activity of carbonic anhydrase (CA) and amount of Rubiso in the Calvin cycle. (**g**) The growth of hyperaccumulators is stimulated by a suitable range of Cd dosage but inhibited at higher dosages, suggesting a higher requirement of Cd for optimal growth

2.2.2.2 ABC (ATP-Binding Cassette) Family

The ATP-binding cassette (ABC) family is one of the largest protein families in living organisms (Higgins 1992). They have been shown to be implicated in a range of processes that encompass polar auxin transport, and xenobiotic and metal detoxification. Among the plant ABC transporters, AtMRP3 (Bovet et al. 2003) and AtATM3 (Kim et al. 2006) have been suggested to transport Cd. Overexpression of *AtATM3* in *Arabidopsis* plants showed enhanced Cd and Pb resistance compared to wild type plants, whereas *AtATM3* knockout plants showed Cd-sensitive phenotypes (Kim et al. 2006). Another ABC transporter in *Arabidopsis*, AtPDR8, is not only involved in the pathogen resistance in *A. thaliana* (Kobae et al. 2006), but it also acts as an efflux pump of Cd^{2+} or Cd conjugates at the plasma membrane of *Arabidopsis* cells (Kim et al. 2007). Regarding yeast and fission yeast, in which Cd is able to form complexes with either glutathione (GSH) or phytochelatins (PCs) subsequently transported into vacuoles via ABC transporters, it is also very likely that some plant ABC transporters are able to transport GS_2–Cd or PC–Cd complexes into sub-cellular compartments of or outside the cell (Bovet et al. 2005).

2.2.2.3 CDF (Cation Diffusion Facilitator) Family

Cation diffusion facilitator (CDF) proteins are a family of heavy metal transporters implicated in the transport of Zn, Cd, and Co that has been identified in bacteria and eukaryotes (Williams et al. 2000). Certain members of the CDF family are known to mediate vacuolar sequestration, storage and transport of metal ions from the cytoplasm to the outer compartment, and some are found in plasma membranes, while others are in intracellular membranes (Paulsen and Saier 1997). MTP transporters in the CDF family have been reported as efflux transporters for the sequestration of Zn to enhance the capability of metal tolerance in plants (Krämer 2005). Additionally, a constitutively high expression of *MTP1* in *A. halleri* (Becher et al. 2004; Chiang et al. 2006) and in an interspecies cross between *A. halleri* and *A. lyrata* has also been reported (Dräger et al. 2004). However, there is no conclusive evidence to date for a functional difference between hyperaccumulator and non-accumulator MTP1 proteins.

2.2.2.4 ZIP (ZRT1/IRT1-Like Protein) Family

ZRT1/IRT1-like protein (ZIP) generally contributes to metal ion homeostasis through the transport of cations into the cytoplasm (Colangelo and Guerinot 2006). It is noteworthy that the involvement of *ZIP* genes in metal accumulation by hyperaccumulators has previously been reported. High *TcZNT1* expression is associated with increased Zn uptake in roots of *T. caerulescens* (Pence et al. 2000). As one of the best characterized members of the ZIP family, AtIRT1 which transports a wide range of divalent transition metal cations, including Cd,

is known as the primary root iron uptake system of *A. thaliana* (Korshunova et al. 1999). IRT-overexpressing *A. thaliana* has shown to accumulate more Zn and Cd in roots under Fe-deplete conditions which stabilize IRT1 protein (Connolly et al. 2002). Such a role of IRT1 is in line with the observation from physiological studies on a number of plant species that Fe limitation leads to an increase in Cd accumulation. A contribution of other ZIP transporters to Cd uptake has also been suggested. *TcZNT1* mediates high-affinity Zn uptake and low-affinity Cd uptake when expressed in *Saccharomyces cerevisiae zhy3* cells (Pence et al. 2000).

2.2.2.5 Natural Resistance-Associated Macrophage Protein

Natural resistance-associated macrophage protein (NRAMP) defines a novel family of related proteins that have been implicated in the transport of divalent metal ions, especially in Fe homeostasis (Curie et al. 2000). Initial work on *AtNRAMP*1, 3, and 4 has shown that they confer Cd uptake activity when expressed in *S. cerevisiae* (Thomine et al. 2000). The authors have shown that *NRAMP* genes in plants encode metal transporters and AtNRAMPs transport both Fe and Cd (Thomine et al. 2000). NRAMP3 and NRAMP4 are responsible for Cd^{2+} efflux from the vacuole and are found to be overexpressed in both shoots and roots of *T. caerulescens* (Oomen et al. 2009; Thomine et al. 2003). It has been indicated that AtNRAMP3 and AtNARMP4 function redundantly in the mobilization of Fe from the vacuole (Lanquar et al. 2005). Also, NRAMP6, a new member of the NRAMP family, is functional and potentially involved in Cd tolerance (Cailliatte et al. 2009). Additionally, the protein shown in yeast to be targeted to intracellular vesicles may mediate the transport of Cd from internal pools.

2.2.2.6 Cation/H^+ Exchangers

Among the transporters thought to be capable of pumping Cd into plant vacuoles, cation/H^+ exchangers (CAXs) have been well characterized (Hirschi et al. 2000). CAX antiporters are a group of proteins that export cations out of the cytosol to maintain ion homeostasis across biological membranes (Pittman et al. 2002). All of the CAXs (CAX1–CAX6) found in *Arabidopsis* have been demonstrated to be capable of transporting Cd (Korenkov et al. 2007a, b). Korenkov et al. (2007a) have suggested that CAX antiporters are not negatively impacted by high Cd and that supplementation of the tonoplast with AtCAX somewhat compensates for a reduced number of tonoplast proton pumps and proton leak, and thereby results in sufficient vacuolar Cd sequestration to provide higher tolerance in tobacco. A recent report has shown that an *Arabidopsis* CAX1 mutant (CAXcd) conferring enhanced Cd transport in yeast is ectopically expressed in *Petunia* which can significantly increase Cd tolerance and accumulation (Wu et al. 2010).

So far numerous evidences have shown that Cd is taken up into plant cells by Fe, Ca, and Zn transporters/channels transporting a broad range of substrates. However,

a potential Cd-specific uptake system has also been suggested in hyperaccumulator plants (Lombi et al. 2001; Zhao et al. 2002), even though it has not been identified. Therefore, there are still lots of unclear areas that need to be investigated in the future.

2.3 Cd Accumulation in Shoots

Following the process of root absorption and transportation, the shoot plays a role as the final destination of Cd.

Cadmium is transferred from the substrate to the above ground parts through xylem transportation, and then reaches the final storage location via xylem unloading. Corresponding to the xylem loading of Cd translocation, there are also two main pathways for Cd xylem unloading: apoplastic bypass and symplastic bypass (Clemens et al. 2002; Karley et al. 2000). In the leaves of *T. caerulescens*, Cd was found in cells lying on the way of water migration from the vascular cylinder to epidermal cells, which is in line with passive Cd transport by the transpiration stream (Wójcik et al. 2005). Leaf cells accumulate solutes differently depending on their cell types (Karley et al. 2000). Several families of transporters may be involved in the xylem unloading of Cd in shoots, such as HMA and CDF (please see Sect. 2.2 for details, Fig. 1d).

The distribution of heavy metals at plant tissue, cellular, and sub-cellular levels is considered as one of the important physiological mechanisms for metal hypertolerance and hyperaccumulation (Cosio et al. 2005; Küpper et al. 1999, 2000; Ma et al. 2005). On the whole, Cd is stored principally in the less metabolically active parts of leaf cells. In *T. caerulescens*, Cd is mainly localized at the edges of the leaves, and also in points of higher concentration spread over the whole limb surface (Cosio et al. 2005). Cadmium is found in leaf epidermal cells in *T. caerulescens*, with concentrations two times higher than that in mesophyll cells (Ma et al. 2005). Besides, Cd is also found in epidermal trichomes of *A. halleri* (Küpper et al. 2000) and *Brassica juncea* (Salt et al. 1995). Most of the Cd in the leaf has been suggested to be localized at the base of trichomes (Küpper et al. 2000; Ma et al. 2005). This preferred storage in leaf epidermal cells may be associated with avoidance of heavy metal damage to photosynthesis. Entrance of Cd into the mesophyll cells may cause phytotoxicity in plants (Hu et al. 2009). Although metals are accumulated to much higher levels in the epidermis, a significant fraction of the total leaf metal accumulation still occurs in the mesophyll, mainly due to low biomass of the epidermis (Ma et al. 2005).

On the sub-cellular level, Cd is found both inside the cells and on the cell walls (Cosio et al. 2005). Similarly, Zhang et al. (2010) have suggested that Cd is mainly distributed in the vascular tissue and localized on the cell wall. In leaves of *T. caerulescens*, Cd and Zn are sequestered predominantly in the epidermal vacuoles (Cosio et al. 2005; Küpper et al. 1999; Ma et al. 2005). Hu et al. (2009) have shown that the major storage sites for Zn and Cd in *P. griffithii* leaves are

vacuoles of epidermis and bundle sheath, and to a lesser extent in cell wall or cytosol, and vacuolar storage may play a major role in strong tolerance and hyperaccumulation of Zn and Cd.

3 Cd Detoxification

3.1 Complexation of Cd by Ligands

3.1.1 Phytochelatins

Plants respond to metal toxicity by triggering a wide range of cellular defense mechanisms. They include immobilization, exclusion, compartmentalization, and synthesis of phytochelatins [PCs, $(\gamma\text{-GluCys})_n\text{-Gly}$], stress proteins, and ethylene (Sanità di Toppi and Gabbrielli 1999). In general, enhanced accumulation of PCs in different plant parts, especially in leaves, has been reported in many plants under Cd stress (Malec et al. 2009; Mishra et al. 2009). However, their role in heavy metal tolerance remains unclear. PC accumulation was higher in non-metallicolocus plants than in hypertolerant plants of *T. caerulescens*, suggesting that PC-based sequestration is not essential for constitutive tolerance or hypertolerance to Cd (Ebbs et al. 2002; Schat et al. 2002). Upon exposure to Cd for 7 days, no PC accumulation was found in any tissues of HE of *S. alfredii* from mine population, while PCs were rapidly synthesized in leaves, stems, and roots of non-mine plants (Sun et al. 2005, 2007a). On the contrary, Zhang et al. (2008) reported that PC formation could be induced in the roots, stems, and leaves of HE of *S. alfredii* exposed to 400 μM Cd for 5 days. Though the plants have been collected from the same Pb/Zn mining area using the same pre-column derivatization method, controversial results have been reported. Hence, it is necessary to explore the entire detoxification mechanisms not only in *S. alfredii*, but also in other hyperaccumulators. Recently, Zeng et al. (2009) have reported that Cd-induced accumulation of PCs occurred only in the roots of the multi-metal hyperaccumulator (Cd, Zn, and Pb) *A. paniculata*. The authors also suggested that the higher PCs in roots may be responsible for more Cd accumulation and complexation in the roots without translocation to the shoots. The rate of metal translocation to the shoot may depend on metal concentration in the root (Hardiman et al. 1984). Prezemeck and Haase (1991) suggested a PC-mediated metal binding in the xylem sap as a possible mechanism for metal translocation. The PCs have also been involved in long-distance symplastic transport of Cd^{2+} in *Brassica napus* (Mendoza-Cózatl et al. 2008).

3.1.2 Glutathione

Apart from metal chelation, GSH is involved in multiple metabolic roles such as intracellular redox state regulation, scavenging of reactive oxygen species (ROS),

transport of GSH-conjugated amino acids, storage of sulfur, and other functions related to cell cycle, plant growth, pathogen-related response, and cell death (Ogawa 2005). As an antioxidant and PC precursor, GSH and its metabolism play an important role in plant response and adaptation to various natural stress conditions (Xiang and Oliver 1999). Unlike PCs, an increase in GSH was observed in all tissues of both ecotypes of *S. alfredii* with increasing Cd and Zn concentrations (Sun et al. 2005, 2007a). Also, the GSH content was higher in non-mine plants than that in the mine plants under Cd exposure for 7 days (Sun et al. 2007a). However, Xiang et al. (2001) have reported that plants with low levels of GSH were highly sensitive to low levels of Cd due to the limited capacity of plants to synthesize PCs. Thus, the most promising replies to the higher Cd tolerance and accumulation in hyperaccumulator *S. alfredii* are the elevated GSH levels in plant tissues and GSH biosynthesis, which seems to be more important. Further research is needed to clarify the possible involvement of GSH in Cd detoxification and tolerance in *S. alfredii* (Jin et al. 2008a) and other Cd hyperaccumulator species.

3.1.3 Other Ligands

Complexation of Cd may play an important role in the internal detoxification of Cd by lowering the free metal ion concentration in the cytosol and vacuoles (Krämer et al. 1996; Hall 2002). It is presumed that an important aspect of metal storage involves metal chelation with organic ligands (Milner and Kochian 2008) such as organic acids, amino acids, peptides, and proteins (Verbruggen et al. 2009b). However, it is still not clear which specific ligands are involved (Milner and Kochian 2008). Küpper et al. (2004) found that a large fraction of the foliar Cd was bound to sulfur ligands in *T. caerulescens* by using X-ray absorption spectroscopy, whereas Ueno et al. (2005) deduced that Cd in the shoot was rather bound to organic acids, mainly malate, with measurements of ^{113}Cd-NMR.

3.2 Antioxidant Defense

Plants possess several antioxidative defense systems to scavenge ROS in order to protect themselves from oxidant stress, including that caused by heavy metals (Benavides et al. 2005). These include both non-enzymatic antioxidants such as GSH and ascorbate (ASA), and enzymatic antioxidants such as superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX), glutathione reductase (GR), and guaiacol peroxidase (GPX). The unique structural properties in GSH such as broader redox potential, abundance, and wide distribution render it as a good non-enzymatic scavenger of ROS in plants. Treatment of the hyperaccumulating ecotype of *S. alfredii* with the GSH synthesis inhibitor L-buthionine-sulfoximine (BSO) resulted in a significant damage in leaves with a concomitant

increase in H_2O_2 and O_2^- production, suggesting that GSH biosynthesis may also contribute to counter the Cd- and Zn-induced ROS production and help to cope the Cd and Zn toxicity (Jin et al. 2008a, b). The natural overproduction of GSH is considered a trait sustaining tolerance to oxidative stress caused by Cd^{2+} in *T. caerulescens* (Boominathan and Doran 2003) and Ni^{2+} in *T. goesigense* (Freeman and Salt 2007). A linear relationship was observed between an increase in SOD and CAT activity with Cd concentration in leaves and roots of the Cd hyperaccumulator *Lonicera japonica* Thunb (Liu et al. 2009). The maintenance of high SOD and CAT activities in *L. japonica* under Cd stress represents an important feature of a metal accumulator tolerating Cd toxicity. Similar results have been reported in several hyperaccumulators such as *T. caerulescens* (Wang et al. 2008), *Solanum nigrum* (Sun et al. 2007b), *Bidens pilosa* L. (Sun et al. 2009), and *A. paniculata* (Qiu et al. 2008).

Polyamines such as putrescine (Put), spermidine (Spd), and spermine (Spm) are polybasic aliphatic amines that play a major role in various physiological and developmental processes in plants. Polyamines also have an antioxidative property by quenching the accumulation of O_2^-, probably through the inhibition of NADPH oxidase, and the effect order is Spm > Spd > Put (Papadakis and Roubelakis-Angelakis 2005). Zhao and Yang (2008) reported that exogenous application of Spd and Spm can alleviate the lipid peroxidation caused by $CdCl_2$ in Pingyi Tiancha, *Malus hupehensis*. Groppa et al. (2001) also reported that Spd and Spm completely restore the GR activity of sunflower leaf discs under Cd^{2+} and Cu^{2+} stress. More recently, Wen et al. (2010) reported that *spermidine synthase*-overexpressing transgenic in vitro shoots of European pear tree slightly alleviated the oxidative stress caused by Cd, Pb, and Zn. Also, a significant positive correlation was observed between Spd and SOD or GR under Cd, Pb, Zn, or Cd/Zn/Pb combination, which indicates that Spd plays a major role in antioxidant activities. Based on these results, the authors suggested that Spd levels are implicated in enhanced heavy metal tolerance, possibly by exerting an antioxidant activity and by the properties of Spd per se including being a metal chelator.

3.3 Cell Wall Binding and Vacuole Sequestration

Plants have a range of potential mechanisms at the cellular level that might be involved in the detoxification and thus tolerance to heavy metal stress (Hall 2002). Cell wall binding and vacuolar sequestration are the two essential detoxification mechanisms that play a vital role in hyperaccumulation of heavy metals (Cosio et al. 2004).

The cell wall is the "first line of defense" against negative factors from the external environment. When captured by root cells, metals are first bound by the cell wall, by an ion exchange of comparatively low affinity and low selectivity (Clemens et al. 2002). Boominathan and Doran (2003) reported that *T. caerulescens* root hairs contained most of the Cd in the cell wall. In roots of *A. halleri* grown

hydroponically, Zn and Cd were accumulated in the cell wall of the rhizosphere as Zn/Cd phosphates (Küpper et al. 2000). Cell wall binding can prevent Cd from being transported across the plasma membrane. This delay in transmembrane uptake may represent an important factor in the defense against Cd poisoning in *T. caerulescens*, allowing time for activation of intracellular mechanisms for heavy metal detoxification (Nedelkoska and Doran 2000).

The vacuole, in turn, is generally considered to be the main storage site for metals in yeast and plant cells (Salt and Rauser 1995). Compartmentalization of metals in the vacuole is also part of the tolerance mechanism of some metal hyperaccumulators (Tong et al. 2004). In *T. caerulescens*, Cd has been found in the apoplast and the vacuole (Vázquez et al. 1992). A 100% and more than 90% Cd in the leaf protoplast were localized in the vacuoles of Ganges ecotype of *T. caerulescens* (Ma et al. 2005) and P. griffithii (Qiu et al. 2011), respectively. These results clearly indicated that internal detoxification of Cd is achieved by vacuolar compartmentation.

After Cd enters the cytosol, some mechanisms may play roles to inactivate it through chelation or conversion to a less toxic form. Phytochelatins could bind Cd^{2+} in the cytoplasm, and Cd transport into the vacuole is an effective way to reduce the levels of Cd toxicity in the cytosol. No doubt, an efficient tonoplast transport of Cd is very important. Several families of transporters may take part in the process of vacuole sequestration, such as YCF1, MTP1, and CaCA (see Sect. 2.2 for details).

It is generally assumed that low molecular weight (LMW) complexes are formed in the cytosol and subsequently transported into the vacuole where more Cd^{2+} and sulfide are incorporated to produce the high molecular weight (HMW) complex, which represents the main storage form of Cd (Clemens 2000). The first molecular insight into the vacuolar sequestration of Cd came from the cloning of HMT1, which complemented a *S. pombe* mutant deficient in the formation of the HMW complex (Ortiz et al. 1995). A constitutively high concentration of malate in the vacuoles, and the formation of the Cd–malate complex may lead to a decrease in subsequent Cd efflux to the cytoplasm (Ueno et al. 2005).

4 Evolution of Cd Hyperaccumulation

Apart from the numerous studies on physiological and molecular mechanisms involved in Cd hyperaccumulation and detoxification, as discussed above, perhaps the most fundamental question is why these unusual species accumulate Cd to concentrations that are toxic to most other organisms. Since 1990s, the ecological and evolutionary significances of metal hyperaccumulation have attracted considerable attention. Five principal hypotheses have been postulated, including drought resistance, metal tolerance/disposal, elemental allelopathy, inadvertent uptake, and defense against herbivores or pathogens (Boyd and Martens 1998). There is little evidence linking hyperaccumulation with drought resistance, metal tolerance/disposal, and elemental allelopathy (Boyd 2004; Mcnair 2003). According to the inadvertent uptake hypothesis, Cd accumulation is possibly mediated by channels

or transporters for essential elements, such as Zn, Ca, and Fe (Lombi et al. 2001, 2002; Lu et al. 2008; Zhao et al. 2002, 2006). This hypothesis, however, has no selective benefits accruing to the hyperaccumulator species (Jiang et al. 2005). Among the five hypotheses, the defense hypothesis has attracted most interest and appears to be supported by many experimental results. In recent years, there is some novel insight into the exploitation of physiological and biological function of Cd in hyperaccumulator species (Liu et al. 2008), as demonstrated in the marine diatom *Thalassiosira weissflogii* (Lane and Morel 2000; Lane et al. 2005). We will now review our current understanding of the most popular defense hypothesis and the potential biological role of Cd in the evolution of Cd hyperaccumulation.

4.1 Elemental Defense

The defense hypothesis suggests that elevated metal concentrations in plant tissues protect hyperaccumulators from certain herbivores or pathogens (Boyd and Martens 1998, Fig. 1e). Different from the more widespread organic (secondary chemical) defense produced by most plants, elemental defense is acquired from the soil without being synthesized by a plant, and thus appears to be less expensive metabolically (Boyd and Martens 1998; Boyd 2007). A large number of trials have been made to test the elemental defense hypothesis, working with a variety of organisms including insects, gastropods, fungi, bacteria, and viruses (see review of Boyd 2007). There is substantial evidence for a protective role of Ni, Zn, and Se against invertebrate herbivory. Nickel was shown to protect the Ni hyperaccumulator *Streptanthus polygaloides* from several leaf-chewing folivores, such as moth, mite, fly, butterfly, and grasshopper (Boyd and Moar 1999; Jhee et al. 2005, 2006; Martens and Boyd, 1994). Similarly, Zn protected *T. caerulescens* from herbivory by grasshopper and butterfly (Behmer et al. 2005; Jhee et al. 1999; Pollard and Baker, 1997). In particular, Se accumulation in *B. juncea* deterred feeding by caterpillars, grasshopper, and aphids at relatively low concentrations of Se in leaves, far below the concentration used to define Se hyperaccumulation (Freeman et al. 2007; Hanson et al. 2003, 2004). There is also evidence that Ni and Se can protect plants from bacteria and fungi (Boyd et al. 1994; Ghaderian et al. 2000; Hanson et al. 2003). On the contrary, no Ni protection has been found against phloem chewers such as aphid and whitefly, probably because phloem Ni in *S. polygaloides* is relatively low (Boyd and Martens 1998; Jhee et al. 2005). Also, neither Se accumulation in *B. juncea* (Hanson et al. 2003) nor Zn hyperaccumulation in *A. halleri* (Huitson and Macnair 2003) deterred herbivory by snails.

In terms of Cd, however, only few studies on plant self-defense by Cd are available (Boyd 2007). Jiang et al. (2005) showed that Cd hyperaccumulation in the leaves of *T. caerulescens* deterred thrips (*Frankliniella occidentalis*) from feeding. Cadmium addition produced a significant reduction in the number of thrips and the leaf feeding damage index (LFDI), suggesting that Cd hyperaccumulation

may offer an adaptive advantage. In another case, Scheirs et al. (2006) demonstrated that only 2.5 mg kg^{-1} Cd in leaves of *Holcus lanatus* was sufficient to decrease attack by the grass miner *Chromatomyia milli* significantly. However, snails seem to be insensitive to the high-leaf Cd or Zn of *T. caerulescens*, whereas glucosinlates appear to decrease the degree of herbivory (Noret et al. 2005). These results, combined with those from Ni, Zn, and Se studies, suggest that protective effects by hyperaccumualted metals are not absolute, but may be related to the ecological interactions between the plant species and the herbivores or pathogens involved (Boyd 2004; Jiang et al. 2005).

4.2 A Biological or Physiological Role of Cd?

Cadmium is generally regarded as a non-essential and toxic trace element. However, a Cd-requiring carbonic anhydrase (CA) isolated from the marine diatom *T. weissflogii* has been characterized as the first Cd-containing enzyme in the biosphere (Lane et al. 2005). Substitution of Zn by Cd to form a Cd-specific CA, particularly under conditions of low Zn, plays a biological role in carbon acquisition and photosynthesis of some marine phytoplanktons (Lane and Morel 2000). It is this important discovery that has generated considerable interest in exploring whether Cd likewise plays a similar physiological or biological function in vascular plants.

Hyperaccumulator species of Cd may be ideal materials to test this hypothesis, because in recent years there is increasing evidence from biological and physiological studies at various levels to support a positive role of Cd in these exceptional species (Table 2, Fig. 1g). At the whole plant level, stimulation of biomass production by addition of Cd in substrates has been observed in most discovered Cd hyperaccumulators (Table 2). The stimulatory effects of Cd on plant growth can vary greatly, depending on the type (ecotype) of species, the growth time, and the substrate tested. For example, Liu et al. (2004) reported by far the greatest increase of approximately 300% of total biomass in *Viola baoshanensis* when treated hydroponically with 267 μM Cd, whereas *A. halleri* showed trivial positive response even at relatively low Cd (5–15 μM) in solution (Zhao et al. 2006). In *S. alfredii*, it is noticeable that while less stimulation (17–25%) of shoot dry weight occurred at 12.5–100 μM Cd in solution (Yang et al. 2004), the total biomass could be increased by about 1.6 or 3.2 times when plants were exposed to 50 or 100 mg kg^{-1} Cd in soil, respectively (Liu et al. 2010a). This difference may be attributed to the different substrate and experimental duration used. Similarly, Liu et al. (2008) found a significant increase in shoot biomass of *T. caerulescens* (Ganges) with increasing addition of Cd (5–500 mg kg^{-1}) in soil, whereas the stimulation was not obvious in hydroponic conditions. In general, this phenomenon is similar to hormesis, a toxicological term referring to low-dose stimulation and high-dose inhibition by toxic agents (Calabrese and Baldwin 1998). However, given that the maximum stimulation by Cd in some Cd hyperaccumulators (e.g., 300% in

Table 2 The stimulatory effects of Cd on hyperaccumulator species

Process	Plant species	Stimulatory dose of Cd and substrate tested	Stimulatory phenomenon associated with Cd	References
Biological character	*Arabis paniculata*	9, 44, 89 μM in solution	(18–41*)% and (12–39)% increase in shoot and root biomass (DW)	Tang et al. (2009b)
		22–89 μM in solution	(22–27)*% increase in total biomass (FW)	Qiu et al. (2008)
	Arabidopsis halleri	5–15 μM in solution	(5–18)$^{\#}$% increase in root biomass	Zhao et al. (2006)
	Picris divaricata	10–25 μM in solution	(13–27)% and (22–78*)% increase in shoot and root biomass (DW)	Ying et al. (2010)
	Potentilla griffithii	44–178 μM in solution	(25$^{\#}$–56*)% increase in total biomass (DW)	Hu et al. (2009)
	Sedum alfredii	12.5–100 μM in solution	(17–25)% and (2–10)% increase in shoot and root biomass (DW) (NR)	Yang et al. (2004)
		50–100 mg kg^{-1} in soil	(60–220)*% increase in total biomass (DW)	Liu et al. (2010a)
	Thlaspi caerulescens (Ganges)	5–500 mg kg^{-1} in soil	(32–57*)% increase in shoot biomass (DW)	Liu et al. (2008)
		1.5 and 3.0 mg kg^{-1} in soil	(70–90)$^{\#}$*% increase in shoot biomass (DW)	Yanai et al. (2006)
	Thlaspi caerulescens (St Félix-de-Pallières)	3 and 30 μM in solution	37% (14 d) and 75% (31 d) in total biomass (DW) at 3 μM Cd	Roosens et al. (2003)
	Viola baoshanensis	44–267 μM in solution	(90$^{\#}$–300)% increase in total biomass (DW) (NR)	Liu et al. (2004)

(continued)

Table 2 (continued)

Process	Plant species	Stimulatory dose of Cd and substrate tested	Stimulatory phenomenon associated with Cd	References
Morphological character	*Sedum alfredii*	50–100 mg kg^{-1} in soil	Root proliferation (over 90%) in Cd-rich patches	Liu et al. (2010a)
	Thlaspi caerulescens (Clough)	0/500 mg kg^{-1} CdS in soil	Root proliferation in Cd-rich patches	Whiting et al. (2000)
	Thlaspi caerulescens (Prayon and Viviez)	&	Root proliferation in hot spots of Cd	Schwartz et al. (2003)
Physiological character	*Arabis paniculata*	9–178 μM in solution	Increase in chlorophyll contents	Tang et al. (2009b)
		22–89 μM in solution	Alleviation of ROS (MDA, O_2^{-1}, H_2O_2) stress in roots	Qiu et al. (2008)
	Picris divaricata	5–50 μM in solution	Increase in CA activity and Rubisco content	Ying et al. (2010)
	Sedum alfredii	200–1,000 μM in solution	Increase in chlorophyll contents	Zhou and Qiu (2005)
	Thlaspi caerulescens (Ganges)	0–50 μM in solution	Increase in CA activity	Liu et al. (2008)
		5–50 μM in solution	Evidence for a high-affinity Cd uptake system	Lombi et al. (2001)

An asterisk denotes that stimulatory effect is significant compared with the control
A symbol # denotes that the data are estimated from the figure of the corresponding reference
A symbol & denotes that Cd levels are not given using inclusions of Cd shots into uncontaminated soil
NR means that significance of stimulatory effect is not reported in reference

V. baoshanensis; 220% in *S. alfredii*; and 90% in Ganges of *T. caerulescens*, Table 2) is much higher than the typical range of 30–60% stimulation that is documented in many hormetic studies (Calabrese and Baldwin 1998), this might not simply be a general case of hormesis – it is possible that Cd has acquired a biological role in some exceptional hyperaccumulator species (Roosens et al. 2003).

From the morphological perspective, a positive response to Cd-enriched hot spots by roots of *T. caerulescens* has been reported (Schwartz et al. 1999, 2003; Whiting et al. 2000). This trait can be much more significant in *S. alfredii*, in which approximately 90% of root biomass is allocated to the Cd-enriched patches, suggesting that a root foraging strategy for Cd similar to that mostly occurring in nutrient acquisition in heterogeneous soils has evolved and plays a role in Cd hyperaccumulation (Liu et al. 2010a). The underlying mechanisms of such non-directional development of root architecture by Cd heterogeneity are yet to be shown, but may be linked with an auxin-regulated signal sensing process, like that observed in root branching by nitrate (Zhang et al. 1999).

Cadmium may also improve physiological processes in hyperaccumulator species (Table 2). Stimulatory effects in leaf chlorophyll synthesis have been reported in *A. paniculata* (Tang et al. 2009b) and *S. alfredii* (Zhou and Qiu 2005). The increase in chlorophyll content in the latter might be a result of Cd-induced increase in Fe uptake (Zhou and Qiu 2005). Meanwhile, the amounts of malondialdehyde (MDA), O_2^{-1}, and H_2O_2 in roots of *A. paniculata* decreased significantly at 22–89 μM Cd levels, suggesting that moderate addition of Cd may alleviate the oxidant stress caused by Cd-induced ROS (Qiu et al. 2008). With respect to photosynthesis, Liu et al. (2008) and Ying et al. (2010) found that the CAs activity in *T. caerulescens* and *P. divaricata* correlated positively with the shoot Cd concentration, demonstrating that Cd may play a physiological role in Cd hyperaccumulation (Fig. 1f). Further study is needed to elucidate the biological function of Cd in hyperaccumulators through enzymological and molecular methods.

5 Future Perspectives

This review has focused on the recent progress in understanding the physiological and molecular mechanisms that may be involved in Cd hyperaccumulation and tolerance. In general, the strategies adopted by hyperaccumulating plants aim to absorb and translocate Cd efficiently, and sequester it mostly in the less metabolic active parts, thus to avoid the onset of toxicity. This is achieved by various mechanisms including root proliferation in Cd-rich substrate, influx into cytosol or vacuole by specific or non-specific transporters, and complexation of Cd by certain ligands in cells. Compared to the studies on Zn, however, the mechanisms behind Cd hyperaccumulation are relatively underinvestigated. There are still lots of unclear areas that need to be investigated in the future. Below, and described in Fig. 1, are perspectives regarding the less explored fields associated with the mechanisms and evolution of Cd hyperaccumulation.

A key point of these perspectives is based on the hypothesis that some special hyperaccumulators may have a higher requirement of Cd for optimal growth (Fig. 1). There is substantial evidence that addition of Cd in substrates does enhance the growth of hyperaccumulator species (Table 2). This stimulatory effect has led to two principal questions as to why and how the higher requirement of Cd may be achieved. On the one hand, a better growth of plant in natural metalliferous soils where Cd hyperaccumulators often colonize may have certain ecological advantages, e.g., higher competition and energy storage. This is obtained by enhanced photosynthesis in which Cd may play a role in stimulating the activity of CA and the amount of Rubisco in the Calvin cycle (Liu et al. 2008; Ying et al. 2010). Whether Cd is beneficial for particular plant taxa as shown in marine diatoms (Lane et al. 2005) remains a mystery. Conventionally, this could be determined by studying the phenotypic differences between plants growing in the absence and the presence of Cd (Pilon-Smits et al. 2009). Furthermore, techniques such as chromatography, dichroism spectra, and fluorimetry are required to check if a Cd-containing CA really exists in such Cd hyperaccumulators.

A higher requirement of Cd may also be linked with an efficient elemental defense against herbivores and pathogens. This appears to be the most accepted hypothesis that provides a reasonable explanation for the evolution of metal hyperaccumulation. However, the vast majority of experimental evidence supporting this hypothesis has focused on hyperaccumulators of Ni, Zn, or Se (Boyd 2007), whereas only a few cases for Cd defense have been tested. Further studies such as non-choice and binary choice both in laboratory and in fields are needed to answer two emerging questions. First, what is the minimum concentration of Cd sufficient for defense? Second, can Cd defense be enhanced by the combination of organic defensive compounds, the so-called synergisitc effects hypothesis (Boyd 2007)?

On the other hand, to meet the high requirement of Cd in plant body, hyperaccumulators must possess highly efficient mechanisms for uptake and tolerance of Cd. There is strong evidence that certain hyperaccumulators have adopted the root foraging strategy to enhance acquisition of Cd in soil. Root foraging is responsible (even though not entirely) for maintaining high levels of Cd in hyperaccumulators. This trait thus has an important implication toward the improvement of plants to be used in practical phytoextraction, since Cd in soil is often heterogeneously distributed. A big challenge with respect to root foraging is to characterize the molecular and physiological responses involved in the changes in root architecture that are observed in hyperaccumulators, e.g., *S. alfredii* and *T. caerulescens* (Liu et al. 2010a; Schwartz et al. 1999, 2003). In terms of the uptake process into roots, numerous evidences have shown that Cd is taken up into root cells by Fe, Ca, and Zn transporters/channels which transport a broad range of substrates. However, a potential Cd-specific uptake system has also been suggested in hyperaccumulator plants (Lombi et al. 2001; Zhao et al. 2002), even though it has not been identified so far. Some other research tools, such as proteomics approaches, could provide a new platform to study the complex biological functions involved in Cd detoxification in hyperaccumulators, such as trafficking, signal transduction, and transport.

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Long-Distance Transport and Plant Internal Cycling of N- and S-Compounds

Cornelia Herschbach, Arthur Gessler, and Heinz Rennenberg

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Abstract A coordinated supply of the whole plant with sulfur (S) and nitrogen (N) requires mechanisms to regulate not only uptake and assimilation but also long-distance transport of both nutrients in the phloem and xylem as well as the plant internal cycling of S and N compounds. In trees, plant internal nutrient cycling which includes bidirectional exchange between phloem and xylem allows to partially uncouple nutrient demand from soil supply and needs to be highly coordinated with seasonal storage and remobilisation of S- and N-compounds. In both annual and perennial plants the pools of N and S compounds cycling within the

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plant provide an integrated signal to adapt the nutrient supply of the plant to the actual demand.

This review discusses the transport of N and S compounds in phloem and xylem, illustrates the quantitative importance and the physiological relevance of different N and S compounds transported and focuses on the exchange between the transport systems. Thereby we demonstrate similarities and differences between N and S in assimilation, transport, storage and the underlying regulatory mechanisms.

1 Introduction

The availability of the macronutrients nitrogen and sulfur is a decisive factor of plant growth and development. Whereas sulfur deficiency is largely restricted to intensive agriculture at sites not exposed to significant atmospheric sulfur deposition (Zhao et al. 1995, 1996; Scherer 2001), nitrogen deficiency is a general phenomenon for plants on cultivated land areas and in natural environments (Vitousek et al. 1997). In agriculture, usage of appropriate fertilizers usually overcomes nitrogen and sulfur deficiency (Zhao et al. 1999a, b; Thomas et al. 2000; Tilman et al. 2002). In natural environments, special features have been developed during evolution to cope with nitrogen limitation (Raven and Andrews 2010). At the ecosystem level, these features include a largely closed ecosystem nitrogen cycle as well as efficient uptake, assimilation, and plant internal distribution of nitrogen at the plant level (Rennenberg and Schmidt 2010).

Nitrogen and sulfur are essential constituents of protein and are thought to be taken up and to be assimilated in a coordinated way (Brunold 1993; Brunold et al. 2003; Kopriva and Rennenberg 2004) in order to meet the species-specific nitrogen-to-sulfur ratio in plant protein (Rennenberg 1984). Uptake of nitrogen and sulfur is thought to be controlled to meet the sulfur and nitrogen demand for growth and development of the entire plant (Hawkesford and De Kok 2006; Gessler et al. 2004). This control includes the allocation of shoot-derived signals to the roots and signal transduction in the roots that results in changes of transporter abundance and activity (Lappartient and Touraine 1996, 1997; Lappartient et al. 1999; Herschbach and Rennenberg 1994; Herschbach et al. 1995a, b; Collier et al. 2003; Gessler et al. 2004), whereas phloem mobile metabolites such as sulfate and glutathione, as well as amino acids such as glutamine, and phytohormones such as cytokinins have been identified as shoot-derived signals for the regulation of sulfur and nitrogen uptake, respectively (Lappartient et al. 1999; Forde 2002; Takei et al. 2002; Collier et al. 2003; Hawkesford and De Kok 2006; Davidian and Kopriva 2010; Gessler et al. 2004). However, the signal transduction pathway leading from the accumulation of these metabolites in the roots to changes in transporter abundance and activity has not been elucidated. In perennial plants, demand-driven control may be insufficient to explain nitrogen and sulfur uptake, because storage and mobilization processes may also determine the uptake of sulfur and nitrogen (Herschbach and Rennenberg 2001a; Millard and Grelet 2010). Under such conditions, more complex regulatory systems

are required that have to be based on seasonal storage, mobilization, and growth rhythms (Jansson and Douglas 2007; Keskitalo et al. 2005; Fracheboud et al. 2009).

Once taken up the chemical form of nitrogen and sulfur acquired, the site of reduction and assimilation as well as the chemical form of nitrogen and sulfur used in cellular processes determine the requirement for long-distance transport. When present in chemical forms appropriate for allocation inside the plant, distribution from source to sink can take place by long-distance transport either in the xylem or in the phloem (Fig. 1). This often requires chemically more inert forms than the original metabolic products. For example, glutathione constitutes a transport form of reduced sulfur (Rennenberg 1984) that is more inert than the original product of sulfate assimilation, i.e., cysteine (Jocelyn 1972). Distribution of nitrogen and sulfur within the plant may be achieved by unidirectional transport in the xylem or the phloem, or by cycling within the plant. Cycling will be obtained by xylem-to-phloem and phloem-to-xylem exchange that can take place along the entire

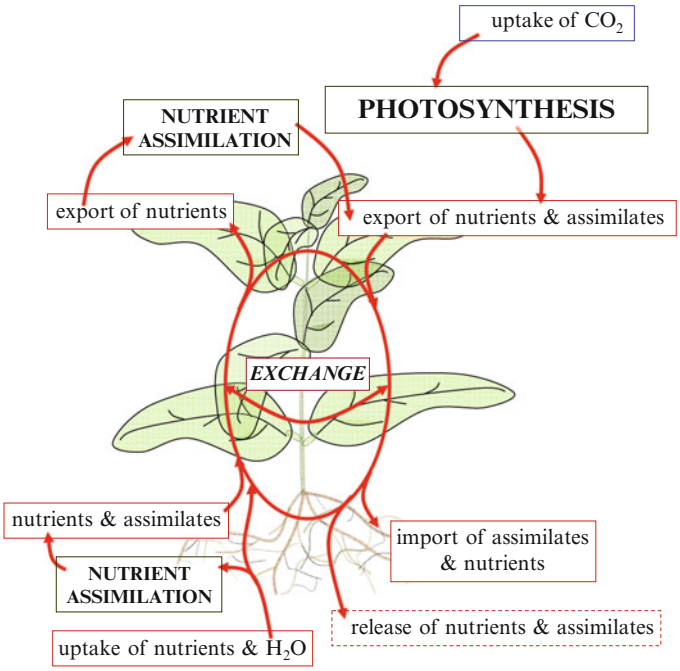

Fig. 1 Model of nutrient cycling in plants. *Red arrows* indicate the flow of nitrogen and sulfur metabolites. Metabolite flow starts with nutrient uptake and assimilation in roots. Then assimilates and nutrients are loaded into the xylem, transported with the transpiration stream and taken up by the leaves. Inside the leaves, nutrients are used and/or assimilated. In addition, carbon assimilates are produced by photosynthesis and are partially used for the assimilation of nutrients. A surplus of assimilates and nutrients in the leaves is loaded into the phloem for its transport to other plant organs, e.g., the roots. Here, the metabolites can be used for root growth and development, retranslocated to the leaves, or can be released into the rhizosphere

transport part and/or in the leaves and roots. This may include metabolic conversions at the sides of exchange between the long-distance transport paths into different mobile, i.e., transportable forms (Gessler et al. 2004). Nitrogen and sulfur metabolite cycling may be necessary for an efficient distribution of these macronutrients particularly in perennial plants that have to cope with changing sources and sinks not only during the seasons, but also upon short-term changes in the environment. The present review summarizes current knowledge on phloem and xylem transport of inorganic and organic nitrogen and sulfur compounds and discusses current evidence for their xylem-to-phloem exchange and cycling within the plant and mainly focus on trees.

2 Phloem Transport

2.1 Sulfur Compounds

In phloem saps and phloem exudates from herbaceous plants, several organic sulfur compounds, namely Cys, Met, γ-EC, glutathione (GSH), γ-glutamylcysteinylserine, and S-methylmethionine (Ohshima et al. 1990; Kuzuhara et al. 2000; Karley et al. 2002; Bourgis et al. 1999; Mendoza-Cózatl et al. 2008), as well as SO_4^{2-}, have been detected (Ohshima et al. 1990; Kuzuhara et al. 2000). Further sulfur compounds such as glucosinolates (Brudenell et al. 1999; Chen et al. 2001) and phytochelatins (Chen et al. 2006; Mendoza-Cózatl et al. 2008) are supposed to be phloem mobile. Sulfate transporters involved in phloem loading of sulfate have been identified in poplar leaves (Dürr et al. 2010), whereas similar studies for transporters of reduced sulfur compounds are lacking. A study on sulfur deficiency with rice revealed sulfate depletion in the phloem sap after 7 days of sulfur starvation (Kuzuhara et al. 2000); again, similar results on the effect of sulfur depletion on organic sulfur in the phloem have not been reported. Indirect evidence for phloem transport of organic sulfur compounds came from grafting experiments (Chen et al. 2006) and mutants (Li et al. 2006). When the bacterial *GSH1* gene was expressed under a light-inducible, shoot-specific expression system in the *cad2-1* mutant of *Arabidopsis* (Cobbett et al. 1998), deficient in the γ-glutamylcysteine synthetase (*GSH1*), heavy metal tolerance was restored (Li et al. 2006). As also the root concentration of γ-EC and GSH increased, this study delivered indirect evidence for γ-EC and GSH transport in the phloem to the roots. Similar results were obtained for the *cad1-3* mutant that has a defect in the phytochelatine synthetase (*PCS1*) gene (Howden et al. 1995). When this mutant was complemented with wheat *PCS1* in a shoot-specific manner, the Cd and arsenic metal sensitivity of the shoot was complemented and the root phytochelatin 2 (PC2) content increased. From these results, the authors concluded that phytochelatins were transported in the phloem from the shoot to the roots (Chen et al. 2006). Girdling experiments, i.e., peeling of the bark just above the root–shoot transition, with poplar plants resulted in a depletion of root-tip GSH and, thus, also indicate GSH transport from the shoot to the roots in the phloem (Herschbach et al. 2010a).

In phloem saps of trees sulfate, Cys, Met, and GSH were detected. Differences in phloem sap organic sulfur composition and contents between species and changes in response to different environmental conditions have been reviewed previously (Herschbach and Rennenberg 2001a, b; Herschbach 2003a, b). Organic sulfur in the phloem differs along the trunk (Herschbach et al. 1998, 2010b) and due to environmental changes (Schulte et al. 2002; Herschbach et al. 2005), but not in response to short-term variations in sulfur fertilization (Herschbach and Rennenberg 2001a). Elevated CO_2 reduced GSH but not Cys contents in phloem exudates of *Quercus ilex* seedlings (Schulte et al. 2002). Flooding of poplars, i.e., anoxia of the root system, enhanced GSH and sulfate contents in phloem exudates (Herschbach et al. 2005). Also H_2S fumigation of the shoot caused an enrichment of GSH in phloem exudates of poplar (Herschbach et al. 2000). Irrespective of these fluctuations, the tripeptide GSH is usually the most abundant organic sulfur compound in the phloem (Herschbach and Rennenberg 2001b).

Whereas sucrose exhibits highest phloem concentrations in stem sections where mature leaves are inserted, Cys, Met, and GSH were highest where young, developing leaves are present, i.e., in the upper part of the stem (Herschbach et al. 1998, 2010b). The reason for this distribution of organic sulfur could either be a strong withdrawal of reduced sulfur out of the phloem into storage tissues of the trunk or a strong dependency on the loading capacity of the leaf source. Both conclusions are supported by published results. ^{35}S-labeled GSH, detected in phloem exudates when ^{35}S-sulfate was fed to a mature poplar leaf, rapidly decreased with increasing distance from the fed leaf and supports phloem unloading of GSH along the transport path (Hartmann et al. 2000). On the other hand, the GSH content in leaves strongly correlates with the GSH content in phloem exudates (Herschbach et al. 2000). As sulfate assimilation operates in both, young developing and mature leaves (Herschbach 2003b; Hartmann et al. 2000), the surplus of reduced sulfur produced in both types of leaves seems to be transported into the phloem.

The peak levels of carbohydrates in phloem exudates in the vicinity of stem section with mature leaves indicate that mature leaves are the main source for sucrose in phloem saps, whereas lower sucrose concentrations in phloem exudates from stem section with young or senescent leaves indicate that these leaves are likely to be minor sources or even sinks (Herschbach et al. 1998, 2010b). This view has been verified with *Populus deltoides* by ^{14}C–CO_2 application (Dickson 1989, 1991). In these studies, mature leaves exported radiolabeled ^{14}C-labeled compounds in both, apical and basipetal directions, whereas young leaves exported ^{14}C-labeled assimilates mostly into the apical plant part and older senescent leaves mostly into basal parts of the poplar plants. Thus, the bulk flow of sucrose in the phloem sap of poplar is bidirectional and is directed to sites of low sugar concentration (Münch 1930), i.e., in the case of poplar to young leaves and to the roots. Other compounds that are present in lower amounts and do not contribute to the osmotic potential are thought to be passively transported within the bulk flow buildup by sucrose (Münch 1930). Thus, from a theoretical point of view, transport of sulfur from developing leaves to the roots seems unlikely, but transport of sulfur from mature to developing leaves can be assumed. Labeling experiments with ^{35}S-sulfur indicated basipetal and apical transport of ^{35}S from mature poplar leaves

(Hartmann et al. 2000). However, sulfur in the form of ^{35}S-sulfate was transported in both acropetal and basipetal direction, while ^{35}S-GSH was exclusively transported in basipetal direction (Hartmann et al. 2000). As distinct specialized phloem loading for apical or for basal transport is highly unlikely, only ^{35}S-sulfate or ^{35}S-GSH transport can be explained by cotransport with sucrose bulk flow in the phloem. As carbon assimilate transport was not analyzed in the same poplar plants, it is not known whether the fed leaves allocated carbon assimilates exclusively into basipetal or into both directions. In addition, it cannot be excluded from these experiments with poplar that apical transport of ^{35}S-sulfate was achieved by a phloem-to-xylem exchange. Considerable amounts of ^{35}S-sulfur were detected only basipetal to the fed leaf in *Fagus sylvatica* (Herschbach and Rennenberg 1995, 1996) and *Quercus robur*, even if a new sprout developed during feeding (Schulte et al. 1998). Thus, in these deciduous trees, sulfur is mostly transported from leaves into the basal trunk and further to the roots. Again, this picture is different to carbon bulk flow that takes place into both, the roots and newly developing sprouts of *Quercus rubra* (Dickson et al. 1990; Dickson and Tomlinson 1996). Here, 90% of the exported ^{14}C-assimilates were allocated to a developing sprout and originated from mature leaves of the previous sprout. In summary, phloem transport of sulfur does not appear to generally follow carbon bulk flow in deciduous trees, but the mechanisms that enable differences between carbon and sulfur transport in the phloem have not been elucidated.

^{35}S-labeled sulfur in the form of sulfate, Cys, and GSH allocated to basal parts of poplar, beech, and oak were largely detected along the entire trunk in bark and wood tissues (Herschbach and Rennenberg 1995, 1996; Schulte et al. 1998; Hartmann et al. 2000). Apparently, sulfate and reduced sulfur compounds are unloaded from the phloem and stored in trunk tissues. In addition, metabolization, i.e., sulfate reduction and assimilation in bark and wood parenchyma cells, has to be considered, because the activity of the key enzyme of the sulfate reduction pathway, APS reductase (Kopriva 2006; Vauclare et al. 2002), has been detected in poplar bark and wood tissues (Herschbach, unpublished results). Environmental changes may influence total sulfur export and/or the sink strength of tissues for exported sulfur. When anaerobic conditions prevail in the root system during flooding, total sulfur export out of old mature poplar leaves was comparable to the export observed in nonflooded controls, but the sink for the sulfur translocated in the phloem shifted from roots to basal bark sections (Herschbach et al. 2005). The total rate of export of ^{35}S-GSH out of *Quercus robur* leaves was enhanced under elevated CO_2, while mycorrhization reduced sulfur transport into roots (Schulte et al. 1998). Irrespective of these factors that modulate sulfur allocation, sulfur transported from the leaves of deciduous trees is mostly incorporated into basal stem sections where it can be stored in the form of acid-soluble or -insoluble compounds (Herschbach and Rennenberg 1995, 1996; Schulte et al. 1998; Hartmann et al. 2000).

In contrast, in conifers, ^{35}S-GSH was not transported from older needle generations (1-year and 3-year old needles) into basal parts of the branch, but almost exclusively into acropetal direction by phloem and/or xylem transport (Schupp et al. 1992; Schneider et al. 1994a; Blaschke et al. 1996). Particular photoassimilates were found to be transported from 2-year old needles to basal stem sections of the branches

and also to the roots, whereas previous year needles export photoassimilates to the current year's needle generation only (Ericsson 1978). Apparently, there is a functional separation of different needle age classes with respect to organic C, but not with respect to organic S export. Transport of reduced S to current year needles is required, because the current year needle generation of spruce is – different to young leaves of deciduous trees – not self sufficient in sulfate assimilation, even in autumn of the current year (Schupp and Rennenberg 1992). Obviously, conifers show a completely different transport pattern for sulfur compared to deciduous trees. But, in both, deciduous trees and conifers, sulfur transport in the phloem differs from photoassimilate translocation. This can only be explained by a separation of the transport paths for sulfur and sucrose.

Irrespective of these observations from studies on sulfur export from different needle age classes, total sulfur in spruce roots increased upon exposure of the shoot to atmospheric SO_2 or H_2S. Apparently, spruce trees are able to switch from pedospheric sulfate to atmospheric sulfur as a sulfur source for growth and development and, in this context, also seem to change the direction of phloem transport of sulfur out of the needles (Tausz et al. 2003). This view is consistent with the observation that the sulfur content of spruce wood reflects the atmospheric SO_2 gas mixing ratio (Barrelet et al. 2008).

2.2 Nitrogen Compounds

The main phloem transport forms of nitrogen are amino acids (e.g., Peuke 2010). In general, the phloem-loading mechanism of amino compounds includes amino acid efflux from the symplast of the source tissue to the apoplast and subsequent carrier-mediated transport, which couples proton gradients to the active amino acid transport and accumulation against concentration gradients. Plant amino acid transporters involved in phloem loading are generally characterized by compound overlapping transport specificities and they originate mainly from two superfamilies (the amino acid-polyamine-choline transporter superfamily, the amino acid supertransporter family 1; for an extensive review see Lalonde et al. 2004).

The active transport of amino compounds into the sieve tubes and the general regulation of long-distance phloem transport of nitrogen are assumed to be at least as complex as sugar transport mainly because the substrates for transport are highly diverse (Lalonde et al. 2004). In addition to the twenty proteinogenic amino acids, a set of nonproteinogenic amino compounds such as γ-aminobutyric acid (e.g., Schneider et al. 1996; Scarano et al. 2005; Pfautsch et al. 2009) and various oligo-peptides (e.g., Fiehn 2003) are transported in the phloem. The relative importance of the supply of different plant tissues with amino compounds via the phloem varies mainly with the location of nitrate and/or ammonium reduction.

In herbaceous species, nitrate taken up by the roots can be either directly reduced in belowground tissues, stored in the root vacuoles, or transported to the leaves for nitrate reduction and/or vacuolar storage (Tischner 2000). A general preference of

shoot over root nitrate assimilation has been postulated for herbs and grasses (c.f. Scheurwater et al. 2002). Ammonium taken up by roots of herbaceous plants is normally assimilated in the roots but also xylem transport of ammonium and assimilation in the shoot has been observed (Finnemann and Schjoerring 1999; Mattsson and Schjoerring 1996). Depending on the nitrogen form supplied and on root-to-shoot partitioning of nitrogen assimilation, phloem transport can play a central role in the nitrogen (re-)distribution within the plant. Under conditions when inorganic nitrogen is primarily reduced in the autotrophic tissues of herbaceous species, the supply of heterotrophic tissues (including developing seeds and fruits) with organic nitrogen has to be completely provided by phloem transport.

In many trees, nitrate and ammonium taken up by mycorrhizal and nonmycorrhizal roots are mainly (under natural conditions, with nonexcess N supply) assimilated to amino compounds in the below-ground tree parts (Gojon et al. 1994). As a result, predominantly organic nitrogen compounds and only traces of NO_3^- or NH_4^+ are allocated from the roots to the shoots via xylem transport (Dambrine et al. 1995; Gessler et al. 1998a; Schmidt and Stewart 1998; Franco et al. 2005; Fotelli et al. 2009). Consequently, roots and trunk tissues in trees do not exclusively depend in their organic nitrogen demand on basipetal phloem transport. Roots might directly consume root-assimilated amino acids and the trunk might at least partially be supplied with organic nitrogen compounds transported in the xylem.

Among the most abundant amino compounds transported in the phloem in herbaceous species are glutamine (Gln), glutamate (Glu), aspartate (Asp), and serine (Ser) (Amiard et al. 2004; Arlt et al. 2001; Lohaus and Moellers 2000). Applying metabolome analysis, Fiehn (2003) characterized many different amino compounds in vascular exudates from leaf petioles of *Cucurbita maxima* at concentrations several orders of magnitude higher than in tissue disks from the same leaves and also found a large number of unknown nitrogen-containing compounds. Main transport amino compounds in the phloem of trees and other perennial woody species are not only Gln, Glu, Asn, and arginine (Arg) (Schneider et al. 1996; Gessler et al. 1998a; Fotelli et al. 2002), but also proline (Pro), which is known to act as osmoprotectant (Palfi et al. 1974), is transported, especially under drought conditions (Peuke et al. 2002a, b; Gessler et al. 2004) and might also be involved in shoot-to-root signaling and regulation of nitrogen uptake under stress conditions (Lee et al. 2009).

During the early phase of foliage development in trees and herbs and before a significant transpiration stream is present, organic nitrogen is transported to the unfolding leaves via the phloem (Jeschke and Pate 1991; Gessler et al. 1998a) in acropetal direction. During this period, increased amounts of Arg, which contains 4 atoms of N per molecule and, thus, is an efficient N-transport substance, were found in the phloem of young and adult beech trees (Fotelli et al. 2002; Nahm et al. 2006). During leaf senescence in turn, reallocation of nitrogen from the leaves to the storage tissues in the stem of deciduous beech also causes Arg concentrations in phloem exudates to increase (Gessler et al. 1998a).

There is not much information on diel variations of amino acid concentrations in the phloem, neither in herbaceous nor in woody species. Whereas concentrations of

phloem carbohydrates are known to vary between day and night (Fiehn 2003; Peuke et al. 2001; Gattolin et al. 2008), the concentration of the most abundant phloem-transported amino acid Gln did not change over a diel course in *Ricinus communis* (Peuke et al. 2001). As concentrations of other amino compounds, however, decreased during night in this species, a compound-specific regulation of phloem loading or variation in amino acid concentrations in the source tissues might be assumed.

3 Xylem Transport

3.1 Sulfur Compounds

In xylem saps, sulfur is present mainly as sulfate, but also reduced sulfur compounds have been frequently detected, though in lower amounts (Herschbach and Rennenberg 2001a, b). Contents and composition of sulfur compounds in the xylem sap differ between species, during the season, with sulfur nutrition, during developmental stages, along the trunk and due to mycorhization. These aspects have been discussed for trees in detail by Herschbach and Rennenberg (2001a, b). In summary: (1) Sulfate and reduced sulfur in the xylem sap are highest during spring, when sulfur is mobilized from the storage tissues of the trunk (Schupp et al. 1991; Rennenberg et al. 1994a, b; Schneider et al. 1994b). (2) In contrast to deciduous trees, peak values of sulfate and GSH in the xylem sap of the conifer *Picea abies* decline only slowly from early spring to early summer (Köstner et al. 1998) indicating a prolonged mobilization phase of sulfur from older needles to supply the current needle generation with sulfur. (3) Cysteine is the main reduced sulfur component in the xylem sap of beech and, depending on the season, increases or decreases with increasing distance to the roots due to storage and mobilization processes (Rennenberg et al. 1994b). The latter observation indicates loading of sulfur from storage tissues into the xylem and unloading of sulfur from the xylem (Rennenberg et al. 1994b). Similar studies for herbaceous plants are lacking.

Sulfur accumulation in the wood has been demonstrated for beech (Eschrich et al. 1988; Herschbach, unpublished results) and for Norway spruce (Barrelet et al. 2006, 2008; Struis et al. 2008; Ulrich et al. 2009). Eschrich et al. (1988) showed that sulfur is enriched in the living cells of beech wood, i.e., sieve element, ray, and pith cells. Norway spruce wood showed that seasonal variations in the sulfur content within the year rings with highest sulfur amounts at the transition from late to early wood (Barrelet et al. 2006). Furthermore, Struis et al. (2008) identified the sulfur in the late wood as sulfate, organic sulfonates, disulfide, and methylthiol. Altogether, these studies demonstrated sulfur storage in the wood of both, conifers and deciduous trees.

Although several sulfate transporters are detected in the vasculature and in the xylem parenchyma of leaf and roots of *Arabidopsis* (Takahashi et al. 2000; Kataoka

et al. 2004), clear evidences whether these transporters release sulfate into the xylem are lacking. Similarly, nothing is known about transporters involved in the efflux of organic sulfur compounds into the xylem from xylem parenchyma cells of the roots and/or from storage tissues along the trunk. Action of such transporters is required, since it is evident from labeling experiments that sulfate taken up by the roots is transported to and accumulated in the leaves (Herschbach and Rennenberg 1994, 1995; and for an example Fitzgerald et al. 1999a, b; Larsson et al. 1991). Beside sulfate, GSH can also be taken up by the roots, loaded into the xylem, and transported to the leaves via the transpiration stream. GSH uptake has been demonstrated for tobacco cell cultures (Rennenberg et al. 1988; Schneider et al. 1992) and with excised mycorrhizal roots from *Quercus robur* (Seegmüller and Rennenberg 2002). Thus, GSH in the xylem sap may not only originate from phloem-to-xylem exchange of GSH allocated from the leaves to the roots or from GSH synthesis in wood and/or bark, but also from the synthesis in, or uptake by the roots.

Knowledge about transporters involved in the uptake of organic sulfur and sulfate from the xylem sap into xylem parenchyma cells of leaves and/or into the living cells of the wood of perennial plants is also scarce. Two sulfate transporter transcripts (*SULTR1;1* and *SULTR3;3a*) are expressed in ray pith cells that are attached to xylem vessels and in parenchyma cells of the primary xylem along the trunk of young poplar plants (Dürr et al. 2010). These sulfate transporters could realize sulfate unloading from the xylem. Further sulfate transporter transcripts were detected in poplar wood that might also be involved in xylem loading and/or unloading (Dürr et al. 2010). Several studies have identified and described putative GSH transporters (Bogs et al. 2003; Cagnac et al. 2004; Zhang et al. 2004; Osawa et al. 2006; Pike et al. 2009). Transport of GSH can be mediated by oligopeptide transporters (OPT) as indicated recently by a complementation study with AtOPT6 in oocytes (Pike et al. 2009). However, OPTs do not specifically transport GSH (Cagnac et al. 2004). For example, the affinity of AtOPT6 to GSH is low and AtOPT6 transports also many tetra- and pentapeptides as well as other peptides up to 12–13 amino acids in length (Pike et al. 2009). As At*OPT6* and other OTPs are expressed in the vasculature of roots, stem, and leaves, it might be assumed that these transporters are involved in long-distance peptide transport and/or peptide distribution throughout the plant (Stacey et al. 2006; Tsay et al. 2007; Pike et al. 2009) and thus these OPT's may also contribute to the distribution of reduced sulfur within the plant (Pike et al. 2009). The cellular localization of the putative GSH transporters, i.e., whether they are expressed in the xylem and/or in the phloem, however, is not obvious from the studies with *Arabidopsis*, but is required to identify their function at the whole plant level. Still, loading of reduced sulfur compounds into the xylem and its unloading from the xylem via specific transporters remains to be proven experimentally, especially for woody plants where information about GSH transporters is not available.

3.2 Nitrogen Compounds

The composition of the nitrogen pool transported in the xylem of plants depends in part on the nitrogen source and on the root–shoot partitioning of the assimilation of inorganic nitrogen (Andrews 1986). When nitrate is the major nitrogen source and when its assimilation is restricted to the shoot as observed in various herbaceous species, nitrate will at least play an important if not the dominant role for nitrogen transport in the xylem. High concentrations of nitrate in the xylem sap have been observed in annual and perennial herbaceous species such as tobacco (Kruse et al. 2002), *Ricinus communis* (Peuke et al. 2001), tomato (Bialczyk et al. 2004), sunflower (Azedo-Silva et al. 2004), and in grasses (Macduff and Bakken 2003). In many tree species, a general assumption is that nitrate taken up from the soil is assimilated in the mycorrhizal roots so that nitrate is transported in the xylem only in traces (e.g., Dambrine et al. 1995; Martin and Amraoui 1989; Gessler et al. 1998a). There are, however, some reports of high concentrations of nitrate in the xylem sap of trees (e.g., Glavac and Jochheim 1993). It was concluded that even though the roots are in general the main sites of nitrate reduction, high nitrate availability and, thus, high uptake rates might exceed the assimilation capacity in the roots and, as a consequence, part of the nitrogen taken up is loaded into the xylem in its inorganic form. This may be considered a process that prevents nitrate leaching into the groundwater at temporarily high nitrate availability to the roots in ecosystems adapted to generally low nitrogen availability (Rennenberg and Schmidt 2010).

For ammonium, it is presumed that assimilation occurs directly in the roots in both, herbaceous and woody species (Miller and Cramer 2005). There is, however, some information for herbaceous plants that ~10% of the nitrogen transported in the xylem can be ammonium when ammonium is the sole nitrogen source (Schjoerring et al. 2002). The authors of the abovementioned study concluded that under conditions of very high ammonium supply, root glutamine synthetase is inhibited and that consequently ammonium is loaded into the xylem. However, even under these conditions, 90% of the nitrogen is transported in the xylem in assimilated forms. Also in nitrate-supplied plants, which assimilate the inorganic nitrogen in the leaves, reduced nitrogen compounds contribute significantly to the N-transport in the xylem. In tobacco, where the actual rate of nitrate reduction in leaves can exceed that in roots by a factor of almost twenty, organic nitrogen compounds contribute ~30% to xylem nitrogen transport (Kruse et al. 2002).

The most important organic nitrogen compounds present in the xylem of plants are amino acids and ureides. Ureids like allantoine or allantoic acid are mainly present in the xylem of legumes actively fixing atmospheric N_2 in symbiosis with *Rhizobia* (Thavarajah et al. 2005). But, also the amides Gln and Asn have been shown to be main xylem transport forms for nitrogen in N_2-fixing plants (e.g., Gessler et al. 2005a). It is known that especially boreal tree species take up considerable amounts of organic nitrogen compounds from the soil (Näsholm

et al. 1998; Lipson and Nasholm 2001), that a significant number of species are capable of amino acid uptake by the roots (Näsholm et al. 2009) and that organic and inorganic nitrogen uptake interact (Stoelken et al. 2010). There is, however, no information if varying proportions of inorganic and organic nitrogen taken up by the roots affect the amino acid composition in the xylem sap.

A highly diverse profile of amino compounds is transported in the xylem that depends on species but also varies with developmental stage, environmental conditions, and during the growing season (Gessler et al. 2004). In species of the Australian woodlands and deciduous monsoon rainforest, for example, Arg was the main nitrogen compound in the xylem sap at the peak of the dry season (Schmidt and Stewart 1998). In the wet season, however, the main proportion of xylem-transported nitrogen was made up from nitrate (monsoon forest species) or amides (woodland species and some monsoon forest species). The authors attributed this switch in transport compounds to the source of nitrogen available for the trees at the different time points. When water availability was high, nitrogen was taken up from the soil and transported either in the form of nitrate or amides. Under dry conditions, nitrogen remobilized from arginine-rich proteins in the stem-storage tissues was assumed to be responsible for the high Arg concentrations (c.f. Rennenberg et al. 2010). Comparable seasonal variations related to storage and remobilization has also been described in temperate forest tree species (Nahm et al. 2006; Gessler et al. 1998a).

As the xylem vessels are part of the apoplastic space, amino compounds transported in the xylem have to be exported from the symplast. Until present, we lack a clear-cut picture on the mechanisms of this export, i.e., if it is driven by carrier-mediated export or by vesicular transport (Lalonde et al. 2004). As not only differences in biochemistry but also selective xylem loading might be responsible for variations in the composition of the xylem amino acid pool, information on export processes and their regulation might help to interpret temporal patterns in the nitrogen compound profile in the xylem sap.

4 Phloem/Xylem Exchange and Cycling

Phloem and xylem transport alone are not adequate to supply the whole plant with nitrogen (Jeschke and Hartung 2000; Peuke 2010) and sulfur (Herschbach et al. 2000; Kruse et al. 2007) and to coordinatively adapt root nitrogen and sulfur uptake to the nitrogen and sulfur demand of the whole plant (Gessler et al. 2004; Herschbach 2003b). In annual or perennial herbaceous plants, signaling of the nitrogen and sulfur status to and the adaptation of nitrogen and S uptake by the roots plays an important role during particular developmental stages, e.g., during seed production (Imsande and Touraine 1994; Hawkesford and De Kok 2006), whereas for long-lived tree species such an adaptation of the nitrogen and sulfur uptake to changing environmental conditions and nutrient supply is crucial during their whole life span (Gessler et al. 2004; Herschbach and Rennenberg 1996). In

addition, pedospheric nitrogen and sulfur uptake of trees has to be tailored not only to the actual nitrogen and sulfur demand, but also to the nitrogen and sulfur-storage capacity at the whole plant level. Communication between shoots and roots and vice versa to achieve a nitrogen and sulfur supply–demand balance at fluctuating nitrogen and sulfur supply can be attained by a pool of amino and sulfur compounds cycling through the plant (Fig. 1). This pool is fed at the sites of uptake of organic or inorganic nitrogen and sulfur or at the sites of remobilization of stored nitrogen and sulfur. Nitrogen and S are released from this pool at the sites of nitrogen and sulfur demand (e.g., cambial or storage tissues). A prerequisite for this cycling is the bidirectional exchange between the xylem and phloem and in the opposite direction (Peuke et al. 2002a, b). In trees, this exchange could take place at the whole trunk and/or in roots as phloem-to-xylem and the shoot as xylem-to-phloem exchange.

4.1 Sulfur Compounds

Xylem-to-phloem exchange of sulfur is evident for the leaves of all plants, but has not been proved along the trunk of trees (Fig. 2). Sulfate that reached the leaves via the xylem can either be stored in the vacuole (Bell et al. 1994), assimilated into GSH (Kopriva and Rennenberg 2004; Rausch et al. 2007), or can be reloaded into the phloem (Lappartient et al. 1999; Herschbach et al. 2000). After metabolization, i.e., after sulfate assimilation, sulfur metabolites such as GSH can also be loaded into the phloem (Herschbach et al. 1998; Lappartient et al. 1999). Thus, the leaves are sites for xylem-to-phloem exchange directly for sulfate and also after interconversion into further sulfur-containing compounds (Chen et al. 2006; Li et al. 2006).

Phloem-to-xylem exchange of sulfur was first demonstrated for spruce. Removal of the bark (i.e., girdling) apical to a fed needle did not alter ^{35}S-sulfur allocation to current year needles applied as ^{35}S-GSH through a needle tip of 1-year-old needles (Schneider et al. 1994a). For ^{35}S transport into current year needles, the ^{35}S-sulfur must be transported first into the phloem for the transport up to the needle base. Then for apical translocation to the current year needles, ^{35}S-sulfur must be exchanged from the phloem to the xylem (Schneider et al. 1994a). The sulfur compound exchanged from the phloem to the xylem was not determined in this girdling experiment, but after feeding ^{35}S-GSH to 1-year-old needle tips only ^{35}S-GSH and traces of ^{35}S-sulfate were detected in the bark (Schneider et al. 1994a). From these results it was concluded that GSH is transported in the phloem to the current year needles that possess a low sulfate-reduction capacity (Schupp and Rennenberg 1992). After feeding ^{35}S-γ-EC or ^{35}S-Cys, the sulfur compounds ^{35}S-sulfate, ^{35}S-Cys, ^{35}S-γ-EC, as well as ^{35}S-GSH were detected in the apical sprout above the fed needle in the wood and in current year needles (Blaschke et al. 1996). Thus, part of the reduced sulfur fed was oxidized to sulfate. Sulfate is the dominant sulfur compound in the xylem sap of the trunk of old-growth spruce trees and is present in concentrations 100-fold higher than GSH (Köstner et al. 1998). From these findings, organic sulfur from storage pools in previous year needles

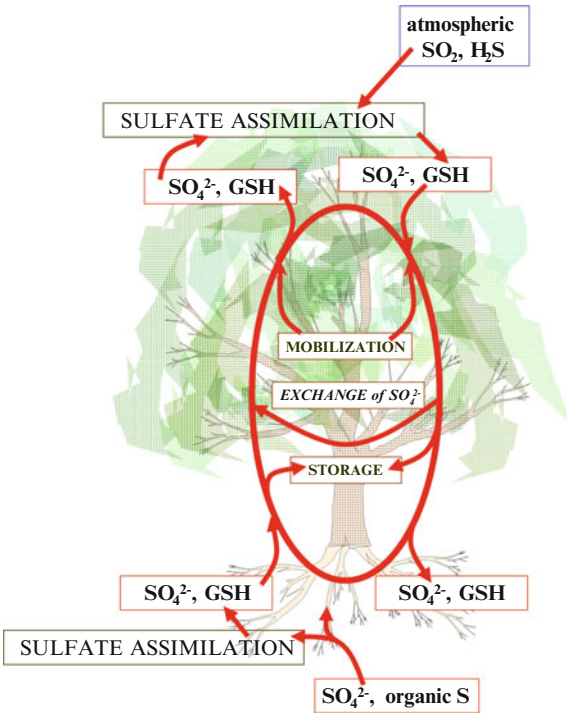

Fig. 2 Model of sulfur cycling in deciduous trees. *Red arrows* indicate the flow of sulfur metabolites. The flow starts from sulfate and probably GSH uptake. Sulfate assimilation in roots is possible, but its significance in sulfur nutrition has not been proved. Sulfate and GSH are loaded into the xylem, transported with the transpiration stream, and taken up by the leaves. Sulfate in the leaves can be assimilated and both sulfate as well as GSH can be loaded into the phloem and transported to the roots. Along the transport path, both compounds can be unloaded and stored in bark and wood tissues. In the roots, GSH can be degraded into its constituent amino acids which can then be used for root growth and development; alternatively, sulfate and GSH may be reloaded into the xylem or released into the rhizosphere

seems to be fed into a large sulfate pool already present in the xylem. This indicates a high availability of sulfate to current year needles. Still, significant sulfate reduction and assimilation in current year needles is unlikely because of an insufficient sulfate-reduction capacity (Schupp and Rennenberg 1992).

From published data, it can be concluded that sulfur cycling in spruce seems more relevant within the individual twig in a time-dependent manner, i.e., during seasonal development, than at the whole plant level. Although, total sulfur in spruce roots increases upon exposure of the shoot to atmospheric SO_2 or H_2S (Tausz et al. 2003) and, although sulfur storage in the spruce wood was indicated by seasonal variations of sulfur contents in year rings (Barrelet et al. 2006; Struis et al. 2008), sulfur mobilization and, thus, phloem-to-xylem exchange along the trunk and in roots has not been proven, even not during seasonal growth. Nevertheless, high amounts of GSH and sulfate were detected during spring in xylem saps of spruce

twigs (Köstner et al. 1998). As the older needles are supposed to be sources for GSH of the current year needles (Schupp et al. 1992; Schneider et al. 1994a; Blaschke et al. 1996), these results support sulfur cycling within individual twigs.

In contrast to conifers, both ^{35}S-sulfate and reduced sulfur, in form of ^{35}S-Cys and ^{35}S-GSH, were detected below the fed leaf in the bark and in phloem exudates down to the roots in poplar (Hartmann et al. 2000; Herschbach et al. 2010a), beech (Herschbach and Rennenberg 1996), and oak (Schulte et al. 1998). It was concluded that reduced sulfur and sulfate are loaded into the phloem and are transported along the trunk down to the root system. Still, the occurrence of ^{35}S-GSH in bark and phloem exudates declined with increasing distance to the fed leaf in poplar (Hartmann et al. 2000) and beech (Herschbach and Rennenberg 1996). Apparently, GSH is unloaded from the phloem but is not exchanged from the phloem to the xylem (Herschbach and Rennenberg 1995, 1996; Schulte et al. 1998; Hartmann et al. 2000). This is evident from lacking ^{35}S acropetal to the fed leaf in beech (Herschbach and Rennenberg 1995, 1996) and in oak, irrespective whether new sprouts developed in oak (Schulte et al. 1998). In poplar, however, ^{35}S-sulfate was detected within the shoot apex and along the transport path apical to the fed leaf in bark and wood (Hartmann et al. 2000). As discussed above, both phloem and xylem transport could be responsible for the sulfate transport to the poplar shoot apex. As xylem sap was not analyzed in this study, a clear answer to this assumption can only be achieved by labeling experiments after stem girdling acropetal to the fed leaf as previously done with spruce (Schneider et al. 1994a).

In deciduous trees, sulfur exchange from the phloem to the xylem can be assumed along the entire trunk during seasonal growth (Fig. 2). This is evident from increasing GSH contents in the xylem sap of poplar (Schneider et al. 1994b) and beech (Schupp et al. 1991; Rennenberg et al. 1994b) during spring and from labeling experiments with beech. Labeled ^{35}S-sulfate was fed to mature beech leaves and the ^{35}S-sulfur distribution was analyzed over one entire growing season (Herschbach and Rennenberg 1996). During late summer and autumn, ^{35}S-sulfur accumulated along the entire trunk in bark and wood as soluble and insoluble ^{35}S-sulfur. In spring, soluble as well as insoluble ^{35}S-sulfur decreased in the trunk and increased in buds and young beech leaves (Herschbach and Rennenberg 1996). GSH, Cys, and sulfate that accumulated during autumn in bark and wood tissues decreased during spring (Herschbach, unpublished results; Dürr et al. 2010). Beside this clear evidence for sulfur storage and mobilization, these findings indicate a time-dependent exchange of sulfur from the phloem to the xylem.

Storage and mobilization of sulfur compounds is regulated at the transcriptional level via distinct transporters. Transcripts of putative sulfate transporters that might be involved in sulfate mobilization from the vacuole (Pta*SULTR4;2*) and in phloem loading (Pta*SULTR3;3a*) increased in the bark of poplar during spring, when simultaneously the sulfate content in the bark declined. In autumn, both sulfate transporter transcripts declined and were finally no longer detectable in the bark tissue when simultaneously the sulfate content in the bark of poplar increased (Dürr et al. 2010). Whether GSH and/or reduced sulfur transport and cycling within deciduous trees are regulated by transporter expression is worth investigating in future.

Phloem-to-xylem exchange may also occur in roots. Phloem transport of GSH to the roots and sulfate reduction in the roots (Herschbach et al. 2010a; Scheerer et al. 2010) together may permit GSH loading into the root xylem and, thus, phloem-to-xylem exchange. Although split root experiments with wheat (Larsson et al. 1991) demonstrated that sulfate, but not reduced sulfur, seems exchangeable between the phloem and the xylem in roots, experiments with tobacco supplied clear evidence that reduced sulfur in form of GSH exchanged from the phloem to the xylem in roots (Kruse et al. 2007) and, thus, contributes to a cycling pool of reduced sulfur. Xylem loading of ^{35}S-GSH was demonstrated with excised mycorrhizal roots of *Quercus robur* at rates of 4–6 nmol g^{-1} fw h^{-1} (Seegmüller and Rennenberg 2002). Thus, reduced sulfur in the form of GSH may also exchange between phloem and xylem in the roots of trees and, as a consequence, may cycle within trees. This assumption must, however, be proven in future investigations.

The sulfur that reaches the roots by phloem transport can contribute to the reduced sulfur demand of the roots and can be involved in the regulation of sulfate uptake. Experiments with tobacco revealed the dependency of tobacco roots on the reduced sulfur in the form of GSH transported from the shoot to the roots in the phloem (Kruse et al. 2007). Girdling experiments showed that poplar roots partially depend on reduced sulfur, i.e., on GSH allocation from the shoot (Herschbach et al. 2010a). Studies with herbaceous plants (Herschbach et al. 1995a, b; Lappartient et al. 1999) and trees (Herschbach et al. 2000) indicate that a surplus of reduced sulfur produced in the leaves is transported via the phloem to the roots and can diminish sulfate uptake.

In conclusion, sulfur cycling at the whole tree level can be described as a process including xylem-to-phloem exchange in the leaves and phloem-to-xylem exchange in the roots, but seems also to proceed in a time-dependent manner during storage and mobilization processes for seasonal growth thereby including phloem-to-xylem exchange of sulfur along the entire trunk (Fig. 2). Basal and apical transport, respectively, during the seasonal growth cycle correlate with sulfur storage during vegetative growth and sulfur mobilization in spring. A comparable pattern was observed in the seasonal course of herbaceous plants when they switched from vegetative to generative growth. In wheat, for an example, sulfur is recycled to the grains under sufficient sulfur supply, but at sulfur depletion sulfur is remobilized from proteins during grain development (Fitzgerald et al. 1999a,b).

Sulfur cycling has been investigated under different nitrogen nutrition regimes and in nitrate-reductase-deficient tobacco plants (Kruse et al. 2007). Here, it was found that the majority of sulfate transported in the xylem from roots to the shoot was reallocated to the roots by phloem transport. In relation to the rate of sulfate uptake, this cycling sulfate pool was lower in ammonium nitrate than in nitrate-fed tobacco plants. This appears to be a result of sulfate accumulation in leaves which probably originates from decreased phloem reallocation in ammonium-nitrate-fed plants. When the nitrate reductase activity was blocked in tobacco roots, a smaller fraction of the sulfate taken up remained in the roots compared to wild-type tobacco, whereas the fraction of the sulfate taken up that accumulates in the leaves did not vary (Kruse et al. 2007). These findings suggest changes in sulfur cycling

depending on the nitrogen supply. As nitrogen nutrition is a major environmental factor that restricts plant growth (c.f. Rennenberg and Schmidt 2010) and nitrate and ammonium taken up by mycorrhizal and nonmycorrhizal roots are mainly assimilated to amino compounds in below-ground tree parts under natural conditions (Gojon et al. 1994), it is worth further investigating, in future studies, the influence of nitrogen fertilization on sulfur cycling in trees.

4.2 Nitrogen Compounds

In young spruce (Gessler et al. 2003), different amino compounds are subject to a bidirectional exchange between xylem and phloem of twigs. Additional experiments with adult spruce and beech (Gessler et al. 1998a, 2003) showed that different amino compounds present in the xylem were also found in the phloem, especially when there were no sink tissues to be supplied. Gln, the main nitrogen transport form in many tree species (Malaguti et al. 2001; Grassi et al. 2003), was shown to be exchanged between xylem and phloem very intensively. For herbaceous species, a similar exchange for Asn between the two transport systems has been observed (e.g., Atkins et al. 1980).

The exchange between xylem and phloem and the reallocation of amino compounds not utilized in the canopy via the phloem to the roots are the basis for the regulatory cycling pool of amino acids in the plant (Fig. 3) (Gessler et al. 2005b). This cycling pool can serve on the one hand as intermediate-term nitrogen storage partially uncoupling the nitrogen demand of the tree from the actual external supply, and fulfils on the other hand regulatory functions (Gessler et al. 2004). When nitrogen uptake and transport of soluble nitrogen compounds to the shoot exceed the demand of above-ground tissues, the phloem allocated pool of amino compounds expands (Youssefi et al. 2000). The subsequent increase in the contents of amino compounds in the roots results in a repression of nitrate net uptake on the transcriptional or post-transcriptional level (Collier et al. 2003; Dluzniewska et al. 2006). In general, phloem-translocated Gln is supposed to be the amino compound primarily involved in this downregulation (Gessler et al. 1998b), but depending on species also other amino acids might play a role (Lee et al. 2009; Merchant et al. 2010).

The assessment of individual amino compounds in xylem and phloem of trees (e.g. Stoermer et al. 1997; Gessler et al. 1998a) showed that not only direct exchange of amino compounds between xylem and phloem, but also metabolic interconversion occurs. In spruce trees, mainly Gln and Asp are transported in the xylem from the roots to the shoots, but Arg accumulates in the needles and in the twig phloem in addition to Gln (Stoermer et al. 1997). As mentioned above, particular nitrogen compounds like Gln transported back to the root via the phloem can exert control over the nitrogen uptake by roots and, thus, metabolic conversion from one compound to another might be crucial for the fine-tuning of whole plant signaling. In addition to xylem-borne amino compounds, shoot assimilated nitrogen can be loaded into the phloem. Nitrogen can also be taken up by the leaves in the form of nitrogen oxides or ammonia and is

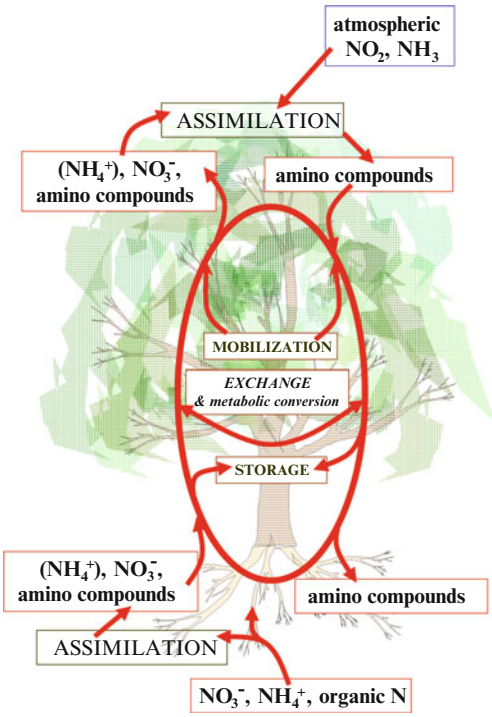

Fig. 3 Model of nitrogen cycling in deciduous trees. The *red arrows* indicate the flow of nitrogen compounds into and within the tree. Nitrogen can be taken up by roots and leaves and assimilation – though mainly located in the mycorrhizal roots – might be partitioned between roots and shoots depending on nitrogen supply. Roots can also take up organic nitrogen, and the various nitrogen compounds are transported in the xylem with the transpiration stream to the canopy. Shoot/leaf-derived amino compounds are loaded into the phloem to supply the trunk and roots with nitrogen together with the part of xylem-borne amino acids which exceeds the demand of the leaves. During transport in either xylem or phloem, nitrogen compounds can be unloaded and stored or are exchanged bidirectionally between the two transport systems

assimilated in the shoot. When loaded into the phloem it also contributes to the cycling pool of amino compounds (Rennenberg and Gessler 1999).

Bidirectional xylem-to-phloem exchange not only takes place in the crown but also occurs in the trunk/stem or root region. Hence, not all amino compounds transported downwards within the phloem are supposed to originate from the top of the plant. For adult spruce trees (Weber et al. 1998), it was observed that Arg only cycled in the below-ground parts of the trees, since this compound could only be detected in the xylem and phloem of roots but was not transported in the xylem of the trunk or in the twigs (Rennenberg et al. 1998). The situation gets even more complex, when nitrogen remobilization in spring and storage in autumn is taken into account. Adult beech trees showed metabolic conversion of transported amino compounds during spring remobilization not only in leaves but also in the trunk (Gessler et al. 1998a). In autumn, during nitrogen storage, mainly Arg is transported

to the storage tissue via phloem transport and is additionally subject to phloem-to-xylem exchange in twigs and trunks (Gessler et al. 1998a).

Total nitrogen fluxes between xylem and phloem and phloem and xylem have been calculated integratively over nine days in *Ricinus communis* (Jeschke and Pate 1991). Nitrogen transfer from the xylem to the phloem in the stem amounted between <1% and 7% of the acropetal nitrogen transport in the xylem. In the roots, ~50% of the nitrogen arriving via phloem transport was retransferred from the xylem. In young poplar, between 4 and 15% of the newly taken up nitrogen cycled between shoots and roots (Dluzniewska et al. 2006). Kruse et al. (2002, 2003) showed that the CO_2 concentration as well as the amount of nitrogen supplied to the roots affected the internal cycling of nitrogen in tobacco and poplar. Increased atmospheric CO_2 concentrations increased root nitrate reduction in tobacco by a factor of ~10 leading to increased transport of reduced nitrogen compounds in the xylem. Even though the main site of nitrate reduction was still the shoot, the increased nitrate reduction in roots was sufficient to supply the below-ground tissues with organic nitrogen (Kruse et al. 2002). Elevated CO_2 concentrations decreased root nitrate uptake in poplar with sufficient nitrogen supply. Increased root-to-shoot ratio at deficient nitrogen supply coincided with enhanced nitrate reduction in the root and elevated CO_2 also enhanced the allocation of newly acquired nitrogen to the youngest leaves. Increased nitrate availability to the roots inhibited nitrate reduction in the roots totally and the entire nitrate taken up was assimilated in the shoots, whereas root nitrate reduction was dominant in the roots under nitrogen deficiency (Kruse et al. 2002) and intermediate nitrogen supply (Dluzniewska et al. 2006). As a consequence, with increasing nitrate supply roots changed from being an exporter of organic nitrogen to being a sink tissue supplied with amino compounds from the shoot via phloem transport (Kruse et al. 2003).

In order to obtain compound-specific nitrogen exchange rates between the two long-distance transport systems combined with information on metabolic interconversion of amino compounds, pulse or steady-state labeling approaches with ^{13}C and ^{15}N-labeled amino acids and metabolic flux analyses on the whole plant level are necessary. The approaches of Kruse et al. (2002, 2003) and Dluzniewska et al. (2006) point in this direction, but they did only differentiate between inorganic and organic nitrogen. In future approaches, a compound-specific resolution is required.

5 Conclusion

The nutritional requirement for a coordinated use of nitrogen and sulfur for protein synthesis and, hence, for growth and development at the whole plant level requires long-distance transport and plant internal cycling as common features. A further common feature is given in trees that have developed a seasonal adaptation in storage and mobilization of nutrients and assimilates (Jansson and Douglas 2007; Keskitalo et al. 2005; Fracheboud et al. 2009). For the two macronutrients sulfur and nitrogen these features are different. This may partially be a consequence of

differences in uptake and assimilation. Whereas sulfate is thought to be the main source of sulfur (Seegmüller and Rennenberg 2002; Hawkesford and De Kok 2006; Davidian and Kopriva 2010), nitrogen is also taken up in its reduced form, either as ammonium or as amino acids (Näsholm et al. 2009; Rennenberg et al. 2010). Only a minor amount of the sulfate taken up seems to be reduced and assimilated in the roots (Herschbach et al. 2010b) and the bulk is transported in the xylem to the leaves where it is reduced and assimilated (Brunold 1990). Thus, sulfate is a regular constituent of the xylem sap of all plants studied (reviewed in Herschbach and Rennenberg 2001b; Rennenberg et al. 2007). Also, reduced sulfur compounds such as GSH and Cys are usually present in appreciable amounts in the xylem sap of herbaceous plants as well as in trees (reviewed in Herschbach and Rennenberg 2001b; Rennenberg et al. 2007). By contrast, in numerous species, inorganic nitrogen taken up by the roots can be reduced and assimilated in both, roots and/or leaves (Tischner 2000). In the xylem sap of species that reduce and assimilate nitrogen almost exclusively in the roots, as observed for many temperate forest tree species, inorganic nitrogen in the form of nitrate is present only in trace amount and the xylem sap is dominated by assimilated nitrogen; particularly by the amino acids glutamine, asparagine, and arginine (Gessler et al. 1998a; Schneider et al. 1996). Inorganic and organic nitrogen as well as organic and inorganic sulfur are subject to phloem transport from the leaves to the roots and other plant organs. The soluble reduced sulfur content of the roots seems to depend on phloem transport of reduced sulfur from the leaves, indicating insufficient sulfate reduction and assimilation for growth and development of the roots (Herschbach et al. 2010a). Whereas sulfate is also phloem mobile, inorganic nitrogen is found in appreciable amounts in phloem exudates only under particular conditions when the atmospheric load of nitrate is excessively high (Gessler et al. 1998a). As a consequence, sulfate as well as organic nitrogen and sulfur, but not inorganic nitrogen, undergo phloem-to-xylem and/or xylem-to-phloem exchange during long-distance transport depending on the developmental state and, thus, on seasonal growth rhythms. Although the tissues involved in, and the processes responsible for this exchange have not been elucidated in detail, it appears that this exchange provides the functional basis for rapid local reactions of plants to changing nutritional requirements, stress and defense reactions. This is of particular significance for large organisms such as trees, where long-distance transport in the phloem from the leaves to the roots could take several days (e.g., Plain et al. 2009).

In perennial plants, long-distance transport of nitrogen and sulfur is also required in the context of storage and mobilization processes. Whereas nitrogen storage and mobilization is dominated by organic nitrogen, i.e., storage protein and amino acids (Wetzel et al. 1989; Rennenberg et al. 2010), with the exception of conifer needles, sulfur storage and mobilization is dominated by sulfate, and organic sulfur is mobilized from storage pools in much smaller amounts (Schupp et al. 1991; Rennenberg et al. 1994b; Schneider et al. 1994b). The latter may explain an efficient sulfate reduction and assimilation in young developing leaves (Hartmann et al. 2000; Herschbach 2003b). Storage and mobilization of nitrogen is strongly regulated by environmental factors such as temperature and day length, but the processes involved

in signal transduction of these factors are largely unknown (Rennenberg et al. 2010). Whether organic sulfur storage and mobilization are controlled by environmental factors remain to be elucidated in future investigations. Nevertheless, first results indicate that sulfate distribution and, thus, its storage and mobilization also seem to depend on day length and temperature (Dürr et al. 2010).

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Blue-Light-Activated Chloroplast Movements: Progress in the Last Decade

Halina Gabryś

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Abstract Chloroplasts accumulate in weakly illuminated regions of the cell and avoid regions exposed to strong light in various plant taxa. These orientation movements of chloroplasts are controlled only by blue light in the mesophyll of vascular land plants. Although efforts to elucidate the mechanisms underlying chloroplast responses to light were undertaken in the whole of the twentieth century, the last decade brought a breakthrough in the knowledge of their molecular basis. The key motive power was the discovery of phototropins, photoreceptors which mediate phototropism, chloroplast relocations, and stomatal movements. In

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Arabidopsis thaliana, phototropin 1 and phototropin 2 control the accumulation response, with the latter active at higher fluence rates. Phototropin 2 is the only photoreceptor to control chloroplast avoidance movement. Whereas the involvement of actin in the movements is unequivocal, its exact role remains controversial. The search for changes in actin organization, induced specifically by blue light, produced divergent results, which depend on the species, detection method, and fluence rate. Short actin filaments formed at the leading edge of the moving chloroplast under a blue irradiation gradient have been postulated to generate the motive force for chloroplast movement. Alternatively, a blue-light-induced relocation of myosin(s) associated with the chloroplast's surface has been suggested to determine the direction of movement. Analyses of mutants showing impaired chloroplast responses resulted in the discovery of several components of the blue light signaling pathway and/or the motor apparatus. CHUP1, a protein identified by analyzing a deletion mutant which exhibits a chloroplast unusual positioning phenotype, has been proposed to connect chloroplasts to actin filaments and regulate actin polymerization on the one hand, and to anchor them to the plasma membrane on the other. Two kinesin-like proteins have been reported to mediate chloroplast movement and to anchor the organelles in the cell membrane. THRUMIN1 has been shown to bundle filamentous actin in vitro, and to associate with the plasma membrane and interact with microfilaments in a phototropin-dependent manner in vivo. Other proteins postulated to play important roles in the signaling pathway are JAC1 and PMI1 and 2. Further investigations are necessary to establish the exact functions of proteins believed to take part in the movement mechanism.

1 Historical Perspective: Blue Light Syndrome and the Identity of Photoreceptors

A general feature of light-controlled chloroplast positioning in various organisms is their accumulation in weakly illuminated regions of the cell and their avoidance of regions exposed to strong light. In the mesophyll of vascular land plants, chloroplasts redistribute in response to blue light. In weak blue light they align themselves along the cell walls which are perpendicular to the light direction (periclinal walls), thereby exposing a maximum cross section to the limiting photon flux. The accumulation response starts at a very low blue fluence rate of about 0.07 μmol m^{-2} s^{-1} and reaches its maximum at about 3.6 μmol m^{-2} s^{-1} in *Arabidopsis thaliana* leaves (Trojan and Gabryś 1996). At higher fluence rates, chloroplasts start redistributing toward the cell walls parallel to the light direction (anticlinal walls). The avoidance response reaches the point of saturation at around 140 μmol m^{-2} s^{-1} and any further increase in the fluence rate does not cause changes in the chloroplast distribution pattern. The accumulation response is accompanied by a decrease of light transmittance through the tissue, whereas the avoidance response brings about an increase in transmittance. This relationship between chloroplast distribution and the level of light transmittance through the tissue has

been used to assess chloroplast movements with a photometric technique. The first photometer, using very weak, modulated red light as a measuring beam, was constructed before 1980 (Walczak and Gabryś 1980). Other constructions followed two decades later (DeBlasio et al. 2003; Williams et al. 2003; Berg et al. 2006). In the dark, chloroplasts are distributed at all cell walls. The distribution depends on endogenous cues, so far unidentified. Although it is often referred to as random, the dark chloroplast distribution pattern strongly depends on environmental light conditions during the growth of plants (Trojan and Gabryś 1996). A later work suggests also an influence of phototropins, blue light sensors which control movement, on chloroplast positioning in *A. thaliana* in the dark (Suetsugu et al. 2005).

Concerted efforts to identify the photoreceptor pigments started as early as the 1970s. "The Blue Light Syndrome", the title of a monograph published in 1980 (Senger 1980) reflected the holistic approach to the photobiology of plants and microorganisms, which was characteristic of that period. Comparison of action spectra measured for numerous light-controlled physiological processes resulted in flavins and carotenoids being competing candidates as short-wavelength photoreceptors. However, action spectroscopy, even supported by photochemical data collected at that time, was insufficient to conclusively identify the photoreceptor. That ambiguity was reflected in the term "cryptochrome", which was adopted as an operational term for a group of blue light photoreceptors absorbing below 520 nm extending into the UV region (Senger 1984).The term cryptochrome has later been restricted to the short-wavelength counterparts of phytochrome, coregulating developmental processes in the blue-UV region (Ahmad and Cashmore 1996). Regarding chloroplast movements, an ingenious conjecture was put forward by Zurzycki (1980) in favor of flavins, based on an analysis of chloroplast responses to polarized light. His hypothesis had to wait over 20 years for experimental verification.

2 Identification of Photoreceptors

The full identification of photoreceptors was rendered possible due to the rapid development of molecular genetic techniques toward the end of the century. Mutants defective in two entirely different types of movement responses elicited by blue light were obtained in the model plant *A. thaliana*. One type of response, phototropism, is based on the differential growth of cells between shaded and illuminated sides of a plant organ. The other one, chloroplast relocation, occurs inside and is limited to the illuminated cell only, or even to the illuminated part of a cell. Two genes, *PHOT1* and *PHOT2*, have been found in the nuclear genome of *A. thaliana*. They encode two very similar proteins associated with the plasma membrane: phototropin 1 (PHOT1) and phototropin 2 (PHOT2). The main functions of phototropins in plant-movement responses to blue light are schematically shown in Fig. 1. Reviews published a few years after their discoveries describe detailed molecular and physiological characteristics of both photoreceptors (Briggs and Christie 2002; Kagawa 2003; Kimura and Kagawa 2006; Christie 2007).

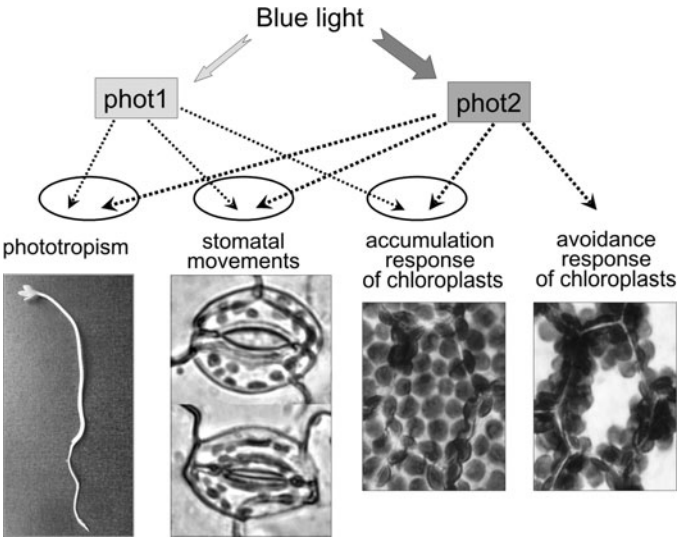

Fig. 1 *Blue light* absorbed by phototropins controls essential movement processes in plants. Phototropins may replace each other in the control of phototropic bending, guard cell movements, and the accumulation response of chloroplasts (marked with *oval shapes*). In contrast, phototropin 2 is indispensable for the avoidance response of chloroplasts

2.1 From nph to Phototropin 1

Phototropin 1 was initially identified as a 120-kDa protein, associated with the plasma membrane in growing regions of etiolated seedlings of pea. Blue light was shown to activate rapid phosphorylation of this protein (Gallagher et al. 1988). Later, the protein was located in the signal transduction pathway for phototropism, and finally, its role as a photoreceptor in this process was established (Christie et al. 1998). The photoreceptor was initially named nph1 for *n*on-*p*hototropic *h*ypocotyl *1*. The function of phototropin 1 in chloroplast redistribution could only be determined after phototropin 2 was identified, and a mutant *phot1phot2* devoid of both photoreceptors was obtained.

2.2 Phototropin 2, the Key Photoreceptor in Chloroplast Movements

In 2001, two research groups have independently identified phototropin 2 as the photoreceptor controlling the chloroplast avoidance response. (Jarillo et al. 2001; Kagawa et al. 2001). *Phot2* null mutants were isolated using two different strategies. Using a reverse genetic approach and screening pools of T-DNA insertion lines, the former group isolated a loss-of-function, null allele mutant, and then looked for

physiological anomalies attributable to the mutation (Jarillo et al. 2001). The approach adopted by the other group involved screening large numbers of chemically mutagenized and/or T-DNA-tagged F2 plants for abnormal avoidance responses of their chloroplasts (Kagawa et al. 2001). Both isolated mutant lines were initially designated *npl1* for n*on*-p*hototropic hypocotyl1*-l*ike*1, later changed to *phot2*. The *phot2* mutants lack the chloroplast avoidance response. Instead, an accumulation response takes place irrespective of the light intensity applied, even at fluence rates saturating for avoidance in the wild type. The expression of the *PHOT2* gene was found to be upregulated by light, in accordance with its role in the control of the chloroplast response to strong light.

In contrast to the striking effect in the *phot2* mutant, the disturbance of chloroplast movement in the *phot1* mutant is much lesser. The threshold of the accumulation response is only slightly shifted toward higher fluence rates as compared with the wild type, while the avoidance response is normal (Kagawa and Wada 2000). This higher light requirement of the mutant suggested the potential role of phot1 as a photoreceptor in the accumulation response. This role has been confirmed in a study of the *phot1phot2* double mutant where the chloroplasts do not respond to light irrespective of its fluence rate (Sakai et al. 2001). Thus, phot1 and phot2 both control the accumulation response even when one is redundant, with phot2 being active in the higher range of fluence rates. The avoidance response is controlled only by phot2. In strong light, phot2 overrides the opposite stimulus (toward periclinal walls) conveyed by phot1. The name of phototropin 2 was put forward based on the similarity of PHOT2 protein to PHOT1 (Briggs et al. 2001). Yet their physiological functions differ. The manifest physiological function of phot1 is the control of phototropic bending while its role in chloroplast movements appears to be auxiliary. The essential function of phot2, however, is the control of chloroplast redistribution, in particular, the avoidance response of chloroplasts, and its role in phototropism is much less conspicuous. Thus, the physiological function of phot2 would be better reflected, e.g., by "photo-avoidin".

The regulation of the stomatal aperture is yet another blue-light-activated movement response controlled by phototropins in *A. thaliana* (Kinoshita et al. 2001). In contrast to chloroplast movements, both phot1 and phot2 share the control over the guard cells, acting redundantly over a broad range of fluence rates. In consequence, single *phot* mutants appear phenotypically normal and only in the double *phot1phot2* mutant is the stomatal response abolished.

2.3 Involvement of Phytochrome B

Red light may enhance the velocity of moving chloroplasts but it does not induce their directional responses in any of the terrestrial angiosperms studied to date, irrespective of its intensity (Walczak and Gabryś 1980; Trojan and Gabryś 1996; DeBlasio et al. 2003; Sztatelman et al. 2010; Gabryś, unpublished results). Nevertheless, phytochromes A and B, the photoreceptors responsible mainly for

red light sensing, appear to be involved in these responses (DeBlasio et al. 2003; Luesse et al. 2010). *PhyA* and *phyB* mutants showed an enhanced avoidance response in a blue light intensity range from 10 to 60 μmol m^{-2} s^{-1}. On the other hand, overexpression of both photoreceptors reduced chloroplast accumulation in weak light. Detailed fluence-response relationships were analyzed for single and double photoreceptor mutants (*phot1*, *phot2*, *phyB* and *photphy*) (Luesse et al. 2010). In conclusion, the authors state that PhyB attenuates the strong light avoidance response acting on both blue light receptors, phot1 and phot2. This function is most probably performed in the signal transduction downstream of phototropins.

3 The Actin Cytoskeleton: Tracks or More?

3.1 Actin Tracks and Baskets

The movement of plant organelles appears to be based predominantly on the actomyosin system (for a review see Krzeszowiec and Gabryś 2011). Inhibition of chloroplast movements by the anti-actin drugs cytochalasins B and D provided the first evidence for the involvement of actin in the mechanism of these movements in seed plants (Izutani et al. 1990; Tlałka and Gabryś 1993; Malec et al. 1996). In several species, light treatments which activated the redistribution of chloroplasts were shown to reorganize concomitantly the actin network. The distinctive structures and arrangements of actin filaments which formed following weak or strong irradiation were first demonstrated in cryptogams (Blatt and Briggs 1980; Kadota and Wada 1992). The relationship between the light-activated spatial reorganization of the actin cytoskeleton and chloroplast redistribution has been investigated in detail in the epidermis of a water angiosperm *Vallisneria gigantea* (Dong et al. 1998). In this species, chloroplasts flow with the circulating cytoplasm and become trapped at the weakly illuminated cell walls. The striking light-dependent reorganization of the actin cytoskeleton entails the rearrangement of the loose network of thin bundles present in dark-adapted cells into a honeycomb array upon several hours of constant irradiation with weak red light. The honeycomb cavities become traps for chloroplasts at the most illuminated walls. The trapping can be reversed by strong blue light: following a few minutes of irradiation, the chloroplasts regain their motility and redistribute toward less illuminated walls (Takagi 2003; Sakurai et al. 2005). Concomitantly, a reorganization of actin filaments takes place, resulting in a more stretched network of thick bundles. Far-red light and/or DCMU [3-(3,4-dichlorophenyl)-1,1-dimethylurea], an inhibitor of electron transport in photosynthesis, inhibit both movements and actin rearrangements. These facts have been interpreted in terms of a joint control of phytochrome and photosynthetic pigments over chloroplast redistribution via actin reorganization.

A structural association between actin cytoskeletal structures and chloroplasts has been demonstrated in *A. thaliana* by means of immunocytochemistry

(Kandasamy and Meagher 1999). The authors hypothesized that some chloroplasts, encapsulated in actin structures and covered with motor proteins, might migrate along actin cables directly, while others might be pulled by fine filaments attached to the cables.

Inspired by the results obtained for *Vallisneria*, Kumatani et al. (2006) investigated the influence of light on the actin cytoskeleton in the pallisade cells of spinach. They reported the occurrence of prominent, long actin bundles specific to strong blue light irradiated cells. This evidence appears to be rather inconclusive because statistical evaluation is lacking.

Another attempt to find light-induced reorganization of the actin cytoskeleton analogous to that observed in cryptogams and water angiosperms was undertaken by Krzeszowiec et al. (2007) under conditions which activate chloroplast redistribution. No blue-light-induced changes in the actin architecture were demonstrated either in *A. thaliana* or in tobacco. However, although the plant material was fixed prior to actin labeling with Alexa fluor phalloidin, even small differences in the shape and distribution of F-actin formations were detectable. Noticeable changes were found in mesophyll cells of *phot2* mutants exposed to strong red light as compared to those exposed to weak red light. As red light does not activate chloroplast responses, these changes cannot be associated with their movement. This line of investigation was continued using live mesophyll cells of transgenic tobacco labeled with GFP fused to a fragment of human plastin, an actin-bundling protein (Anielska-Mazur et al. 2009). Also in this model, which allows an examination of cytoskeleton reorganization in real time, no blue-light-specific actin rearrangement could be detected. Strong light of 40 μmol m^{-2} s^{-1} caused a reversible widening of actin bundles. This intensity of blue light resulted in an incomplete avoidance response (about 20% below saturation). The widening effect was neither directional nor blue light specific. In general, similar effects were observed both in blue light, which activates chloroplast movements in tobacco, and in inactive red light.

The chloroplasts were surrounded with dense cocoons built of thin actin filaments (Anielska-Mazur et al. 2009). These structures, which have been called baskets, were tightly bound together around adjacent chloroplasts and attached to cortical actin bundles. The baskets were remarkably stable and survived not only strong light but also EGTA treatment, the latter being much more damaging to cortical actin than to the fine filaments surrounding the chloroplast. On the basis of qualitative and quantitative analysis, the authors concluded that actin baskets and their interactions with the cortical actin cytoskeleton play a key role in chloroplast positioning in higher land plants. A more general conclusion drawn from this study is that the directionality of chloroplast responses is not based on specific blue-light-induced changes of F-actin.

3.2 Short Actin Filaments

Based on a different experimental model, a particular role for actin in light-induced chloroplast redistribution has recently been proposed. Kadota et al. (2009) analyzed

actin dynamics in transgenic *A. thaliana* plants expressing GFP-mouse talin fusion protein. Using microbeam irradiation with strong light, they identified short actin filaments (cp-actin) situated at the chloroplast periphery on the plasma membrane side. The amount of these filaments at the front and the rear of the chloroplasts, measured as the intensity of GFP fluorescence, depended on light intensity. In response to strong light, the filaments on irradiated chloroplasts transiently disappeared and then reappeared at the leading side of the moving organelle. Cp-actin behaved differently in the mutants deficient in phototropins. Its dynamics were similar to wild type in the *phot1* mutant where both types of chloroplast responses take place. In *phot2*, which shows the accumulation instead of avoidance response in strong light, cp-actin filaments were abundant, and denoted biased, under these conditions. In contrast, they were absent from the *chup1* mutant (see Sect. 3.3). The authors conclude that phototropins mediate directional photomovement and anchoring of chloroplasts by regulating cp-actin filaments. They propose a model of blue-light-controlled chloroplast positioning based entirely on rearrangements of short actin filaments on the surface of chloroplasts. In their opinion, plants have evolved a unique, actin-based mechanism for chloroplast movements, different from those identified to date for other organelles.

However significant these findings may turn out to be, it is worth mentioning that cp-actin filaments have been demonstrated at light intensities by far exceeding the saturation of the avoidance response. They were observed at continuous blue irradiation of 377 μmol m^{-2} s^{-1}, and a still higher intermittent (blue) GFP excitation which amounts to 1,875 μmol m^{-2} s^{-1} when integrated over time. The biased relocalization of cp-actin took place mainly at the rim of microbeam fleck. Thus, the relevance of the biased short actin effect to chloroplast redistribution under physiological light conditions has yet to be substantiated.

3.3 Discovery of CHUP1

The presence of short actin filaments was shown to depend on an actin-binding protein, *c*hloroplast *u*nusual *p*ositioning 1 (CHUP1), localized at the chloroplast envelope (Kasahara et al. 2002; Oikawa et al. 2003). In the *A. thaliana* mutant *chup1*, chloroplasts are completely immobile in the palisade mesophyll: they aggregate at the bottom of cells. CHUP1 was originally proposed as a possible factor linking chloroplasts to actin filaments on the basis of molecular genetic analysis. It contains an actin-binding domain; interaction between actin and CHUP1, irrespective of the polymerization state of the actin was demonstrated in vitro (Oikawa et al. 2003; Schmidt von Braun and Schleiff 2008a). Besides the actin-binding domain, CHUP1 contains a profilin-binding motif. Interaction between both proteins and the direct influence of CHUP1 on the formation of actin–profilin complexes was reported in vitro (Schmidt von Braun and Schleiff 2008a). A model has been proposed according to which CHUP1 plays the role of a docking site for actin filaments (Wada and Suetsugu 2004; Schmidt von Braun

and Schleiff 2008b). The model predicts that the protein recruits profilin and actin to the chloroplast and initiates actin polymerization by the profilin-regulated addition of actin monomers to the barbed end of the filament. A physiological analysis of transgenic *A. thaliana* plants expressing truncated CHUP1 in a *chup1* mutant background, supported by the effects of cytoskeletal experiments, complemented this interpretation by showing that the coiled-coil region of the protein may anchor chloroplasts at the plasma membrane (Oikawa et al. 2008). Thus, CHUP1 appears to play a key role in the mechanism of chloroplast movement and anchorage.

4 Myosin or Kinesin-Based Mechanism?

4.1 Blue-Light-Induced Relocation of Myosin(s) at the Chloroplast Surface

Myosins, previously shown to localize at the surface of the *A. thaliana* chloroplast (Malec et al. 1996, Wojtaszek et al. 2005), were tested as a potential target for light signals. Immunocytochemistry using polyclonal antibodies against plant myosin VIII and against animal myosins provides evidence that these proteins may serve as a target for a blue-light chloroplast-orientation signal (Krzeszowiec and Gabryś 2007). Arrangements of myosins observed in the mesophyll of wild type *A. thaliana* differed depending on light intensity. Myosins were associated with chloroplast envelopes in tissue irradiated with weak blue light. By contrast, in tissue irradiated with strong blue light chloroplasts were almost myosin free. The effect was absent in red light and in the *phot2* mutant deficient in phototropin 2. However, the rearrangement of myosins was similar to wild type at the surface of chloroplasts in the *phot1* mutant. A working hypothesis has been developed that myosins are the final element of the blue light signal transduction pathway starting with phototropin 2 and leading to chloroplast movements. This hypothesis requires further experimental verification. The term myosin(s) adopted for the protein(s) showing specific redistribution upon blue light treatment has to be treated operationally until the identity of the protein has been unambiguously proved.

A recent study was aimed at the identification of myosins linked with the surface of *Nicotiana benthamiana* organelles and involved in their movements (Avisar et al. 2008). The authors used overexpression of headless myosin tails, which is expected to inhibit transport of organelles either by interfering with the capacity of the organelles to bind myosins, or by reducing their motor activities. This approach, supported by the RNAi technique, demonstrated that the class XI myosin XI-K plays a major role in the movement of mitochondria, peroxisomes, and Golgi stacks in the leaf cells. None of the tested tails of five XI myosins and the myosin VIII-1 affected light-induced chloroplast movements in *N. benthamiana* epidermal cells. This does not preclude the involvement of yet another myosin in the mechanism of chloroplast movements.

4.2 Kinesin-Like Proteins

In line with the cp-actin concept, two kinesin-like proteins have recently been identified in *A. thaliana*. They are KAC1 and KAC2, which are linked with chloroplast responses (Suetsugu et al. 2010). KAC1 was known previously as a protein interacting with various proteins implicated in the cell cycle and with the functioning of microtubules. Chloroplast responses to microbeams and/or 2 h continuous irradiation were tested in *kac1*, *kac2*, and the double *kac1kac2* mutant. While irregularities have been shown to occur in chloroplast responses in the mutant plants, their occurrence does not seem to add up to any consistent pattern. For example, only the *kac1* mutant was found to be "partly defective" in the accumulation response whereas *kac2* behaved similar to wild type. Also *kac2* showed an avoidance response typical of wild type in white light. On the other hand, in the double mutant the distribution of chloroplasts both in weak and strong light was similar to that of wild type in strong light. It appears therefore that the KAC proteins, particularly KAC1, regulate chloroplast attachment to the cell membrane rather than the responses themselves. As the accumulation response appears to rely more strongly on chloroplast anchorage than does the avoidance one (cf. Zurzycki 1980), disturbances of this response reported for *kac* mutants could be more evident.

5 Signaling in Chloroplast Movements

The existence of two separate pathways leading to either an accumulation or an avoidance chloroplast response was postulated a long time ago on the basis of a model describing chloroplast responses to strong blue light pulses (Walczak et al. 1984). Subsequently, evidence in favor of this model has accumulated (Tlałka and Gabryś 1993; Grabalska and Malec 2004). A recent detailed analysis of dose-response relationships in phototropin mutants has brought a valuable confirmation of these concepts (Luesse et al. 2010). Phototropins have been shown to operate via integrated but antagonistic pathways to control the accumulation response.

The most significant progress observed in the area of light signal transduction stems from discoveries of new proteins, all in *A. thaliana*. In 2005 and 2006, three proteins were described, and they appear to play the role of signal transduction constituents for chloroplast responses. One of these proteins, a J-domain protein required for the chloroplast accumulation response (JAC1), was identified following isolation of a mutant defective in the accumulation response (Suetsugu et al. 2005). The *JAC1* gene was shown to encode a C-terminal domain which shares about a 30% similarity with an analogous region of the clathrin uncoating factor auxilin. The gene was found to be expressed in leaves and stems (stronger in leaves) but not in roots. A transient expression assay of *35 S-GFP-JAC1* showed the presence of fusion protein all over the cell, including the nucleus. Two other proteins have been called PMI1 and PMI2. Their names: *p*lastid *m*ovement *i*mpaired 1 and 2, come

from two mutants isolated during one screen (DeBlasio et al. 2005; Luesse et al. 2006). Although these proteins have been given names differing only by number, the characterization of the genes by which they are encoded suggests that they may have dissimilar functions. *PMI1* deficiency is reflected in the attenuation of chloroplast movements at all tested fluence rates. This suggests that the protein is necessary for both accumulation and avoidance responses. Sequence analysis of the protein implies that it may be involved in calcium-mediated signal transduction, possibly by protein–protein interaction (DeBlasio et al. 2005). On the other hand, plants with *PMI2* deficiency show the accumulation response over a fluence-rate range which goes far into the range which induces the avoidance in the wild type: the chloroplasts start moving toward anticlinal walls only above 75 μmol m^{-2} s^{-1}.

A very interesting mutant, *thrumin1-1*, has been isolated recently in a photometric screen of *pmi* mutants, on the basis of the poor kinetics of its chloroplast responses (Whippo et al. 2011). Molecular genetic investigations led to the identification of the relevant protein which belongs to a glutaredoxin family of proteins found in plants and animals. THRUMIN1 has been shown to bundle filamentous actin in vitro. Using THRUMIN1-YFP reporters, the authors localized the protein to the plasma membrane and provided evidence for its light-dependent, phototropin-mediated relocalization to microfilaments in vivo. Thus, THRUMIN1 was suggested as a potential link between phototropin photoreceptor activity at the plasma membrane and the functioning of actin in chloroplast movements (Whippo et al. 2011).

Evidence has been presented for the involvement of phosphoinositide kinases in the transduction of the light signal as factors specifying the direction of chloroplast movements. Wortmannin, an inhibitor of phosphoinositide-3-kinases, was shown to inhibit chloroplast responses to blue light first in the water angiosperm *Lemna trisulca* (Grabalska and Malec 2004) and later in *A. thaliana* mesophyll cells (Anielska-Mazur et al. 2009). In *L. trisulca*, 100 nM wortmannin specifically inhibited the accumulation response whereas the avoidance response was insensitive to the inhibitor at this concentration. A complete inhibition of chloroplast avoidance was obtained at 1 μM wortmannin.

A difference in sensitivity to wortmannin was also visible in the respective responses of tobacco chloroplasts, although a much higher concentration of the inhibitor (10 μM) was required to obtain full inhibition of the accumulation and 50% inhibition of the avoidance response. The inhibition was largely reversible by Ca^{2+} ions administered exogenously (Anielska-Mazur et al. 2009). The recovery of wortmannin-inhibited movements obtained with Ca^{2+} suggests that these ions play at least two roles in the mechanism of the movements: they control the motor apparatus and transmit the light-generated signal downstream of the phosphoinositide kinases.

Exogenous Ca^{2+} also restored chloroplast movements inhibited by trifluoperazine, a calmodulin blocker (Anielska-Mazur et al. 2009). The fact that calcium restored the vectorial movement of chloroplasts when introduced nondirectionally means that it is not a carrier of information about the direction of chloroplast motion. Interestingly, Mg^{2+} was twice as effective as Ca^{2+} in restoring both chloroplast responses in samples treated with trifluoperazine. In general, the restorative power of Mg^{2+} was equal to, or higher than, that of Ca^{2+} in all cases where

a disturbance of calcium homeostasis disrupted chloroplast movements, and in the case of movement inhibition with wortmannin. The higher effectiveness of Mg^{2+} may suggest that this ion cooperates with Ca^{2+} in the signaling pathway of blue-light-induced tobacco chloroplast movements.

Obviously, signaling pathways other than those generated by light must have an impact on chloroplast redistribution. Cross-talk between environmental stress signals and the light signals controlling chloroplast movements is unquestionably of key importance. In the last decade, this aspect of cell signaling has not aroused much interest. Only one study was focused on redox signaling which may be indicative of both abiotic and biotic stress (Wen et al. 2008). Exogenous H_2O_2 at a concentration of 10^{-4} M was shown to enhance the avoidance response of *A. thaliana* chloroplasts. On the other hand, strong blue light induced H_2O_2 generation at the plasma membrane and the chloroplast. The light-induced H_2O_2 generation and the chloroplast avoidance response were largely decreased by the exogenous addition of the H_2O_2-specific scavenger catalase and other antioxidants (ascorbic acid and mannitol). Other compounds shown to reduce H_2O_2 production are DCMU and diphenyleneiodonium (DPI), an NADPH oxidase inhibitor. The authors conclude that strong blue light can induce H_2O_2 generation, and that the production of H_2O_2 is involved in the chloroplast avoidance response. This conclusion needs more experimental support for validation. Mannitol was reported to slightly enhance both chloroplast responses in another study (Banaś and Gabryś 2007). Moreover, DCMU does not affect chloroplast movements in *A. thaliana* (Slesak and Gabryś 1996) which puts in question the significance of its H_2O_2-scavenging properties for their mechanism.

Sugars were the focus of another work (Banaś and Gabryś 2007). Sucrose and glucose were shown to inhibit chloroplast responses in a concentration and time-dependent manner when brought into direct contact with the mesophyll tissue of *A. thaliana*. 3-O-methylglucose did not affect chloroplast movements suggesting the participation of a hexokinase-dependent pathway. The prolonged exposure necessary to inhibit the movements implies the contribution of altered gene expression. The sugar effect was counteracted by overexpression of phototropin2. This may suggest that sugars modify some component(s) of the phot2-mediated signal transduction pathway. The significance of the observed effects remains a matter of speculation. An elevated sugar level may be sensed as a stress condition resulting from a disturbed source/sink balance (Rolland et al. 2006). Sugars have been reported to accumulate in plant tissues during pathogen attacks (Berger et al. 2007). Movement responses are energy consuming, and plants may preferentially direct energy into more vital processes under stress conditions.

6 Physiological Significance of the Movements Corroborated

In one of his pioneering works, Zurzycki (1955) demonstrated that the light-activated redistribution of chloroplasts helps to maximize light harvesting under energy-limiting conditions. This statement, based on experiments done with *Lemna*

trisulca, was treated as a textbook truth and never contested. Only after 50 years was it experimentally confirmed for *A. thaliana* and attributed to phototropin mediation (Takemiya et al. 2005).

The significance of the avoidance response in strong light was not that clear because no correlation could be demonstrated between the course of chloroplast redistribution toward anticlinal walls and the dose-response curve of photosynthesis (Zurzycki 1955). Later, the avoidance response was shown to protect the photosynthetic apparatus from excess energy in strong light (Park et al. 1996; Kasahara et al. 2002; Sztatelman et al. 2010). Experimental evidence for the photoprotective function of chloroplast movements in higher plants was provided by the observation that *Tradescantia albiflora* was less prone to high light damage than *Pisum sativum* (Park et al. 1996). The mesophyll cells of these two plants are similar at the biochemical level, but only *T. albiflora* shows light-controlled chloroplast redistribution. After the identification of *A. thaliana* photoreceptor mutants, it was possible to investigate their light stress susceptibility. Kasahara et al. (2002) demonstrated the photoprotective function of chloroplast relocations. The growth and chlorophyll fluorescence parameters indicative of photosystem II performance were compared for wild-type plants and *phot2* mutants of *A. thaliana*. This approach was later refined in a study taking into account potential changes in processes other than chloroplast movements which might be caused by pleiotropic effects of the *phot2* mutation (Sztatelman et al. 2010).

Yet another role of the avoidance response was transiently discussed: this movement would position the chloroplasts closer to the intercellular air spaces, thereby enhancing CO_2 diffusion under strong light when carbon dioxide is limiting. However, photo-acoustic analysis provided no evidence for enhanced gas diffusion resulting from chloroplast redistribution (Gorton et al. 2003).

All these studies were done under laboratory conditions. Williams et al. (2003) were the first to assess chloroplast redistribution in field conditions. Using a custom-made portable instrument, they measured red light transmittance changes which reflect chloroplast redistribution in white-light-irradiated leaves. Movements in four investigated species of terrestrial angiosperms: *Alocasia brisbanensis*, *Helianthus tuberosum*, *Eustrephus latifolius*, and *Cissus hypoglauca* were qualitatively similar, with varying magnitudes and kinetics. Interestingly, chloroplasts were in motion most of the time in all four species and they rarely achieved extreme positions at the periclinal or anticlinal cell walls.

7 Questions to Be Resolved

The relatively rapid accumulation of new data on the mechanisms of chloroplast responses in higher terrestrial plants stimulates new questions reaching deeper into these mechanisms. One current fundamental question is whether the short actin filaments reported to build up at the leading edge of the moving chloroplasts indeed play a role in the generation of the motive force. Such a role of cp-actin has

been postulated on the basis of experiments carried out under conditions differing from those typical of the induction of chloroplast movements in mesophyll cells. Palisade cells have been the object of most observations (Kasahara et al. 2002; Oikawa et al. 2003). Chloroplasts do not populate the cell wall adjacent to the epidermis in this tissue after dark adaptation (Trojan and Gabryś 1996). Other experiments demonstrated cp-actin in petiole cells (Kadota et al. 2009; Suetsugu et al. 2010) which may have a distinctive mechanism of chloroplast movements. More important, most reported effects have been observed inside/in the vicinity of areas irradiated with microbeams of very strong blue light, exceeding by far the saturation of the chloroplast avoidance response. It is worth mentioning that blue light which saturates the avoidance response of chloroplasts exerts a more stressing influence on *A. thaliana* leaf tissue than red light of an 18 times higher fluence rate (Sztatelman et al. 2010). Thus, two questions arise, that is, whether all aspects of the proposed mechanisms are applicable to chloroplast redistribution taking place (1) under ordinary physiological conditions, and (2) in all types of mesophyll cells.

Another key problem is whether myosins or kinesin-like proteins play the role of a motor for the movements. Results obtained with the immunocytochemical technique cannot be considered as decisive evidence because of the risk that the anti-myosin antibodies were not fully specific. Therefore, the identification of a putative myosin is essential. Similarly, much experimental work has to be done before the exact role of KAC proteins is understood.

An unresolved question is the relationship between chloroplast movements and cytoplasmic streaming in vascular land plants. This problem has been discussed elsewhere in the context of the diverse mechanisms of chloroplast movements in various plant taxa (Krzeszowiec and Gabryś 2011). A recent analysis of organelle motility in *N. benthamiana* leaf cells has revealed independent movement patterns for Golgi stacks, mitochondria, and peroxisomes (Avisar et al. 2008). The authors concluded that the notion of coordinated cytoplasmic streaming is not generally applicable to higher plants. This conclusion is particularly important for directional movements of chloroplasts in the mesophyll cells of higher land plants which appear to start only at the onset of illumination. However, some link between chloroplast movement and cytoplasmic streaming has to be taken into account, particularly under strong light.

Another problem to be resolved is the identification of any likely feedback from photosynthesis. This aspect of chloroplast movement regulation has hardly been addressed so far for higher land plants. Only one study has been reported in which chloroplast responses in *A. thaliana* were assessed with photosynthetic electron transport blocked by DCMU or methyl viologen (Slesak and Gabryś 1996). While neither treatment affected the responses activated by continuous light, such feedback is to be expected if chloroplast redistribution indeed serves the optimization of photosynthesis.

This brief discussion of some open questions does not cover all the areas which need to be worked on to fully understand the mechanisms of light-directed chloroplast redistribution in the leaves of vascular land plants. The rapid accumulation of data, connected mainly with the isolation and analysis of new mutants, gives grounds for optimism about progress in this field in the not too distant future.

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Role of Chloroplast Thylakoid Lumen in Photosynthetic Regulation and Plant Cell Signaling

Cornelia Spetea

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Abstract The aqueous lumen enclosed by the thylakoid membrane network of the chloroplast is the compartment where molecular oxygen is produced from water during photosynthetic light-dependent reactions. The thylakoid lumen has been thought for a long time to contain mainly plastocyanin and oxygen-evolving

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complex-associated proteins, playing important roles during these reactions. In the last decade, the functional characterization of thylakoid lumenal proteins from *Arabidopsis thaliana* has brought insights into the complex role of this subcellular compartment. The aim of this chapter is to provide an updated view of the protein composition of the thylakoid lumen and its emerging roles in photosynthetic regulation and plant cell signaling. Recent research has uncovered redox signaling and a new paradigm about the role of nucleotides in the thylakoid lumen.

1 Introduction

The chloroplast is the defining organelle of photoautotrophic plant cells. It performs photosynthetic light-dependent reactions in the thylakoid membrane and light-independent reactions in the soluble stroma. In addition to these two compartments, the chloroplast is surrounded by a double envelope membrane, which plays a main role in intracellular communication. The thylakoid membrane encloses a lumenal space, which has been thought for a long time to play only the role of a sink for protons (H^+) generated during the light-dependent reactions (Fig. 1). This relatively small chloroplast compartment is the focus of this chapter.

The chloroplast is also known as the photosynthetic plastid, and its biogenesis from undifferentiated pro-plastids requires light. Although chloroplasts have their own genome (plastome), originating from that of the cyanobacterial ancestor, the

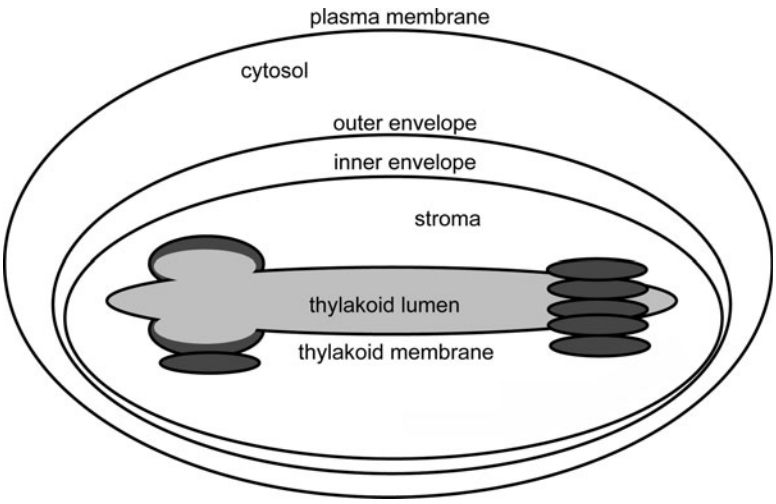

Fig. 1 Organization of the chloroplast within the plant cell. The chloroplast is composed of several compartments: the outer and inner envelope enclosing an intermembrane space and surrounding the organelle, the soluble stroma, and the thylakoid membrane, enclosing a lumenal space. The plasma membrane surrounding the plant cell, and the cytosol where the chloroplast is located are also shown

vast majority of about 3,000 genes encoding chloroplast proteins have been transferred to the nucleus. The plastome codes for only about 80 chloroplast proteins, having essential functions in photosynthesis and chloroplast protein synthesis. Therefore, chloroplasts must efficiently import nucleus-encoded and cytosol-synthesized proteins. A subset of proteins, including those encoded by the plastome, must be further sorted to the thylakoid membrane for assembly into the photosynthetic complexes. An efficient regulation of synthesis and assembly of photosynthetic complexes is essential for their optimal function in the thylakoid membrane (Bauer et al. 2001).

Microscopic studies of chloroplasts have contributed to our knowledge about the structural and functional organization of thylakoid membranes. Freeze fracture, electron tomography, and immunoelectron microscopy have revealed a nonrandom distribution of photosynthetic complexes between appressed (grana core) and non-appressed (grana margins and stroma lamellae) thylakoids, and a continuous lumenal space between these two regions (Fig. 1; Shimoni et al. 2005; Mustárdy et al. 2008; Daum et al. 2010). It appears that the distribution of functional complexes between the two regions is dynamic and affected by changes in the light environment (Anderson et al. 2008).

2 Photosynthetic Apparatus: Structure, Function, and Regulation

The oxygenic photosynthetic apparatus consists of four major multi-subunit complexes involved in the light reactions, namely, the water-oxidizing photosystem II (PSII), cytochrome b_6f (cytb_6f), photosystem I (PSI), and the H^+-translocating ATP synthase (CF_0F_1), all four located in the thylakoid membrane of cyanobacteria, green algae, and higher plants (Fig. 2). The first three photosynthetic complexes are involved in sunlight absorption, electron extraction, and transfer from water to $NADP^+$, whereas the fourth complex uses the H^+ gradient created across the thylakoid membrane during the electron transfer to power ATP synthesis (Merchant and Sawaya 2005). PSII is mostly found in the grana, and PSI and ATP synthase in the stroma lamellae, whereas cytb_6f is evenly distributed between the two types of membranes (Shimoni et al. 2005; Mustárdy et al. 2008; Daum et al. 2010). It is obvious that the bulky part of the ATP synthase prevents it from localizing to the grana. In the case of the photosystems, the lateral segregation is driven by electrostatic and lipid-driven interactions. The three-dimensional structure of all the four complexes has been resolved at various resolutions, and great progress has been made in the past 10 years in elucidating the structure and function of various subunits using spectroscopy, molecular genetics, and biochemistry (Nelson and Ben-Shem 2004; Nelson and Yocum 2006). Comparative analyses of the available structures from cyanobacteria, green algae, and plants have shed light on the evolutionary pathways leading to oxygenic photosynthesis.

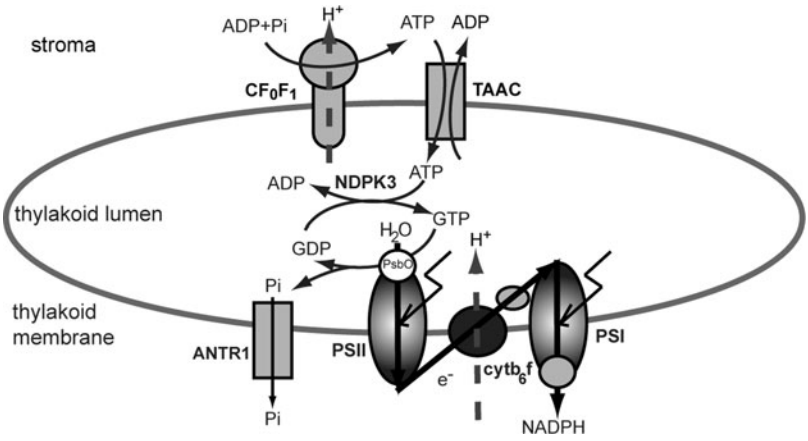

Fig. 2 Photosynthetic complexes, light-dependent reactions, and nucleotide metabolism within the thylakoid membrane. ATP is synthesized from ADP and Pi by the H^+-translocating ATP synthase (CF_0F_1) on the stromal side of the thylakoid membrane, and transported inside the lumenal space by the thylakoid ATP/ADP carrier (TAAC). Here it can be converted to GTP by the nucleoside diphosphate kinase 3 (NDPK3). The PsbO extrinsic subunit of the water-oxidizing photosystem II (PSII) complex binds and hydrolyzes GTP. The resulting phosphate (Pi) is exported back to the stroma by the thylakoid Pi transporter (ANTR1). The GTP signaling optimizes the function and turnover of PSII, the first multisubunit complex involved in the electron transfer chain. The electron transport continues via cytochrome b_6f (cytb_6f) and photosystem I (PSI), yielding NADPH, and is coupled to H^+ translocation into the lumen. The latter contributes to the trans-thylakoid H^+ gradient, which drives ATP synthesis

Among photosynthetic complexes, PSII catalyzes the first set of photosynthetic reactions, resulting in the oxidation of water and production of molecular oxygen and reducing equivalents (Fig. 2). PSII is actually the only biological system on Earth that can provide us the oxygen we breathe. The reducing equivalents are ultimately used to drive CO_2 fixation in the chloroplast stroma. The active PSII in plants is a supercomplex consisting of a dimeric core with over 25 subunits/monomers and of a trimeric outer light-harvesting antenna, connected to the core via a monomeric inner antenna. So far, only the structure of PSII from a cyanobacterium has been resolved by X-ray crystallography (Kamiya and Shen 2003; Ferreira et al. 2004; Loll et al. 2005; Guskov et al. 2009). Moreover, the mechanisms underlying PSII biogenesis, assembly, and turnover are poorly understood. PSII can be inactivated by light at all intensities alone or in combination with other stress factors, affecting the plant's daily productivity (Powles 1984; Mattoo et al. 1989; Takahashi and Murata 2008). This process is known as photoinhibition and has reversible (photoinactivation) and irreversible (photodamage and repair) phases. The main reason for photodamage is that PSII produces oxygen, and under stress conditions, this could be converted to reactive oxygen species (ROS) with harmful effects. The extent of photoinhibition is the result of a dynamic balance between photodamage and repair of PSII (Ohad et al. 1984; Aro et al. 1993, 2005). To avoid photoinhibition, plants use photoprotective mechanisms either to suppress

the damage or to facilitate repair of damaged PSII. Therefore, photoinhibition occurs only under conditions when the rate of photodamage exceeds the rate of repair. ROS have been shown to block the synthesis of PSII proteins, and thus suppress repair (Takahashi and Murata 2008). The repair of damaged PSII consists of multiple steps, aimed to monomerize and partially disassemble the complex to allow degradation of the D1 protein and its replacement by a new copy, followed by reassembly and reactivation of the complex (Aro et al. 2005). This multistep process must be rigorously regulated, and crucial for this is an array of 'auxiliary' proteins, such as kinases, phosphatases, proteases, stress-induced and heat-shock proteins, and solute and metabolite transporters. Compared to the available knowledge concerning photosynthetic machinery, there is still a lot to be done in the field of auxiliary and transport proteins, to understand their importance not only for chloroplast biology, but also in the functioning of other plastid types (Mulo et al. 2008; Spetea and Schoefs 2010). Nevertheless, we know that the mechanisms used for PSII biogenesis, assembly, and repair are predominantly of prokaryotic origin, as, for example, the proteases involved in the repair cycle (Nixon et al. 2010).

3 Thylakoid Lumenal Proteins: Classification and Role in Photosynthetic Regulation

The thylakoid lumen is a narrow aqueous space within the thylakoid membrane that contains around 80 proteins, as based on proteomics studies (Schubert et al. 2002; Peltier et al. 2002). During chloroplast and thylakoid membrane biogenesis, all lumenal proteins are nuclear-encoded, synthesized in the cytosol, imported into the chloroplast, and then sorted to the lumenal space, based on the presence of specific targeting sequences. An important fraction of lumenal proteins displays increased expression levels following exposure to light, indicating functions related to photosynthetic activity (Granlund et al. 2009a, b). This chapter focuses on thylakoid lumenal proteins from *Arabidopsis thaliana (Arabidopsis)*, which have been identified and characterized in the recent years (Table 1). The majority of these proteins have been identified by mass spectrometry-based proteomics, but some of them could be detected only in genetic screens. Phenotypic analyses of *Arabidopsis* mutants have shed light on the function of lumenal proteins, and hence on the complex role the lumen plays within the chloroplast and the plant cell. The identified functions are related to photosynthetic electron transfer, oxygen evolution, protein folding, proteolysis, nucleotide metabolism, and redox signaling. These functions imply an important role of the thylakoid lumen in biogenesis, optimal function, acclimation, and repair of the photosynthetic apparatus, to ensure its efficiency under ever-changing and even stressful environmental conditions.

Table 1 Summary of characterized thylakoid lumenal proteins from *Arabidopsis thaliana*

Family name (lumenal members)	AGI number	Protein function	References
Plastocyanin			
PC1	At1g76100	Electron carrier between cytb_6f and PSI, copper homeostasis, redox signaling	Gupta et al. (2002), Abdel-Ghany (2009) and Pesaresi et al. (2009)
PC2 (main PC)	At1g20340		
Oxygen-evolving complex-associated proteins			
PsbO1 (main PsbO)	At5g66570	Mn stabilizing, Ca binding, carbonic anhydrase	Suorsa and Aro (2007)
PsbO2	At3g50820	GTPase, PSII disassembly	Lundin et al. (2007b, 2008)
PsbP1	At1g06680	Ca^{2+}, Cl^-, Mn binding, GTP signaling, grana stacking, formation of PSII supercomplexes	Ifuku et al. (2004, 2005, 2008), Anderson et al. (2008), Ido et al. (2009) and Yi et al. (2009)
PsbQ1	At4g21280	Cl^- binding, grana stacking	Yi et al. (2006) and Anderson et al. (2008)
PsbQ2 (main PsbQ)	At4g05180		
Psb27		OEC assembly with PSII, PSII repair	Chen et al. (2006), Nowaczyk et al. (2006) and Roose and Pakrasi (2008)
PsbP-like proteins			
PPL1	At3g55330	OEC assembly with PSII, PSII repair	Ishikawa et al. (2005) and Ishihara et al. (2007)
PPL2	At2g39470	NDH accumulation	Ishihara et al. (2007)
PsbQ-like proteins			
PQL1	At1g14150	NDH component	Suorsa et al. (2010)
PQL2	At3g01440	Cyclic electron transfer	Yabuta et al. (2010)
PQL3	At2g01918		Yabuta et al. (2010)
Violaxanthin de-epoxidase			
VDE	At1g08550	Xanthophyll cycle, photoprotection	Baroli and Niyogi (2000), Arnoux et al. (2009) and Han et al. (2010)
High chlorophyll fluorescence proteins			
HCF136	At5g23120	PSII assembly and repair (D1 precursor stabilizing), redox signaling	Meurer et al. (1998), Plücken et al. (2002), Motohashi and Hisabori (2006) and Komenda et al. (2008)

LPA19	At1g05385	PSII assembly (D1 precursor processing)	Wei et al. (2010)
Immunophilins			
Cyp38	At3g01480	PSII assembly	Fulgosi et al. (1998), Vener et al. (1999), Fu et al. (2007) and Sirpiö et al. (2008)
Cyp20-2	At5g13120	PSII and NDH assembly, redox signaling	Edvardsson et al. (2007), Ingelsson et al. (2009) and Sirpiö et al. (2009)
FKBP13	At5g45680	Main PPIase, Redox signaling	Gopalan et al. (2004), Edvardsson et al. (2007) and Ingelsson et al. (2009)
FKBP20-2	At3g60370	PSII assembly, Rieske complex formation, redox signaling	Lima et al. (2006)
Proteases			
CtpA	At4g17740	D1 processing at the C-terminus for PSII assembly	Anbudurai et al. (1994)
Deg1	At3g27925	D1 proteolysis, chaperone activity for PSII assembly	Kapri-Pardes et al. (2007) and Sun et al. (2010)
Deg5	At4g18370	D1 proteolysis	Sun et al. (2007)
Deg8	At5g39830	D1 proteolysis	Sun et al. (2007)
Nucleoside diphosphate kinases			
NDPK3	At4g11010	Inter-conversion of ATP to GTP	Spetea et al. (2004)
Peroxiredoxins			
PrxQ	At3g26060	Unclear	Petersson et al. (2006)
Unclassified proteins			
TLP18.3	At1g54780	PSII assembly, D1 protein degradation	Sirpiö et al. (2007)
TL29	At4g09010	Associated with PSII	Granlund et al. (2009a, b)

The accession numbers in TAIR database (*Arabidopsis* gene index, AGI) and the function of each member are indicated
The source of these data is given in references

3.1 Plastocyanin

Plastocyanin (PC) is a lumenal mobile copper-binding protein, mediating electron transfer between the cytb_6f and PSI complexes. There are two paralogous genes in *Arabidopsis* (AGI 2000), *PETE1* and *PETE2*, coding for PC1 and PC2, the latter being the more abundant isoform. Mutants obtained using the RNA interference (RNAi) approach lack detectable levels of PC in the lumen, and are reduced in height by 50% compared to wild-type plants (Gupta et al. 2002). Most recently, Pesaresi et al. (2009) reported that null mutants of either of the isoforms displayed 60–90% lower PC content, however without drastic effects on the photosynthetic electron flow, indicating that they have similar function and that the PC concentration in the lumen is not limiting in these mutants. Nevertheless, according to the report by Abdel-Ghany (2009), the two PCs respond differently to copper availability, and the absence of either of them affects copper homeostasis in the lumen. Other proposed roles for PC are in relation to a cytochrome c-like protein and redox regulation of lumenal activities (Gupta et al. 2002); however, no direct evidence for their interaction has been found (Pesaresi et al. 2009).

3.2 Oxygen-Evolving Complex-Associated Proteins

The mechanism of oxygen evolution by PSII has remained conserved during evolution from cyanobacteria to higher plants. The oxygen-evolving complex (OEC) is a Mn_4CaCl_2 ion cluster (Loll et al. 2005) whose binding environment is optimized by several lumenal extrinsic PSII subunits. The accepted view is that plants and green algae have PsbO, PsbP, and PsbQ as OEC-associated proteins, whereas cyanobacteria have PsbO, PsbP, PsbQ, PsbV, and PsbU, the last two being lost during evolution of higher plants (Thornton et al. 2004). Numerous auxiliary functions have been suggested for these proteins in addition to stabilizing OEC. Excellent reviews on the OEC proteins, their interactions, and functions during PSII life cycle have been published by Seidler (1996), Suorsa and Aro (2007), Roose et al. (2007), and Ifuku et al. (2010). Furthermore, the sequencing of the *Arabidopsis* genome, and subsequent identification and characterization of putative lumenal proteins have revealed the presence of multiple isoforms and homologs of the OEC-associated proteins (Table 1), indicating a functional diversification of this group of proteins (Ifuku et al. 2010). In this section, we review the current knowledge about PsbO, PsbP, PsbQ, and Psb27, the latter proposed as an OEC transiently associated protein.

PsbO is conserved in all oxygenic photosynthetic organisms and is mainly known to stabilize the Mn_4CaCl_2 ion cluster. Other functions are related to Ca^{2+} binding and GTPase and carbonic anhydrase activity (Suorsa and Aro 2007). PsbO has also been proposed to regulate the water supply to OEC and the resulting H^+ transfer (Shutova et al. 2005).

Cyanobacteria, green algae, or spinach have a single-copy *psbO* gene. Sequencing of the *Arabidopsis* genome (AGI 2000) revealed the existence of two genes coding for two distinct proteins (PsbO1 and PsbO2), which differ only by ten amino acid residues in the mature forms. The expression of these proteins in *Arabidopsis* was confirmed by proteomic analyses of the thylakoid lumen (Peltier et al. 2002; Schubert et al. 2002). Both genes are expressed in wild-type plants, while the PsbO1 protein is about fourfold more abundant than PsbO2 (Murakami et al. 2002; Lundin et al. 2007b). The lower expression of PsbO2 indicates a regulatory rather than a structural role for this isoform in the function of PSII. Simultaneous RNAi-based suppression of *psbO* gene expression in *Arabidopsis* has confirmed the importance of PsbOs for PSII stability and photoautotrophy in plants (Yi et al. 2005). Notably, *Arabidopsis* mutants with a disrupted *psbO1* gene display an upregulation of *psbO2* expression without reaching wild-type PsbO levels (Lundin et al. 2007b, 2008; Liu et al. 2008). According to these reports and the one by Allahverdiyeva et al. (2009), the mutants are retarded in growth, have pale green leaves, contain less PSII than the wild-type plants, and display defects in PSII electron transport. The phenotype of *psbo2* mutants is also different from that of wild-type plants: they have retarded growth, dark-green elongated leaves, and a higher PSII content (Lundin et al. 2007b). Notably, under high light conditions, especially the growth and fitness of the PsbO2-lacking mutant were dramatically decreased, explained by malfunction of the PSII repair cycle at the disassembly step(s). Taken together, the findings from the phenotypic analyses of the mutants deficient in either of the *psbO* genes point to the importance of PsbO1 for the optimal function of PSII, and of PsbO2 in the repair cycle of this complex. Notably, between the two isoforms, PsbO2 has a higher GTPase activity than PsbO1 (Lundin et al. 2008), which has implications for GTP signaling during PSII repair (discussed in Sect. 4.2).

PsbP has been thought for a long time to be specific to higher plants and green algae, but more recent analyses have identified orthologs in cyanobacteria (Thornton et al. 2004). Its function is to optimize Ca^{2+} and Cl^{-} concentration for water oxidation, and also to provide Mn^{2+} ions to PSII during the light-dependent assembly of the Mn_4CaCl_2 cluster, a process known as photoactivation (Seidler 1996; Suorsa and Aro 2007; Roose et al. 2007). A high-resolution structure is available for tobacco PsbP, which revealed an electrostatic surface resembling that of cyanobacterial PsbV (Ifuku et al. 2004). Interestingly, in the same work, homology searches have indicated that the folding of PsbP is similar to that of a regulatory protein for a Ran GTPase. Therefore, a novel function in GTP signaling of PSII activity was suggested, which could be related to the reported GTPase activity of PsbO (Spetea et al. 2004; Lundin et al. 2008). Mutant analysis using RNAi approach in tobacco revealed a crucial role for PsbP in PSII activity and stabilization of the Mn_4CaCl_2 cluster in vivo (Ifuku et al. 2005). More recently, detailed analyses of these mutants indicated a specific role in the accumulation of active PSII supercomplexes and also in the grana stacking of thylakoids (Ido et al. 2009; Yi et al. 2009). There are two PsbP paralogs in *Arabidopsis*, PsbP1 and PsbP2, the latter one being most likely a pseudogene which has lost its function during evolution (Ifuku et al. 2010).

PsbQ has been reported to increase the binding affinity of Cl^- ions to PSII (Seidler 1996). However, RNAi in tobacco indicated that PsbQ is not essential for PSII activity in vivo (Ifuku et al. 2005). *Arabidopsis* contains two expressed PsbQ proteins, PsbQ1 and PsbQ2, which have 67% identical sequences and are encoded by two distinct genes (Peltier et al. 2002; Schubert et al. 2002). Simultaneous RNAi-mediated suppression of PsbQ expression in *Arabidopsis* indicated the importance of PsbQ for OEC stabilization, PSII function, and photoautotrophic growth under low light conditions (Yi et al. 2006). PsbP and particularly PsbQ were proposed to interact directly across the grana lumen, and hence to contribute to the integrity of the grana stacks (Anderson et al. 2008). However, RNAi-based mutant analyses confirmed that only PsbP is required for the thylakoid architecture (Yi et al. 2009).

Psb27 is a highly conserved component of the PSII complex whose structure has been resolved in cyanobacteria (Mabbitt et al. 2009). Since this component has not been previously detected in any of the available PSII crystal structures, it has been suggested to bind only transiently to PSII, by occupying the binding position of one of the OEC-associated proteins (e.g., PsbO), and thus facilitate the assembly of the Mn_4CaCl_2 cluster (Roose and Pakrasi 2008; Cormann et al. 2009). Psb27 was also suggested to be involved in PSII repair, since it was detected as a component of preassembled PSII complexes and because mutants lacking this protein displayed problems in recovery following photoinhibition (Chen et al. 2006; Nowaczyk et al. 2006).

3.3 PsbP-Like Proteins

Genomic and proteomic analyses identified a number of PsbP homologs in plants, cyanobacteria, and algae. In *Arabidopsis*, there are two PsbP-like proteins (PPLs) and seven PsbP-domain containing proteins (PPDs) (Ifuku et al. 2010). The two PPLs show homology to the cyanobacterial PsbP (Ishihara et al. 2007). PPL1 is expressed under stress conditions, and mutants lacking this protein display higher sensitivity to high light and delayed recovery of PSII activity compared to the wild type. Therefore, PPL1 was suggested to assist in the efficient repair of photodamaged PSII complexes. PPL2 is co-expressed with subunits of NAD(P)H dehydrogenase (NDH) and is, therefore, proposed to be required for accumulation of this complex. When it comes to PPDs, the closest neighbor based on phylogeny is PPL1, indicating that they may be paralogs with a so-far unknown function (Ifuku et al. 2010).

3.4 PsbQ-Like Proteins

There are three proteins paralogous to PsbQ in *Arabidopsis*, named PsbQ-like proteins (PQLs), PQL1, PQL2, and PQL3 (Ifuku et al. 2010). The mutants lacking PQLs are not affected in oxygen evolution, instead the NDH complex-mediated cyclic electron flow is impaired, and the levels of its subunits are decreased (Suorsa

et al. 2010; Yabuta et al. 2010). Reversibly, the PQL levels are decreased in mutant lines deficient in certain NDH subcomplexes. Therefore, Suorsa and coworkers suggested that the three PQLs are novel subunits of the NDH complex, each of them with distinct localization and type of interactions.

3.5 *Violaxanthin De-epoxidase*

Violaxanthin de-epoxidase (VDE) converts violaxanthin, a carotenoid bound to the thylakoid light-harvesting proteins, to zeaxanthin, which has the ability to dissipate excess light as heat and also to scavenge ROS (Baroli and Niyogi 2000). VDE is free in the lumen at neutral pH and attaches to the thylakoid membrane upon acidification of the lumen, thus accessing its substrate violaxanthin. VDE uses ascorbate as a co-substrate. The structure of its lipocalin domain has been determined at both acidic and neutral pH (Arnoux et al. 2009). Interestingly, the enzyme is a monomer with occluded binding site at neutral pH and is a dimer with two linked open binding sites at acidic pH, the latter revealing an adaptation of an asymmetric structure to a symmetric substrate. Based on the crystal structure, two residues located in the putative binding sites for violaxanthin and ascorbate have been suggested to participate in the catalytic mechanism (Saga et al. 2010). Overexpression of VDE was found to increase the function of the xanthophyll cycle and to alleviate photoinhibition of PSII and PSI in tomato during high light and a combination of chilling stress with low irradiance (Han et al. 2010).

3.6 *High Chlorophyll Fluorescence Proteins*

High chlorophyll fluorescence 136 (HCF136) was originally discovered based on its mutant high chlorophyll fluorescence phenotype due to problems with biogenesis of PSII (Meurer et al. 1998). The protein has been shown to localize strictly to the lumen of stromal thylakoid membranes. HCF136 is expressed in dark-grown *Arabidopsis* seedlings, indicating that it must be present when PSII complexes are assembled (Plücken et al. 2002). HCF136 has orthologs in cyanobacteria (YCF48) and is proposed to be a stability and/or an assembly factor of PSII, since it stabilizes the newly synthesized D1 protein and its binding to the reaction center pre-complex (Komenda et al. 2008). It has also been suggested that HCF136 participates in the redox-mediated reduction of proteins in the lumen (Motohashi and Hisabori 2006).

Low PSII accumulation 19 (LPA19) is an *Arabidopsis* paralog of Psb27, which, like HCF136, was identified based on its mutant high chlorophyll fluorescence phenotype due to low PSII accumulation (Wei et al. 2010). This protein was shown to interact with the C-terminus of precursor D1, thus facilitating its processing during PSII biogenesis.

3.7 Immunophilins

Immunophilins are present in all subcellular compartments of *Arabidopsis* and are expected to participate in protein folding, degradation, and signal transduction, and thus to be crucial during development and stress response (Romano et al. 2004). The thylakoid lumen of plant chloroplast contains 16 immunophilins, namely, five cyclophilins (Cyp) and eleven FK506-binding proteins (FKBPs), which are predicted to be involved in protein folding. However, only two of them have been shown to possess peptidyl-prolyl cis/trans isomerase (PPIase) activity, indicating that the other immunophilins may have lost this activity during evolution (Edvardsson et al. 2007). Below, I will review the knowledge about two cyclophilins (Cyp38 and Cyp20-2) and two FKBPs (FKBP13 and FKBP20-2) due to their location in the thylakoid lumen and recently documented function in photosynthetic regulation.

The first characterized immunophilin associated with the thylakoid membrane was spinach TLP40, also known as the thylakoid lumen PPIase (Fulgosi et al. 1998). It was predicted to contain a cyclophilin-like C-terminal domain, an N-terminal leucine zipper, and a phosphatase-binding domain. TLP40 was mainly found to be located in the lumen of the stroma-exposed thylakoid membranes, where proteins are integrated in photosynthetic complexes. This immunophilin could be either present free in the thylakoid lumen or bound to the membrane, forms which were proposed to activate and inactivate, respectively, a putative PP2A-like phosphatase that dephosphorylates PSII core proteins (Vener et al. 1999). It has, therefore, been proposed to be a component of a signal transduction chain during the repair of photodamaged PSII complexes. The *Arabidopsis* ortholog has been identified as cyclophilin 38 (*Cyp38*) using biochemical and proteomic approaches (Schubert et al. 2002; Peltier et al. 2002). This protein does not display any cyclophilin activity and lacks the phosphatase-binding domain. The mutants deficient in Cyp38 displayed problems with assembly and stability of supercomplexes during biogenesis of PSII, and also showed malfunction of its donor side, increased susceptibility to photoinhibition, and decreased phosphorylation of PSII proteins (Fu et al. 2007; Sirpiö et al. 2008).

Cyclophilin *Cyp20-2* predominates in the lumen of the stroma-exposed thylakoid membranes and is proposed to be an auxiliary protein of the NDH complex, since it comigrates with this complex in native gels (Sirpiö et al. 2009). Its expression was found to be strongly reduced in mutant lines deficient of a membrane-embedded component of the NDH complex, and it was therefore suggested to participate in the assembly of the hydrophobic domain of the complex. On the contrary, no malfunction of the NDH complex was observed in *cyp20-2* knockout mutants (Sirpiö et al. 2009). Furthermore, the PPIase activity in *cyp20-2* mutants was similar to that of the wild type and is attributable to FKBP13, and no clear phenotypic difference in double mutants for Cyp20-2 and FKBP13 was observed (Edvardsson et al. 2007; Ingelsson et al. 2009). Taken together, these

findings indicate redundancy of lumenal immunophilin functions and suggest that the Cyp20-2 and FKBP13 do not act as PPIases in the thylakoid lumen.

FKBP13 is a major immunophilin in the thylakoid lumen, shown to contain a pair of disulfide bridges located in the N- and C-termini of the protein (Gopalan et al. 2004). These bridges are essential for PPIase activity and appear to be specific to chloroplast FKBPs since they are not present in animal and yeast orthologs. The FKBP13 protein is active in the oxidized form and inactive in the reduced form. This activation pattern is opposite to the one present in the stroma, where enzymes are activated following reduction by thioredoxin (Trx) (Gopalan et al. 2004, 2006). Characterization of the FKBP13 protein revealed a new mechanism of regulation in the thylakoid lumen, namely, redox regulation (see also Sect. 4.1). To better understand this mechanism, the crystal structure of the reduced form of *Arabidopsis* FKBP13 was determined (Gopalan et al. 2006). Based on that structure, redox-linked changes in the secondary structure of the catalytic PPIase domain were proposed to be responsible for the functional difference between the oxidized and reduced forms. Based on the redox dependence of its PPIase activity, FKBP13 has been proposed to catalyze protein folding under oxidative stress and oxygen evolution in the thylakoid lumen (Shapiguzov et al. 2006). Mutants deficient in FKBP13 display almost no PPIase activity, without however showing differences in growth and photosynthetic parameters from the wild type, when grown under either optimal or stress conditions (Ingelsson et al. 2009).

FKBP20-2 is an immunophilin containing one disulfide bridge located at the C-terminus, which was found to be reduced by Trx (Lima et al. 2006). FKBP20-2 itself displays only poor PPIase activity, which is not affected by Trx. Knockout mutations in the corresponding gene caused reduced plant growth due to lower rates of PSII activity (Lima et al. 2006). Based on the characterization of the same mutants using native gels and immunoblots, FKBP20-2 was shown to be required for the accumulation of PSII supercomplexes in *Arabidopsis* via a yet unknown mechanism.

3.8 Proteases

Following co-translational insertion in the thylakoid membrane, the D1 subunit of PSII complex is processed at the C-terminus by the CtpA protease. This information is supported by the fact that disruption of the corresponding gene in the cyanobacterium sp. *Synechocystis* PCC 6803 resulted in accumulation of a D1 protein that is 2 kDa long (Anbudurai et al. 1994). Although the mutant contains functional reaction centers, it lacks water-oxidizing PSII activity. This indicates that the proteolytic activity of CtpA is absolutely required to achieve full PSII activity and water oxidation.

Among chloroplast proteases, three ATP-independent serine Deg proteases are present inside the thylakoid lumen, namely, Deg1, Deg5, and Deg8. *Deg1* is attached peripherally at the lumenal side of the thylakoid membrane, and its location made it an attractive candidate for proteolysis of the photodamaged D1 protein

(Kapri-Pardes et al. 2007). The D1 protein contains five transmembrane helices (A to E), and there are several known cleavage sites exposed at the luminal side of the thylakoid membrane, namely, located in the C-terminus, in A–B loop and C–D loop. Mutants depleted of the Deg1 protein are smaller, more sensitive to photoinhibition, and accumulate lower amounts of fragments (of 16 and 5.2 kDa) and correspondingly larger amounts of photodamaged D1, which is an inactive form. Overexpressed Deg1 is capable of inducing production of the 16-kDa D1 protein fragment. Most recently, a chaperone activity of Deg1 was demonstrated and showed that Deg1 assists in the assembly of PSII by interaction with the D2 protein (Sun et al. 2010).

Deg5 and Deg8 form a hexamer in the lumen, which is proposed to cleave the D1 protein in the C–D loop synergistically, generating a 16-kDa fragment (Sun et al. 2007). Mutants lacking either of the two enzymes, and specially the double mutant are more sensitive to photoinhibition, since they are not able to produce this specific D1 fragment.

3.9 Nucleoside Diphosphate Kinase 3

Nucleoside diphosphate kinases (NDPKs) catalyze the transfer of the gamma-phosphate group of ATP to nucleoside diphosphates (NDPs), thus generating nucleoside triphosphates (NTPs) other than ATP. NDPKs are encoded by a small gene family and are present in both prokaryotic and eukaryotic organisms. NDPKs may not only function as regulators of intracellular nucleotide pools (housekeeping enzymes), but may also be involved in signal transduction by activating GTP-binding proteins. At present, at least three NDPK paralogs have been identified in *Arabidopsis,* NDPK1, NDPK2, and NDPK3, with orthologs in other plant species. Among them, special attention has been paid to NDPK2 due to its involvement in phytochrome and UV-B light signaling (Shen et al. 2005). Later on, Shen et al. (2008) have demonstrated a role of NDPK2 as a GTPase-activating protein, thus linking phytochrome-mediated light signaling and G-protein-mediated signaling.

Although predicted as a mitochondrial protein, the spinach NDPK3 was characterized as a thylakoid lumenal protein (Spetea et al. 2004). A pea NDPK ortholog of the spinach protein was found in the intermembrane space of mitochondria, tightly bound to the inner membrane through an interaction with the mitochondrial ADP/ATP carrier, and was suggested to supply GTP for the activation of a GTP-binding protein and/or for export to the cytosol (Sweetlove et al. 2001; Knorpp et al. 2003). *Arabidopsis* NDPK3 has a dual location, namely, in the thylakoid lumen (Spetea et al. 2004) and in the mitochondrial intermembrane space (Sweetlove et al 2001; Heazlewood et al. 2004). NDPK3 activity in the lumen was shown to be specifically involved in ATP to GTP interconversion, and proposed to link ATP transport across the thylakoid membrane with GTP signaling during PSII repair cycle (Spetea et al. 2004; Fig. 2). Attempts to perform phenotypic analyses of *ndpk3* mutants have failed due to the inability to identify homozygous

plants, which may indicate the lethal effect of this mutation (Knorpp and Spetea, unpublished observations).

3.10 Peroxiredoxins

Peroxiredoxins (Prx) are present in many organisms from bacteria to mammals and have function in oxidant defense and signal transduction. Among the ten peroxiredoxins found in *Arabidopsis*, the 2-Cys Prx, which is attached at the stromal side of the thylakoid membrane, is the best studied and suggested to be involved in antioxidant defense and protection of photosynthesis (Pulido et al. 2010). The thylakoid lumen contains PrxQ, for which no clear role has been assigned, since the mutant lacking this protein displayed a normal photosynthetic performance (Petersson et al. 2006).

3.11 Unclassified Proteins

TLP18.3 was identified in proteomic studies of thylakoid-associated polysome nascent chain complexes and shown to be a lumenal protein evenly distributed between grana and stroma membranes. The PSII complexes in mutants lacking this protein were found to be more susceptible to photoinhibition, because of a poor assembly of monomers into dimers within the grana stacks due to an impaired D1 protein degradation in the stroma regions (Sirpiö et al. 2007).

TL29 is one of the most abundant proteins in the lumen. It shares sequence homology to an ascorbate peroxidase and was, therefore, suggested to participate in defense against ROS. However, no such activity in the lumen could be associated with TL29. Knockout mutants for the corresponding gene displayed wild-type levels of lumenal peroxidase activity, when grown under high light stress. On the contrary, TL29 was found to be a component of PSII complex (Granlund et al. 2009a, b).

4 Role of Thylakoid Lumen in Plant Cell Signaling

Functioning and efficient cell signaling are essential for cell survival. Many mechanisms allow for rapid switching of signals, on or off, e.g., redox status, GTP, Ca^{2+}, and protein phosphorylation (Clark et al. 2001). These mechanisms have been poorly studied in the thylakoid lumen in the past. Below, I will review the most recent reports, which have brought new insights into the thylakoid signal transduction.

4.1 Redox Signaling

Redox signaling includes modification of amino acids via reactions with nitric oxide and oxidation of cystein residues (Hancock 2009). Such oxidative reactions depend on the redox status of the intracellular environment in which the protein resides and require the presence of a ferredoxin/thioredoxin system. Target enzymes of such a system have been identified in both chloroplast stroma and the thylakoid lumen, but it appears that a reduced and an oxidized form of enzymes are active, respectively, in the two compartments (Buchanan and Luan 2005). As a lumenal example, the case of FKBP13 has been described in Sect. 3.7.

C-type cytochrome thiol disulfide transporter CcdA is a thylakoid-specific membrane transfer system mediating disulfide bond reduction of proteins in the chloroplast thylakoid lumen (Motohashi and Hisabori 2010). More specifically, CcdA supplies reducing equivalents from the stroma to HCF164 (Sect. 3.6), considered to be a lumenal thioredoxin-like protein. Interestingly, 19 thioredoxin targets have been found using proteomics in the *Arabidopsis* thylakoid lumen (Hall et al. 2010). Among these targets are the two PsbOs and VDE. It has, therefore, been proposed that PsbO1 and PsbO2 may be degraded in a redox-dependent manner and that the VDE activity could be dependent on the thiol redox state of the enzyme.

4.2 GTP Signaling

GTPases represent the most important component of signaling cascades. Requirement for GTP has been reported for proteolysis of the D1 protein following light-induced inactivation of PSII complex (Spetea et al. 1999, 2000). In a search for potential GTPases mediating this crucial event in plant survival during light stress, the PsbO has been characterized as a GTPase (Fig. 2; Spetea et al. 2004; Lundin et al. 2007a). The isolated PsbO protein has a low intrinsic GTPase activity, which is enhanced considerably in its PSII-associated form. The GTPase activity induces the release of PsbO from its binding site on the lumenal surface of PSII and enhances the rate of D1 protein degradation. Notably, *Arabidopsis* PsbO2 differs, has a higher GTPase activity than PsbO1, and plays a specific role in D1 protein degradation (see Sect. 3.2).

These findings have opened a new area of research related to GTP signaling across the thylakoid membrane. Furthermore, it has raised the question about the presence of nucleotides in the lumenal space. A constant pool of GTP or other nucleotides does not exist in the thylakoid lumen (Kieselbach et al. 1998). Furthermore, no nucleotide-binding proteins have been identified during proteomics of the lumen (Schubert et al. 2002; Peltier et al. 2002). Nevertheless, functional genomics in *Arabidopsis* has allowed identification and characterization of lumenal NDPK3 (Sect. 3.9) and of an exchanger of ATP against ADP across the thylakoid

membrane, named thylakoid ATP/ADP carrier (TAAC) (Spetea et al. 2004; Thuswaldner et al. 2007). TAAC-mediated translocation of ATP and conversion to GTP by the NDPK3 must be highly regulated processes so that the produced amounts are sufficient only for signaling. The lumenal GTP may activate GTPases, such as the PsbO protein (Fig. 2), initiating signaling within and across the thylakoid membrane, including the light-dependent turnover of the D1 protein.

Detailed phenotypic analyses of two *taac* knockout mutants indicated a reduced ATP trans-thylakoid transport and an increased sensitivity to prolonged high light stress compared to that in the wild type (Thuswaldner et al. 2007; Yin et al. 2010). The D1 protein turnover in these mutants was found to be impaired due to a malfunctioning PSII repair at the disassembly steps, requiring the GTPase activity of PsbO. A thylakoid phosphate transporter (ANTR1) has been identified at the gene level and is suggested to export back to the chloroplast stroma the phosphate resulting from nucleotide metabolism in the lumen (Ruiz Pavón et al. 2008). Due to their transport activity, TAAC and ANTR1 may provide the link between ATP synthesis on the stromal side of the thylakoid membrane and nucleotide-dependent reactions in the lumenal space (Fig. 2).

The above-described GTP signaling may be a plant-specific pathway which has evolved due to the unique structural organization of the thylakoid membrane for the following reasons: (1) D1 protein degradation requires GTP only in plant thylakoid but not in cyanobacterial membranes (Spetea et al. 1999); (2) TAAC is a eukaryote-specific transporter; (3) four G-domains in classic GTPases were found to be conserved in plant PsbO sequences, but not in green algae and cyanobacterial sequences (Lundin et al. 2007a); and (4) PsbP1 is plant specific and its structure resembles that of a GTPase-activating protein (Ifuku et al. 2004). The GTP signaling may regulate the on/off cycle of PsbO. The next step in the research on GTP signaling would be to elucidate the precise role of GTP during PSII repair cycle and its role in other processes in the thylakoid lumen.

4.3 Ca^{2+} Signaling

Ca^{2+} signals are known to regulate the physiology and responses to environment of the plant cell, and to be able to induce multiple pathways simultaneously (Kudla et al. 2010). This signaling requires transport systems to move Ca^{2+} ions between compartments, and also Ca^{2+}-binding proteins to activate enzymes, such as Ca^{2+}-dependent protein kinases. The picture of Ca^{2+} signaling in the thylakoid membrane is fragmentary, as described below. There is biochemical evidence for a Ca^{2+}/H^{+} antiporter in the pea thylakoid membrane (Ettinger et al. 1999), but the corresponding gene has not yet been identified. The thylakoid ATP/ADP carrier is an ortholog to a rabbit peroxisomal Ca^{2+}-dependent carrier, which activity is strictly regulated by Ca^{2+} concentration (Thuswaldner et al. 2007). A Ca^{2+} sensor protein (CAS) has been characterized in the thylakoid membrane and shown to be involved in the regulation of stomata opening (Vainonen et al. 2008; Weinl et al. 2008). Ca^{2+}

binding to the lumenal side of subunit III of the ATP synthase has been reported to gate the H^+ flux, controlling lumenal pH and VDE activity, and hence photoprotection (Dilley 2004). There have been previous reports on the regulation by Ca^{2+} ions and calmodulins of thylakoid protein phosphorylation and of D1 protein turnover (Li et al. 1998, 2010). Finally and most importantly, the OEC complex contains Ca^{2+} ions, and the associated OEC proteins PsbO and PsbP bind Ca^{2+} ions and can therefore function as calmodulins. During PSII biogenesis and turnover, Ca^{2+} ions associate and dissociate from the OEC, which could lead to the induction of Ca^{2+} signaling in the lumen and across the membrane. Interestingly, the *Arabidopsis* genome contains several predicted chloroplast Ca^{2+}-dependent protein kinases (Spetea, unpublished observations), awaiting identification and functional characterization.

4.4 Protein Phosphorylation

Protein phosphorylation is one of the most important posttranslational modifications in eukaryotic cells and mediates almost all basic processes. Chloroplast phosphoproteomics in *Arabidopsis* revealed novel target proteins, phosphorylation sites, and kinase activities, hence novel cascades that may be part of networks for different functions (Baginsky and Gruissem 2009). There is no doubt about the existence and role of phosphorylation of thylakoid proteins on the stromal side of the membrane (Pesaresi et al. 2011), but so far only sporadic reports on phosphorylation of lumenal proteins are available (Rinalducci et al. 2006; Wagner et al. 2006).

5 Concluding Remarks

The chloroplast thylakoid lumen was initially thought as a proton sink, but the genomic- and proteomic-based findings obtained in *Arabidopsis* have revealed a much more complex role than that previously anticipated. Most of the up-to-date characterized lumenal protein components have a wide range of functions related to photosynthetic linear and cyclic electron transfer, PSII assembly/turnover, and photoprotection. Notably, there is increasing evidence for the existence of proteins participating in redox, GTP, and Ca^{2+} signaling across the thylakoid membrane. There is still a lot to be done to elucidate these pathways, and many lumenal proteins are still awaiting functional characterization. Nevertheless, it is obvious that our knowledge about the thylakoid lumen is rapidly expanding beyond the simple bioenergetic perspective.

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Connecting Environmental Stimuli and Crassulacean Acid Metabolism Expression: Phytohormones and Other Signaling Molecules

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Abstract Plasticity in Crassulacean acid metabolism (CAM) expression has long been recognized to occur both within and between species, and a range of environmental cues is implicated in both long- and short-term regulation of CAM operation. Important insights into the signal transduction chains between environmental stimuli and CAM expression are now available, and our discussion is focused on these recent findings. The role of plant hormones, nitric oxide, reactive oxygen species, intracellular calcium, protein phosphatases, and kinases in the signaling cascades leading to changes in CAM expression in response to water availability, salinity, light intensity, and photo- and thermoperiod is discussed. Whenever possible, differences and similarities among the signaling elements controlling CAM expression in distinct CAM plant models are highlighted. Conceivably, many other aspects of the signaling processes leading to CAM expression are still

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to be elucidated; therefore, some potential strategies to improve our knowledge in this field are briefly discussed.

1 Introduction

Crassulacean acid metabolism (CAM) is essentially a mechanism of CO_2 concentration at the site of ribulose-1,5-biphosphate carboxylase/oxygenase (RUBISCO) and occurs in many plants, including submerged freshwater plants. For aquatic species, this method of CO_2 concentration improves CO_2 uptake, while for terrestrial ones, this photosynthetic adaptation represents a mechanism for conserving water when compared to C_3 photosynthesis. In fact, by carrying out atmospheric CO_2 uptake mainly at night, when evapotranspiration rates are much lower than during daytime, water loss is considerably reduced, and, therefore, water use efficiency (WUE) in CAM plants is markedly improved (Cushman and Borland 2002; Winter et al. 2005). The enhanced WUE provided by CAM photosynthesis represents a distinctive ecological advantage for plants living in environments characterized by constant or intermittent limitations in water supply, such as desertic and epiphytic niches, respectively. Not surprisingly, the abundance of CAM species is notably higher in these water-limited sites (Cushman 2001; Cushman and Borland 2002).

Biochemical, ecophysiological, anatomical, taxonomic, and evolutionary aspects of the CAM pathway have been extensively reviewed by many authors (Borland and Taybi 2004; Cushman and Bohnert 1999; Keeley and Rundel 2003; Lüttge 2004a; Osmond 1978; Silvera et al. 2010; Ting 1985), whereas the signaling events controlling the expression of this type of photosynthesis have received relatively less attention (Taybi et al. 2002). In this review, we will emphasize the current knowledge of CAM signaling in our discussion and also highlight some future perspectives in this field.

1.1 Multiple Origins of CAM

The CAM pathway has evolved polyphyletically many times in the plant kingdom and is currently found in approximately 16,800 species of 343 genera in 35 families, including terrestrial and aquatic angiosperms (monocots and eudicots), in addition to gymnosperms (Silvera et al. 2010). The more representative families of this syndrome are Aizoaceae, Asclepiadaceae, Asteraceae, Bromeliaceae, Cactaceae, Crassulaceae, Euphorbiaceae, Portulacaceae, and Orchidaceae. A survey of Bromeliaceae species showed that both CAM syndrome and epiphytic habit have evolved at least three times (Crayn et al. 2004). For Clusiaceae and Orchidaceae, multiple, independent CAM evolutions also occurred (Gehrig et al. 2003; Silvera et al. 2010). Although the original ecological pressure for the evolution of CAM was probably

the challenge of CO_2 acquisition from the atmosphere, it is currently accepted that aridity must have been the main driving force responsible for the polyphyletic evolution of CAM in terrestrial environments (Keeley and Rundel 2003).

1.2 Biochemical Aspects of the CAM Pathway

Basically, in CAM plants, CO_2 uptake from the environment occurs during the night (phase I, after Osmond 1978) and sometimes during the light period when there is higher air humidity (early morning – phase II, and late afternoon – phase IV). In certain cases, internal CO_2 produced by respiration can also be used. In the cytosol, the CO_2 is captured in oxaloacetate (OAA) by the enzyme phosphoenolpyruvate carboxylase (PEPC). This enzyme is activated by phosphorylation through a dedicated kinase, which is under circadian control and synthesized *de novo* each night (Taybi et al. 2000, 2004). OAA is reduced to organic acids, mainly malic acid, which are stored in the large vacuole of the chlorenchyma cells during the night. Therefore, nocturnal acid accumulation is one of the principal diagnostic features of CAM. Subsequent daytime decarboxylation of organic acids releases CO_2 in the cytoplasm, which is assimilated into carbohydrates by RUBISCO (C_3 cycle – phase III). This increased intracellular CO_2 concentration suppresses stomatal opening during most of daytime.

Given the expressive number of independent origins of CAM, great metabolic diversity can also be found among distinct CAM groups. Two major decarboxylase enzymes are responsible for releasing CO_2 from malate during daytime: PEP carboxykinase (PEPCK) and malic enzyme (NADP-ME and NAD-ME). Plants from the families Asclepiadaceae, Bromeliaceae, Euphorbiaceae, and Portulacaceae perform malate decarboxylation preferentially through PEPCK, while species from the families Aizoaceae, Cactaceae, Crassulaceae, and Orchidaceae have ME as the major decarboxylase. Thus, families with either type of enzymes occur within monocots and eudicots (Christopher and Holtum 1996).

The CAM pathway is also characterized by a daily carbohydrate cycle, which comprises the night-time synthesis of PEP to form C_4 acids (e.g., malic acid) and the daytime acid decarboxylation, followed by the return of three carbon compounds to the carbohydrate storage pools (Borland and Dodd 2002; Borland and Taybi 2004). Approximately 25–30% of total leaf dry mass of CAM plants may turnover each day in the acid/carbohydrate cycle that provides RUBISCO with CO_2 (Black and Osmond 2003). The type of carbohydrate accumulated during daytime varies among species, generating basically two major groups: the starch formers (e.g., *Mesembryanthemum crystallinum*) and extrachloroplastic carbohydrate formers (e.g., *Ananas comosus*) (Christopher and Holtum 1996).

Besides the day/night fluctuations in organic acids, other parameters such as the abundance of CAM-related enzymes, and the diurnal pattern of stomatal conductance and CO_2 uptake have also been used to determine the occurrence of CAM,

allowing the identification of several permutations in this photosynthetic pathway and emphasizing both intra- and interspecific plasticity in CAM expression.

2 CAM Physiotypes and Plasticity

CAM photosynthesis is both inducible and constitutive. The majority of CAM species is believed to exhibit this photosynthetic adaptation in a constitutive mode at adulthood since net CO_2 uptake by these plants occurs almost exclusively at night even under well-watered conditions, resulting in conspicuous diurnal fluctuations in organic acids. These constitutive or obligate CAM species can be differentiated according to strong and weak CAM depending on the degree of nocturnal acid accumulation (Cushman and Borland 2002; Herrera 2009; Silvera et al. 2005). Under severe drought conditions, many CAM plants shift to a CAM idling mode, where stomata remain closed day and night, and nocturnal recycling of respiratory CO_2 into organic acids allows the occurrence of some photosynthetic activity during daytime (Ting 1985). CAM idling is considered a very strong form of CAM and is interpreted as a strategy to minimize photoinhibition, maintain a positive carbon balance, and allow a rapid recovery of the photosynthetic activities when the environmental conditions become more favorable (Ting 1985). In contrast, a weak form of CAM named CAM cycling has also been described, which displays a classical C_3 gas exchange pattern associated with some diurnal fluctuation in organic acids resulting from the nocturnal recycling of respiratory CO_2 (Patel and Ting 1987; Ting 1985). Besides minimizing photoinhibition, the CAM cycling behavior also leads to a reduction in daytime stomatal aperture, increasing water conservation.

On the contrary, inducible CAM species, also called C_3–CAM facultative (or C_3–CAM intermediate) plants, are able to shift from C_3 to CAM in response to changes in environmental conditions, including drought, salinity, light intensity, photo- and thermoperiod, and nutrient deficiency (Cushman and Borland 2002; Lüttge 2004a). Among these environmental stimuli, water stress is probably the predominant factor regulating CAM expression in terrestrial C_3–CAM facultative species (Cushman 2001; Cushman and Borland 2002). Under optimal, well-watered conditions, facultative CAM plants maximize growth by performing C_3 photosynthesis, but when challenged by water shortage, they rapidly undergo a C_3-to-CAM transition, decreasing daytime gas exchange and consequently increasing WUE. Therefore, a remarkable plasticity in adjusting CO_2 uptake and transpirational water loss is perhaps the most obvious adaptive advantage of expressing CAM in a facultative way.

Inducible CAM might also provide other mechanisms to help optimize physiological performance under drought conditions (Herrera 2009). First, dark CO_2 fixation and respiratory CO_2 recycling can contribute to maintain a positive carbon balance under water deficit. Second, the CAM cycle may facilitate water uptake through a nocturnal decrease in the leaf osmotic potential due to acid accumulation (Herrera et al. 2008; Ruess et al. 1988). Third, CAM induction by drought may also

represent a strategy for extending resource allocation to reproductive biomass and maximize reproduction before the end of the life cycle (Herrera 1999; Taisma and Herrera 2003; Winter and Ziegler 1992). Taken together, these potential adaptive traits provided by CAM photosynthesis under drought conditions may compensate the higher energy requirement for carbon fluxes and the more limited CO_2 uptake capacity of CAM photosynthesis when compared to C_3 pathway.

The time span required for the induction of CAM is strongly dependent on the species and environmental stimuli considered (Taybi et al. 2002). For instance, CAM induction in response to water deficit or salt stress is usually rapid (within hours or a few days) and, in most cases, can be reversed to C_3 mode after re-watering (Cushman and Borland 2002; Freschi et al. 2010a, b; Lüttge 2006). In contrast, photoperiodic induction of CAM has been shown to be gradual (throughout months), largely irreversible, and probably associated with an acceleration in developmental progression, as clearly demonstrated for both *M. crystallinum* and *Kalanchoë blossfeldiana* (Brulfert et al. 1982; Cheng and Edwards 1991; Gregory et al. 1954). These temporal differences in the C_3–CAM progression not only implicate mechanistic particularities at the biochemical level (e.g., upregulation of housekeeping enzymes *versus* expression of CAM-specific enzyme isoforms) but also suggest that distinct regulatory and signaling processes may also be involved in the control of CAM expression by different environmental factors.

3 Signaling Transduction and CAM Regulation

The switch from C_3 to CAM photosynthesis is perhaps one of the most complex plant adaptive responses to environmental challenges, implying a coordinated reprogramming of gene expression and regulatory mechanisms to ensure the establishment of a new metabolic homeostasis at cellular, tissue, and whole-plant levels. Changes in circadian system, carbohydrate partitioning, intracellular transport of metabolites, osmotic adjustment and the synchronization between two competing carboxylation systems (PEPC and RUBISCO) inside the same cell are triggered within a time frame that may vary from a few hours to several weeks. To maintain a metabolic equilibrium during this dramatic physiological shift, an integrated series of transcriptional, translational, and posttranslational regulatory processes is required (Cushman and Bohnert 1999). Therefore, based on the physiological complexity and high responsiveness to environmental stimuli, it is plausible to expect the existence of equally complicated signaling routes controlling the C_3–CAM transition.

Currently, most of our understanding about the signaling cascades implicated in the induction of CAM photosynthesis is based on studies conducted on common ice plant (*Mesembryanthemum crystallinum*), which has become a favored CAM plant model not only for signaling research but also for biochemical, genetic, and molecular genetic studies (Bohnert and Cushman 2000; Cushman and Bohnert 1999; Cushman and Borland 2002; Taybi and Cushman 1999). However, it is

pertinent to mention that other CAM plants also have a great potential for investigations of the signal transduction networks controlling CAM expression, including *Clusia* and *Kalanchoë* species (Lüttge 2006; Taybi et al. 1995) and commercial CAM plants such as pineapple (*Ananas comosus*) (Freschi et al. 2010a). Therefore, although the data discussed in this review will be predominantly based on studies conducted with *M. crystallinum*, whenever possible, comparisons will be made with results obtained using other CAM plants.

3.1 Signaling Events During CAM Modulation by Water Availability

Virtually all C_3–CAM facultative plants respond to water stress with a rapid increase in the abundance of transcripts and proteins of CAM-related enzymes, which in most cases is followed by the induction of functional CAM (Brulfert et al. 1993; Cushman and Borland 2002; Freschi et al. 2010a; Lüttge 2004a; Taybi et al. 1995). Depending on the intensity and duration of the water stress, typical CAM or CAM idling can be induced (Brulfert et al. 1996; Freschi et al. 2010b; Guralnick and Ting 1986; Lüttge 2006; Sipes and Ting 1985). During investigations about CAM induction by water stress, it has often been discussed whether the water deficit signal is perceived directly by a reduction in the mesophyll cell turgor pressure, or endogenous signals are actually required to connect this environmental stimulus and the CAM induction response (Eastmond and Ross 1997; Piepenbrock et al. 1994; Taybi and Cushman 1999; Winter and Gademann 1991). Favoring the hypothesis that a decrease in leaf cell turgor exerts a direct control on CAM expression, it has been demonstrated that CAM induction in well-watered plants (Piepenbrock et al. 1994; Winter and Gademann 1991) or detached leaves (Taybi and Cushman 1999) of *M. crystallinum* is preceded by a reduction in leaf turgor. However, by carrying out a split-root experiment with this same species, where only half of the roots were deprived of water, Eastmond and Ross (1997) clearly demonstrated that CAM induction can be triggered before any change in mesophyll cell turgor pressure, suggesting that a signal generated at water-stressed roots is conveyed to the shoot, inducing CAM expression in the leaf tissues. Although the identity of this endogenous signal was not established in this particular work, evidence obtained by other studies has indicated some possible candidates (Chu et al. 1990; Dai et al. 1994; Peters et al. 1997; Taybi and Cushman 2002; Taybi et al. 2002). Indeed, reinforcing the hypothesis that CAM induction by water stress does not depend on changes in the leaf water content, we have recently demonstrated that the drought-induced upregulation of CAM expression in the tank C_3-CAM facultative bromeliad *Guzmania monostachia* occurs exclusively at the apical leaf portion, which curiously is the only region of the leaf to display no significant changes in its relative water content (Freschi et al. 2010b). In this specific case, besides the root system, more basal leaf regions, which experience intense decrease in their relative water content under drought, might also be involved in the production

of signal(s) responsible for inducing CAM expression in the turgid apical leaf portions.

As with other environmental stresses, water limitation can promote changes in absolute concentration and distribution of plant hormones, which in most cases results in significant changes in the balance between distinct hormonal classes. Generally, water deficit stimulates abscisic acid (ABA) synthesis and inhibits cytokinin (Ck) production in the roots, thus affecting the supply of these hormones to the leaves via transpiration stream (Pospisilova et al. 2005). As a result, the balance between ABA and Cks in foliar tissues represents an important mechanism controlling physiological responses (e.g., the stomatal closure) that culminate in higher adaptive fitness for the whole plant (Hare et al. 1997; Pospisilova et al. 2000, 2005; Tanaka et al. 2006). According to the available data, ABA and Cks are also considered the two major hormonal classes controlling CAM expression, and it is currently speculated that these two hormones might indeed act as root-derived messengers responsible for modulating the CAM activity in response to changes in soil water availability (Chu et al. 1990; Schmitt and Piepenbrock 1992; Thomas and Bohnert 1993; Thomas et al. 1992).

Distinct lines of evidence indicate that ABA acts as a stimulatory signal for CAM expression (Taybi et al. 2002). Studies conducted on several CAM plants, including *M. crystallinum*, *K. blossfeldiana*, and *A. comosus*, have demonstrated that exogenous ABA can trigger increases in activity and/or transcript accumulation of CAM-related enzymes either when applied to intact plants (Chu et al. 1990; Dai et al. 1994; Forsthoefel et al. 1995a, b; Freschi et al. 2010a; McElwain et al. 1992; Taybi et al. 1995; Tsiantis et al. 1996) or directly supplied to detached leaves (Taybi and Cushman 1999, 2002; Taybi et al. 1995). In most cases, the ABA-induced upregulation of CAM enzymes is followed by an initiation, or upregulation, of functional CAM, leading, in some cases, to levels of night-time acid accumulation equivalent to those observed in water-stressed plants (Chu et al. 1990; Dai et al. 1994; Freschi et al. 2010a; Ting 1981). Depending on the treatment conditions and the plant species, either typical CAM or CAM idling can be induced by exogenous ABA (Taybi et al. 1995). Differences in the efficacy of the ABA treatment have also been observed depending on the plant age (Chu et al. 1990), light intensity (McElwain et al. 1992; Thomas et al. 1992) and organ treated, i.e., feeding ABA to the roots is usually more effective than foliar application (Chu et al. 1990; Edwards et al. 1996).

Corroborating the stimulatory effect of exogenous ABA on CAM expression, the osmotic stress-induced upregulation of CAM generally shows a positive correlation with increased leaf content of ABA, as demonstrated in *M. crystallinum* (Taybi and Cushman 2002), *K. blossfeldiana* (Taybi et al. 1995), and, more recently, in young pineapple plants (Freschi et al. 2010a). These increases in endogenous ABA usually precede the appearance of CAM (Freschi et al. 2010a; Taybi et al. 1995), whereas a concomitant reduction in both leaf ABA abundance and CAM expression can also occur during water stress recovery (Freschi et al. 2010a). Therefore, increased ABA production and/or accumulation seems to represent a signal to promote and, subsequently, maintain high levels of CAM expression under water stress conditions.

Investigations to determine whether ABA accumulation is indispensable for the water deficit-induced initiation of CAM have demonstrated that a parallel, non ABA-dependent, signaling route also accounts for the induction of this photosynthetic pathway (Freschi et al. 2010a; Taybi and Cushman 2002). When ABA biosynthesis was inhibited in detached leaves of *M. crystallinum* or intact plants of pineapple, no considerable changes could be detected in the drought-induced upregulation of CAM enzymes (Freschi et al. 2010a; Taybi and Cushman 2002) and nocturnal acid accumulation (Freschi et al. 2010a). Therefore, as demonstrated for other plant responses to environmental stresses (Ishitani et al. 1997; Riera et al. 2005), the control of CAM expression by water deficit might involve both ABA-dependent and -independent signaling pathways (Freschi et al. 2010a; Taybi and Cushman 2002).

The occurrence of CAM induction by drought without ABA accumulation implies that other endogenous signals may substitute for this hormone in interconnecting water availability and CAM expression. In this sense, the existence of negative endogenous signal(s) capable of repressing CAM operation has been suggested by several authors (Eastmond and Ross 1997; Peters et al. 1997; Schmitt and Piepenbrock 1992). For instance, it has been shown that the degree of water loss required to induce PEPC transcript accumulation in detached leaves of *M. crystallinum* is lower than that observed daily in leaves of intact individuals performing C_3 photosynthesis (Piepenbrock and Schmitt 1991; Winter and Gademann 1991). Therefore, it has been hypothesized that putative regulatory substances produced outside the leaf might repress CAM expression by overriding daily transient conditions of leaf water deficits (Eastmond and Ross 1997; Schmitt and Piepenbrock 1992). Under water deficit, however, this putative repressor signal would no longer be produced and/or transported to the leaves, thereby de-repressing CAM induction (Schmitt and Piepenbrock 1992).

Based on available data, Cks are currently the best candidates to fulfill a role as negative root-derived signals responsible for inhibiting CAM expression in leaves of well-watered plants (Eastmond and Ross 1997; Schmitt and Piepenbrock 1992). Among the evidence supporting this view, considerable decreases in endogenous Cks have been observed during the water stress-induced upregulation of CAM in excised leaves of *M. crystallinum* (Peters et al. 1997) and intact plants of pineapple (Freschi et al. 2010a). In water-stressed pineapple plants, the upregulation of genes encoding key enzymes of the CAM pathway (e.g., PEPC and PEPCK) coincided specifically with the period of major decrease in the leaf Ck concentration (Freschi et al. 2010a). In contrast, the downregulation of CAM by water stress recovery is associated with an increase in endogenous Cks (Freschi et al. 2010a; Peters et al. 1997). Moreover, although initial reports have described some stimulatory effect of 6-benzylaminopurine (BA) on the expression of the CAM-specific PEPC isoform of *M. crystallinum* (*Ppc1*) (Thomas and Bohnert 1993; Thomas et al. 1992), more recent studies have demonstrated that exogenous Cks are indeed suppressors of CAM initiation in response to stress conditions (Freschi et al. 2010a; Peters et al. 1997; Schmitt et al. 1996; Schmitt and Piepenbrock 1992). Working with *M. crystallinum*, Schmitt and Piepenbrock (1992) demonstrated that micromolar

concentrations of BA not only inhibited the upregulation of CAM by environmental stresses but also accelerated the decline in PEPC transcript abundance in detached leaves from stressed plants subjected to rehydration. Moreover, when the root system, which is the main site of Ck production, is removed from *M. crystallinum* plants, the induction of PEPC by water stress is clearly intensified (Schmitt and Piepenbrock 1992). A certain level of specificity between the cytokinin chemical structure and the CAM inhibition response might also occur since zeatin-type Cks are relatively more efficient than isopentenyladenine-type ones in suppressing the water stress-induced upregulation of CAM in young pineapple plants (Freschi et al. 2010a).

Resembling the effect of exogenous Cks, methyl jasmonate (MJA) has also been shown to limit PEPC induction and CAM operation in water-stressed *M. crystallinum* plants (Dai et al. 1994; Schmitt et al. 1996). Moreover, MJA reinforces the decrease in PEPC transcript abundance in detached leaves subjected to stress recovery and seems to act synergistically with Cks during the downregulation of PEPC and CAM expression (Schmitt et al. 1996). In contrast, ethylene and auxin do not seem to participate in the modulation of CAM expression in response to water stress since exogenous treatments with these growth regulators did not affect CAM activity either in *A. comosus* (Freschi et al. 2010a) or in *M. crystallinum* (Dai et al. 1994; Hurst et al. 2004). Moreover, pineapple plants challenged with changes in water availability showed no marked oscillations in endogenous indole-3-acetic acid (IAA) or in the emission of ethylene during either the upregulation or the attenuation of CAM expression (Freschi et al. 2010a). In the CAM plant *Aptenia cordifolia*, changes in endogenous salicylic acid (SA) also did not occur following water stress imposition (Cela et al. 2009); however, the relationship between this stress-related signaling molecule and CAM expression still needs more investigation.

Some second messengers potentially involved in transducing the water stress stimulus into changes in CAM expression have already been identified in both *M. crystallinum* (Taybi and Cushman 1999, 2002; Taybi et al. 2002) and *A. comosus* (Freschi et al. 2010a). Among these downstream signaling events, changes in intracellular calcium concentration seem to represent an important point of convergence among external and internal factors controlling CAM expression. Pre-treatment of detached leaves of ice plant (Taybi and Cushman 1999) or pineapple (Fig. 1) with EGTA, an extracellular calcium chelator, revealed that mobilization of this ion from apoplastic space is crucial for ABA- and drought-induced initiation of CAM, suggesting that the influx of extracellular Ca^{2+} is a key downstream event during the regulation of CAM expression via both ABA-dependent and -independent signaling pathways. Conversely, treatment of unstressed leaves with ionomycin, a calcium ionophore, triggered the expression of the CAM-specific PEPC isoform (*Ppc1*) in *M. crystallinum* and resulted in up to fourfold increases in the activities of PEPC, PEPCK, and malate dehydrogenase (MDH) in *A. comosus* (Fig. 1). Moreover, unstressed leaves of ice plant treated with the thapsigargin, an intracellular Ca^{2+} releaser, also stimulated *Ppc1* transcript accumulation, while experiments with Ca^{2+}-channel blockers indicated that L-type Ca^{2+} channels are

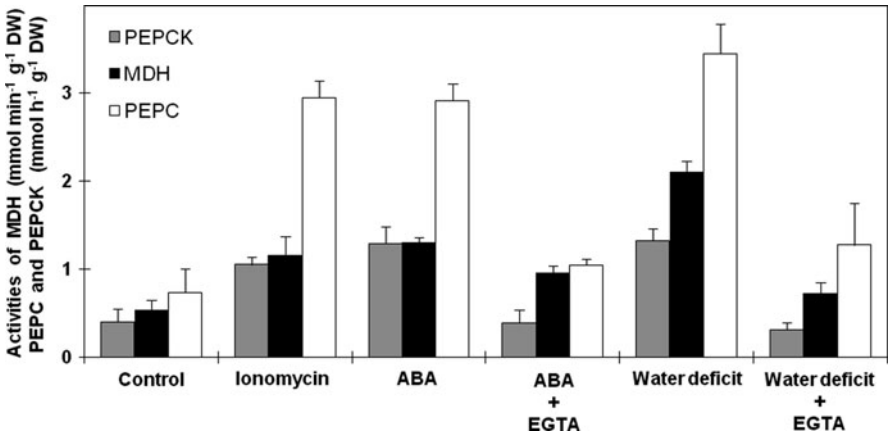

Fig. 1 Changes in cytosolic calcium mediate ABA- and water deficit-induced upregulation of CAM enzymes in young pineapple plants. Detached leaves of 3-month-old plants were treated for 15 d with ionomycin (50 μM), ABA (100 μM), ABA (100 μM) + EGTA (10 mM), water deficit (30% polyethylene glycol), or water deficit + EGTA (10 mM), and, subsequently, the activities of PEPC, MDH, and PEPCK were determined as described in Freschi et al. (2010a). Data are means ± SD of three replicates from two independent experiments. *DW* Dry weight

likely involved in the signaling events leading to CAM expression in this species (Taybi and Cushman 1999). Evidence has also revealed that inositol-1,4,5-triphosphate (IP3), an important plant cell signal involved in the mobilization of Ca^{2+} from intracellular storages, might also play a role in the ABA-dependent signaling cascade leading to *Ppc1* expression in *M. crystallinum*; however, this second messenger is likely not required for the stress-induced initiation of CAM via the ABA-independent pathway (Taybi et al. 2002).

In plant cells, one of the most prominent mechanisms responsible for transducing oscillations in intracellular [Ca^{2+}] into cellular responses is the activation of Ca^{2+}-dependent protein kinases (CDPKs). By treating detached leaves of *M. crystallinum* with specific inhibitors of Ca^{2+}/calmodulin-dependent enzymes, Taybi and Cushman (1999) demonstrated that CDPKs might also participate in the signaling events leading to *Ppc1* transcript accumulation in response to osmotic stress, dehydration, or exogenous ABA. Indeed, accumulating evidence suggests that at least in *M. crystallinum*, events of protein phosphorylation/dephosphorylation may represent a significant step in the signal transduction network connecting external stimuli and CAM induction (Taybi and Cushman 1999; Taybi et al. 2002). Inhibition of protein phosphatases by okadaic acid or cantharidic acid strongly blocked *Ppc1* transcript accumulation in response to stress (e.g., osmotic stress, dehydration, and salinity) or ABA (Taybi and Cushman 1999; Taybi et al. 2002). However, as highlighted by Cushman and Bohnert (2004), special caution is needed in drawing conclusions from results obtained through treatments with inhibitors of protein kinases and phosphatases, since nonspecific impairment of other metabolic and regulatory processes can also be expected to occur. However, studies focused on

the characterization of the expression dynamics of specific protein kinases and phosphatases during the modulation of CAM in facultative species might provide more conclusive data on this subject.

By using this approach, Miyazaki et al. (1999) have isolated and characterized the tissue- and stress-specific expression pattern of 10 genes (*MPC1-10*) homologous to protein phosphatases of the PP2C family from ice plant. In this study, they have demonstrated that one of these PP2Cs, termed *MPC6*, is expressed at high levels in young leaves and displays a gradual decrease in transcript abundance as the leaves age. Drought and salinity stress can accelerate the decline in *MPC6* transcript abundance, while, in contrast, these same stresses resulted in the upregulation of other *MPC*s (e.g., *MPC2* and *MPC8*) (Miyazaki et al. 1999). Together, these developmental and stress-induced modifications in *MPC*s' expression indicate that some PP2Cs of *M. crystallinum* may play a role as negative (e.g., *MPC6*) or positive (e.g., *MPC2* and *MPC8*) modulators of CAM expression, either by directly regulating components of the CAM machinery or more indirectly, by changing the phosphorylation state of other signaling proteins, such as transcription factors involved in the control of CAM-specific gene expression. For instance, the day/night evaluation of transcript abundance of five *MPC*s (*MPC2*, *5*, *6*, *8*, and *9*) in ice plants performing CAM revealed that their expression is under strict circadian control (Sato et al. 2007), suggesting that these PP2C isoforms could also be involved in diurnal regulation of the phosphorylation state of CAM-related proteins. Finally, a tentative suggestion of a role of *MPC2* in the ABA-signaling pathway has also emerged (Miyazaki et al. 1999) based on the fact that this drought-responsive gene is clustered in the same PP2C group where several negative regulators of ABA signaling are found, e.g., *ABI1*, *PP2CA*, and *ABI2* (Leung et al. 1997; Merlot et al. 2001; Meyer et al. 1994; Rodriguez et al. 1998). However, more studies are required to determine exactly how protein phosphatases participate in the signaling of CAM induction.

Based on the premise that CAM expression is particularly affected by water deficit, signaling molecules associated with plant adaptive responses to this abiotic stress represent a logical target for new studies on the signaling mechanisms that induce and/or maintain the CAM pathway. Illustrating this premise, we have shown that the free radical nitric oxide (NO), which has recently emerged as a mandatory participant in several plant responses to external stresses, might also play a role in CAM modulation, representing a positive signal for the establishment of this specialized mode of photosynthesis (Freschi et al. 2010a). Exogenous NO, applied as donors or gaseous mixtures, was shown to be sufficient to trigger increases in CAM expression at levels comparable to those detected in plants subjected to water deficit or ABA treatment. In agreement with these pharmacological data, the upregulation of CAM expression in plants exposed to water deficit was accompanied by increases in both NO emission and endogenous levels (Freschi et al. 2010a). Removal of NO from the leaf tissues by a specific NO scavenger revealed that this free radical might be involved in the ABA-induced upregulation of CAM in pineapple, probably acting as a second messenger of this hormone. Nitrate reductase (NR) was identified as the most likely biosynthetic origin of NO during

ABA-triggered CAM induction, since the inhibition of this enzymatic activity completely abolished NO production and strongly blocked CAM initiation in response to exogenous ABA (Freschi et al. 2010a). Together, these data indicate that NO probably acts as a key downstream component in the ABA-dependent signaling pathway leading to the upregulation of CAM in pineapple plants.

Considering the multiple, independent origins of the CAM pathway in the plant kingdom, it is quite interesting to observe that two phylogenetically distant species, such as the eudicot *M. crystallinum* and the monocot *A. comosus*, probably share several signaling elements during the regulation of CAM expression in response to changes in water availability. In both species, two signaling pathways, one ABA dependent and other ABA independent, converging into changes in intracellular [Ca^{2+}], appear to be the major stimulatory routes responsible for the upregulation of CAM in response to drought stimulus (Fig. 2). In contrast, endogenous Cks apparently represent a parallel inhibitory signal, suppressing CAM expression when water availability is not limiting. In *A. comosus*, NO probably acts as a downstream component in the ABA-dependent signaling cascade, while in *M. crystallinum*, CDPKs may play a decisive role in transducing oscillations in cytosolic calcium into CAM expression.

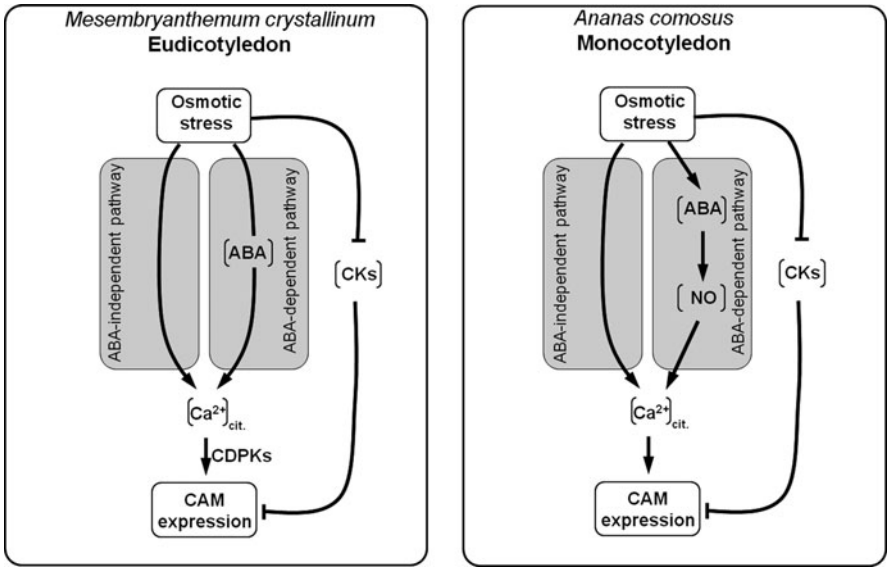

Fig. 2 Model of potential signaling transduction events mediating osmotic stress-induced upregulation of CAM in *M. crystallinum* (based on Taybi et al. 2002) and *A. comosus* (based on Freschi et al. 2010a). *ABA* abscisic acid, *Cks* cytokinins, *NO* nitric oxide, $[Ca^{2+}]_{cit}$ cytosolic calcium concentration, *CDPKs* Ca^{2+}-dependent protein kinases

3.2 *Signaling Events During CAM Modulation by Salinity*

One of the main consequences of salinity is osmotic stress; therefore, the higher WUE provided by the CAM syndrome could be interpreted as an obvious adaptive advantage for plants living in high salinity conditions. However, contrary to expectation, constitutive or inducible CAM is not commonly found among true halophytes (Lüttge 2004b). Currently, the best known example of CAM halophyte is *M. crystallinum*, which displays intense C_3–CAM switch when challenged with high salinity conditions (Cushman and Bohnert 1992b, 2004; Cushman et al. 1990; Lüttge 1993, 2004b; Winter 1973). Therefore, the current knowledge of the endogenous signals associated with the CAM induction by salt stress is restricted to this plant model.

Although salinity results in osmotic stress, a direct influence of ionic stress on CAM expression has been evidenced in ice plant. For example, when detached leaves of this species are exposed to salt stress, the induction of genes encoding CAM-related enzymes can be detected within a timeframe of a few hours (<3 h), during which only minimal water loss is observed (Cushman and Borland 2002; Taybi and Cushman 1999). Under these conditions, the CAM initiation may result, at least in part, from a direct ionic effect of salinity stress. Therefore, separate signaling transduction pathways might be involved in controlling the manifestation of CAM in response to salt and water stress.

Whereas endogenous ABA accumulation is commonly observed during the drought-induced C_3–CAM transition (Freschi et al. 2010a; Taybi and Cushman 2002; Taybi et al. 1995), much less clear is the relationship between this phytohormone and the CAM induction by salt stress (Taybi et al. 2002; Thomas et al. 1992). Working with hydroponically grown *M. crystallinum* plants, Thomas et al. (1992) observed that increases of about tenfold in the leaf ABA content coincided with the rise in PEPC enzyme triggered after 30 h of salt stress treatment. In contrast, when detached leaves of this same species were exposed to only 9 h of salt stress, no significant changes in endogenous ABA were observed, even though a marked increase in *Ppc1* transcript abundance could be detected (Taybi and Cushman 2002). Moreover, inhibition of ABA biosynthesis did not prevent the induction of PEPC expression by salt stress (Taybi and Cushman 2002; Thomas et al. 1992), indicating that ABA might not be required for the initiation of CAM in response to salinity. In this sense, the salt stress-triggered ABA accumulation in *M. crystallinum* leaves reported by Thomas et al. (1992) could possibly be a result of salt-induced water deficit rather than the effect of salt per se, since significant water losses are commonly observed following long-term exposure to salinity.

The involvement of endogenous Cks in the signaling of CAM induction by salt stress is also not clearly understood. Although Thomas et al. (1992) observed no significant changes in the zeatin-type cytokinin content in plants of *M. crystallinum* maintained for about 3 days in the presence of salt stress, Peters et al. (1997), working with this same species, detected a marked decrease in a variety of free and conjugated forms of this hormone after 6 days of salt stress imposition.

Additionally, co-treatments with Cks and salt stress have also provided divergent results (Schmitt and Piepenbrock 1992; Thomas and Bohnert 1993; Thomas et al. 1992). When supplied to the roots, exogenous BA did not affect the salt-induced CAM expression (Thomas and Bohnert 1993; Thomas et al. 1992); however, the supply of Cks directly to the leaves has been reported to repress CAM induction by high salinity (Schmitt and Piepenbrock 1992). Differences in the experimental conditions make it difficult to establish a consensus among these results (Schmitt and Piepenbrock 1992; Thomas and Bohnert 1993; Thomas et al. 1992), leaving many doubts about the actual role of Cks during CAM induction by salt stress.

In salt-stressed plants of *M. crystallinum*, marked changes in the content of free and conjugated polyamines and enhanced ethylene production have also been observed during the period that comprises the induction of CAM (Kuznetsov et al. 2007; Stetsenko et al. 2009), leading to the hypothesis that these two classes of stress-related endogenous signals could possibly mediate the CAM induction by high salinity. However, additional experiments demonstrated that both ethylene and cadaverine (a free polyamine), when exogenously applied to non-stressed plants, do not stimulate either *Ppc1* expression or nighttime acid accumulation (Kuznetsov et al. 2007). Nevertheless, the accumulation of cadaverine, among other polyamine species, is assumed to have an active role in the adaptation of ice plant to salt stress, probably acting as free radical scavengers (Kuznetsov et al. 2007).

In the last decade, a number of studies have characterized the antioxidant responses in plants of *M. crystallinum* exposed to salt stress (Broetto et al. 2002; Miszalski et al. 2001; Niewiadomska et al. 2004; Slesak et al. 2002, 2003, 2008). Besides analyzing the potential effects of the CAM induction on the antioxidant metabolism per se, part of these studies have also investigated whether the cellular redox status and/or reactive oxygen species (ROS) would play a signaling role during the C_3–CAM switch in response to high salinity. In general, considerable changes in transcript abundance and activity of antioxidant enzymes occur in parallel with the C_3–CAM transition induced by salt (Broetto et al. 2002; Niewiadomska et al. 1999; Slesak et al. 2002). In many cases, increases in distinct isoforms of superoxide dismutase (SOD) (Broetto et al. 2002; Miszalski et al. 1998; Slesak et al. 2002) and decreases in catalase (Broetto et al. 2002; Niewiadomska et al. 1999; Niewiadomska and Borland 2008) have been observed during the salt-induced C_3–CAM shift, suggesting enhanced hydrogen peroxide (H_2O_2) generation. H_2O_2, as other ROS, is continuously produced as a byproduct of several plant metabolic pathways; however, the intracellular levels of this potentially damaging compound are tightly controlled by a set of antioxidant compounds and antioxidative enzymes. On the contrary, under stressful conditions such as salt stress, this delicate equilibrium between H_2O_2 synthesis and scavenging may be unbalanced, resulting in the accumulation of this compound, which, among other consequences, might serve as an endogenous signal to trigger adaptive plant responses. In *M. crystallinum*, Slesak et al. (2008) reported that the induction of functional CAM in salt-stressed plants was preceded by significant increases in leaf H_2O_2 concentration. Moreover, a positive correlation between photo-production of H_2O_2 inside chloroplasts and the CAM induction in ice plant has been demonstrated,

further suggesting a role for H_2O_2 in CAM expression (Slesak et al. 2003). In addition, exogenous H_2O_2 was also found to upregulate CAM expression in this species when applied at relatively high concentrations (100 or 400 mM) (Slesak et al. 2003). Treatments with oxidative stress factors different from H_2O_2, such as ozone, have also resulted in the upregulation of important metabolic components of the CAM pathway, such as the expression of the CAM-specific PEPC isoform (*Ppc1*) and its dedicated kinase (*Ppck*) (Borland et al. 2006) and the activity of fumarase, NAD-ME, and NADP-ME (Niewiadomska et al. 2002). However, this gaseous oxidant failed to induce functional CAM expression, as evidenced by the lack of significant nocturnal acidity accumulation in ozone-treated individuals of this species (Borland et al. 2006; Hurst et al. 2004; Niewiadomska et al. 2002), suggesting that not all components of the CAM machinery are responsive to this oxidative stress factor. Consequently, further studies are required to elucidate the possible roles of oxidative stress and ROS on CAM induction by salt stress. Also, it would be interesting to investigate a possible interaction between ROS and some downstream events (e.g., intracellular $[Ca^{2+}]$ and protein kinases and phosphatases) that seem involved in the regulation of CAM expression by both drought and salt stress (Taybi and Cushman 1999; Taybi et al. 2002).

At the molecular genetic level, the modulation of CAM gene expression in response to salinity can also rely on the existence of several *cis*-acting elements for salt-responsive gene expression in the 5′ flanking regions of CAM-related genes such as *Ppc1* and *GapC1* (isogene encoding a stress-inducible NAD-dependent glyceraldehyde-3-phosphate dehydrogenase) (Cushman and Bohnert 1992a, 2004). Moreover, increased levels of CC(A/T)GG methylation in the ice plant genome have also been observed during the salt stress-induced C_3–CAM shift, resulting in hypermethylation of satellite DNA, which might be associated with the acquisition of a specialized chromatin structure capable of modulating the transcriptional activity of a large set of CAM-related genes (Dyachenko et al. 2006). In addition, more recently, Huang et al. (2010) reported increases in methylation of specific cytosines of the *Ppc1* promoter region, which coincided with the enhanced transcriptional activity of this gene during the C_3–CAM transition triggered by salt stress. Therefore, the transcriptional activation of *Ppc1* by salinity might be, at least in part, regulated at the epigenetic level (Huang et al. 2010). These recent findings suggest that salinity-induced changes in DNA methylation are possibly implicated in the transcriptional control of CAM-related genes, opening a significant window of opportunity for new investigations regarding the relevance of epigenetic control on induction of the whole CAM machinery.

3.3 Signaling Events During CAM Modulation by Light

Supplying both energy for photosynthesis and relevant environmental information, light modulates many aspects of the CAM pathway. First, because carbohydrate availability is a major constraint for nocturnal CO_2 uptake in CAM plants (Borland

and Dodd 2002; Borland and Taybi 2004), the abundance of photosynthetically active radiation receipt in the previous photoperiod determines CAM activity on a daily basis (Borland and Dodd 2002; Lüttge 2004a). Second, enzymes and regulatory proteins required for the functioning of the CAM cycle might be modulated by light at the transcriptional, translational, and/or posttranslational levels. Third, and more essential for our discussion, some light attributes such as intensity, quality, and temporal distribution (photoperiod) can modulate the degree of CAM expression on a long-term basis (Brulfert et al. 1975; Maxwell et al. 1999; Taybi et al. 2002). Some clues about the nature of the photoreceptors at the beginning of the light-driven CAM-induction cascades are currently available (Grams and Thiel 2002); however, the endogenous signals involved in the signal transduction chain between the light stimulus and CAM expression remain largely unknown.

Under natural conditions, high light intensities are commonly associated with water stress. Therefore, it has been argued whether light stress affects CAM per se or acts exclusively in combination with changes in water availability (Lüttge 2000). Whereas some studies have shown that high irradiance is ineffective in upregulating CAM expression when applied as the only environmental stress (Barker et al. 2004; Kornas et al. 2010), other reports have indicated that high photosynthetic radiation itself might represent a stimulatory signal for the intensification of CAM activity in C_3–CAM intermediate plants such as *Clusia minor* (Franco et al. 1991; Grams and Thiel 2002; Herzog et al. 1999), *M. crystallinum* (Broetto et al. 2002), and *Guzmania monostachia* (Maxwell et al. 1994, 1999; Maxwell 2002). In *C. minor*, which displays rapid and reversible C_3–CAM switches in response to high irradiation, Grams and Thiel (2002) have demonstrated that a UV-A/blue light receptor is likely involved in controlling CAM expression in response to high light intensities. According to Herzog et al. (1999), when exposed to high irradiance under well-watered conditions, the C_3–CAM transition in *C. minor* is not associated with increases in endogenous ABA levels.

Perhaps the most evident and widely reported effect of light as a signal for the control of CAM expression is the gradual C_3-to-CAM progression promoted by photoperiodic stimuli, as observed in the annual species *K. blossfeldiana* and *M. crystallinum* (Cheng and Edwards 1991; Gregory et al. 1954). The photoperiod requirement to promote CAM in these species is quite distinct, i.e., short days for *K. blossfeldiana* (Brulfert et al. 1975; Gregory et al. 1954) and long days for ice plant (Cheng and Edwards 1991; Guralnick et al. 2001); however, in both cases, phytochrome has been characterized as the photoreceptor responsible for sensing the light stimulus (Brulfert et al. 1975; Cockburn et al. 1996). In ice plant, the ratio of red: far red light has been shown to modulate the magnitude of PEPC and CAM induction in response to salt stress (Cockburn et al. 1996), suggesting that the signal transduction cascades triggered by phytochrome and salinity may interact somehow to promote CAM photosynthesis.

In all cases described, the C_3–CAM shift in response to changes in the photoperiod seems to be associated with an enhancement in the plant developmental progression, as indicated by a significant acceleration in the flowering onset, which is taken as a classical indicator of the plant developmental stage (Cheng and

Edwards 1991). In this sense, at least two aspects might be taken into consideration regarding the link between plant ontogeny and CAM induction. On the one hand, the influence of the developmental phase achieved by individual leaves or whole plants on the capacity to express CAM has been accepted almost as consensus for many C_3–CAM facultative species, in which CAM induction cannot be fully induced by environmental cues until a critical stage of development is attained (Cushman et al. 1990). On the other hand, an intense dispute has arisen whether the CAM expression can be triggered in some inducible CAM plants exclusively by the developmental progression, occurring regardless of the presence or absence of environmental stresses when the plant reaches a specific phase of ontogeny (Herppich et al. 1992; Piepenbrock et al. 1994). Currently, it is not clear if the photoperiodic stimulation of CAM expression results from the acceleration in the plant developmental progression or, alternatively, if CAM and flowering induction are independently triggered by the same changes in photoperiod. At least for *M. crystallinum*, recent evidence clearly demonstrated that CAM induction is under environmental rather than developmental control (Winter and Holtum 2007); therefore, the photoperiodic induction of CAM operation and the onset of floral development in this species might possibly represent separate responses to the same environmental cue.

Gibberellins (GAs) are considered one of the hormonal classes more closely related with photoperiodic responses in plants. For instance, GAs can replace the long day requirement in several plant events, including the induction of floral transition in rosette species (Halliday and Fankhauser 2003). In *M. crystallinum*, foliar application of GA induced a significant PEPC increase and also stimulated nighttime acid accumulation, which were accompanied by earlier branching, flowering, and leaf senescence when compared to that in control plants (Guralnick et al. 2001). Therefore, the acceleration of general plant development and the C_3–CAM transition either by GA under short day conditions or by long-day photoperiod without exogenous GA inevitably lead to the hypothesis that this hormonal class is probably implicated in the signaling cascades connecting photoperiodic signal and CAM expression (Guralnick et al. 2001). However, endogenous evidence of a role for GA during CAM signaling is still missing. Indeed, the only correlation between endogenous hormones and photoperiodic induction of CAM consists of the observation that elevations in leaf ABA levels preceded PEPC increase in intact plants or isolated leaves of *K. blossfeldiana* exposed to inductive photoperiodic conditions (Taybi et al. 1995).

3.4 Signaling Events During CAM Modulation by Temperature

In general, differences in the day/night temperature (thermoperiod) are more effective in modulating CAM performance than variations in absolute temperatures. Although the CAM cycle can satisfactorily occur under constant temperature (Lüttge 2004a; Lüttge and Beck 1992), lower night-time temperatures associated

with warmer daytime conditions have been described to be favorable for CAM operation in both facultative and obligatory CAM species (Haag-Kerwer et al. 1992; Neales et al. 1980; Nievola et al. 2005). In the inducible CAM species *C. minor*, for instance, the C_3–CAM switch is clearly promoted when the differences between day and night temperatures are amplified (Haag-Kerwer et al. 1992). During the modulation of CAM performance, the first target of temperature seems to be the control of translatable mRNA abundance and activity of individual enzymes and regulatory proteins, such as that reported for PEPC kinase (Borland et al. 1999). Moreover, temperature can also directly affect the fluidity and, therefore, the permeability of cell membranes, altering the capacity of compartmentation of metabolites, which is especially critical for CAM plants because nocturnally produced organic acids must be accumulated in the vacuole until the subsequent photoperiod (Friemert et al. 1988; Kluge et al. 1991; Kliemchen et al. 1993; Behzadipour et al. 1998). Finally, changes in air humidity are a classic consequence of alterations in temperature; therefore, the modulation of some elements of the CAM syndrome by temperature might indeed be attributed to changes in the air water vapor pressure, as previously discussed for the diurnal pattern of stomatal aperture in CAM plants (Lüttge 2004a). The involvement of plant hormones or other endogenous signals in the regulation of CAM by temperature remains almost completely unexplored. Perhaps the only exception is a preliminary study carried out with young pineapple plants exposed to constant or thermoperiodic conditions (Nievola et al. 2005). According to this study, when compared with individuals maintained under a constant temperature of 28°C, pineapple plants exposed to thermoperiodic conditions (28°C day/15°C night) exhibited increased nocturnal acid accumulation and higher levels of endogenous ABA and IAA during most of the day/night cycle (Nievola et al. 2005); however, further studies are necessary to clarify whether these changes in the leaf hormonal composition induced by thermoperiodic conditions are in fact associated with the upregulation of CAM expression in this species.

4 Conclusions and Perspectives

In this chapter, we aimed to cover the major advances in defining the signaling mechanisms controlling CAM expression in facultative species, with an emphasis on the interplay between plant hormones and second messengers. The signaling cascades implicated in transducing environmental cues such as water availability, salinity, light intensity, and photo- and thermoperiod into changes in CAM expression were discussed separately in an attempt to highlight differences and similarities among the signal transduction routes triggered by each of these external stimuli. As noted in our discussion, important aspects of the molecular and signaling processes that underpin the induction of CAM by water deficit have been recently discovered, revealing a coordinated and complex set of parallel, yet overlapping signaling pathways, which apparently involve plant hormones, intracellular calcium, nitric

oxide, protein phosphatases, and kinases. Comparatively less is known about the signaling events implicated in the modulation of CAM expression by salinity, light, and temperature. Even scarcer is the available information regarding the signaling events mediating the regulation of CAM by other environmental factors such as nutrient availability, extremes in temperature, or anoxia.

Taking into consideration that, in the field, the distinct environmental factors that modulate CAM operation never act individually but always interactively, a significant challenge for the future will be to uncover how different external stimuli are integrated to adjust CAM expression at levels compatible with the environmental conditions. On the contrary, since changes in the distinct environmental parameters are frequently challenging to separate, even in laboratory experiments, special attention is also required to clarify whether a given external stimulus is able to modulate CAM expression per se or acts indirectly by affecting other environmental parameters (e.g., effect of temperature and salinity on water availability).

Besides discovering new components of the signaling cascades leading to CAM expression, another future challenge will be to compare the signaling networks implicated in the environmental control of CAM expression in facultative species with contrasting C_3–CAM switch dynamics. For instance, the signaling cascades implicated in the gradual and virtually irreversible establishment of CAM under certain conditions (e.g., photoperiod induction of CAM in ice plant) may possibly differ from those required for the rapid and freely reversible C_3–CAM switches observed under some circumstances (e.g., drought-induced CAM expression in some *Clusia* spp.). Such studies, if conducted in phylogenetically distant species, will also be essential to evaluate the degree of conservation in the signaling mechanisms controlling CAM expression in plants with independent evolutionary origins of the CAM pathway.

Another important goal for the future will be the generation of mutants and transgenic CAM plants with alterations in perception or biosynthesis of specific signaling elements (e.g., plant hormones, NO, ROS, protein phosphatases, and kinases), which could represent an important tool to investigate complex questions such as the molecular and biochemical processes required to promote CAM expression in response to developmental and external factors. In this context, efficient mutant screening protocols, and transformation and regeneration systems for CAM plant models are sorely needed.

Moreover, it appears to be highly worthwhile to study how environmental and developmental factors interact at the signaling level in order to determine the degree of CAM expression. In this sense, comparisons between the changes in endogenous signaling compounds in facultative CAM plants at different developmental stages, and, consequently, with distinct responsiveness to environmental signals that stimulate CAM, could possibly improve our understanding of the regulatory cascades that govern interactions between the developmental and environmental control of CAM photosynthesis.

Ultimately, given the ample range of environmental stimuli implicated in the modulation of CAM and the virtually infinite ways that they can interact, not only

among themselves but also with developmental and tissue-specific inputs, many other aspects of the signaling processes leading to CAM expression are still to be elucidated. Therefore, a combination of biochemical, genetic, and molecular strategies in different CAM models might be needed to improve our understanding of the signal transduction events required to orchestrate the complex array of physiological modifications necessary for fine-tuning CAM expression according to environmental conditions.

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Part IV
Systematics

Systematics of the Green Algae: A Brief Introduction to the Current Status

Thomas Friedl and Nataliya Rybalka

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Abstract Green algae are a monophyletic lineage of Archaeplastida, a supergroup of photosynthetic eukaryotes exclusively originated from primary endosymbiosis. The green algae are divided into two clades, the Chlorophyta and Streptophyta. The Chlorophyta comprises the vast majority of green algae, with three major lineages (Trebouxiophyceae, Ulvophyceae, and Chlorophyceae) forming the "crown group" of Chlorophyta and, as their early offspring, an assemblage of various monophyletic lineages of unicellular prasinophyte algae. Chloroplast phylogenomics and multigene phylogenetic analyses not only largely confirmed previous assumptions based on single gene (18S or rbcL) analyses, but also revealed novel robust groupings. While a bifurcation of the Chlorophyceae and a clear distinction of four major lineages within the Ulvophyceae have been supported, the internal phylogenetic

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structure and circumscription of Trebouxiophyceae still remain ambiguous. The Streptophyta comprises relatively few algal lineages and embryophytes. The embryophyte land plants are a sister group of streptophyte green algae (Zygnematophyceae or a lineage comprising Zygnematophyceae and Coleochaetophyceae), but transitions of the green algae to the land have taken place several times, even outside the Streptophyta. Progress in green algal systematics at the species level revealed ITS2 rDNA as an appropriate candidate for DNA barcoding and species distinction. Approaches to integrate sequence analyses of several genes with morphology are now state of the art for species delimitations. An improved taxon sampling with consideration of traditional gathered expert knowledge combined with multigene phylogenetic analyses, and improved phylogeny inference methods will be required to clarify areas of ambiguity in the green algal phylogenies. A revisit of morphology is essential to establish synapomorphies for the novel clades in molecular groupings.

1 Introduction

Green algae constitute a morphologically and ecologically diverse monophyletic lineage of Archaeplastida (Adl et al. 2005) which also includes embryophytes. Archaeplastida (also referred to as Plantae; Keeling 2004, 2010) is the only supergroup of photosynthetic eukaryotes exclusively originated from primary endosymbiosis. The plastid origin through primary endosymbiosis has been reviewed by a large number of articles, e.g., Bhattacharya et al. (2004), Reyes-Prieto et al. (2007), Baldauf (2008), Gould et al. (2008), Archibald (2009b), and Keeling (2010).

The Viridiplantae, to which all green algae and the embryophytes belong, the Rhodophyta, and the Glaucophyta form the Archaeplastida. The phylum Rhodophyta is often regarded as a sister group to Viridiplantae. The monophyletic origin of Archaeplastida has been supported by nuclear and chloroplast multigene analyses (Rodríguez-Ezpeleta et al. 2005; Archibald 2009b). Green algae share most of their features with embryophytes, including chloroplast structures, pigmentation, cell wall composition, and a characteristic stellate structure in the transition zone between flagellum and basal body (for review, see Lewis and McCourt 2004; Leliaert et al. 2011). Apart from being mostly not of true multicellular organization (see Coleochaetophyceae and Charophyceae; Leliaert et al. 2011) and often confined to aqueous habitats, it is often difficult to delimit the green algae from embryophytes without consideration of their very diverse gross morphologies. Morphological variation of green algae spans from the smallest known eukaryote (the prasinophyte *Ostreococcus*) and tiny flagellates to giant unicells with multiple nuclei or multicellular forms reminiscent of bryophyte gametophytes (*Coloechaete*). The majority of green algae thrive in freshwater or terrestrial habitats, but some microscopic forms (prasinophytes) are abundant in marine phytoplankton. Macroscopic marine forms are only known from the Ulvophyceae. It is typical for green algae to live in various terrestrial habitats (Holzinger 2009). Among green

algae, there are many "land plants," i.e., transitions to the land happened many times in the evolution of Chlorophyta and Streptophyta (Lewis and Lewis 2005; Lewis 2007). Examples for terrestrial green algal life are the biofilms of building facades (Barberousse et al. 2006, 2007), biological soil crusts (Smith et al. 2004; Büdel et al. 2009), or algal crusts on tree bark (Lüttge and Büdel 2010). Excellent reviews on the peculiarities of terrestrial green algae have been presented by López-Bautista et al. (2007) and Rindi (2011). Green algae, especially a variety of members of Trebouxiophyceae, are frequently involved in symbioses with ciliates and metazoa (for review, see Pröschold et al. (2011) and references therein) as well as lichenized fungi, where they are the dominating photobionts (review: Friedl and Büdel 2008).

The Viridiplantae are divided into two major clades, the phyla Chlorophyta and Streptophyta (Lewis and McCourt 2004; Petersen et al. 2006; Pröschold and Leliaert 2007). The vast majority of green algae belongs to the Chlorophyta, whereas the Streptophta comprise relatively few algal lineages and embryophytes. The embryophytes are a sister group to certain assemblage of streptophyte green algae (recent reviews: Turmel et al. 2007; Becker and Marin 2009; Wodniok et al. 2011). With respect to the phylogeny of the Chlorophyta, recent work confirmed that an array of distinct lineages of prasinophyte green algae (formerly assigned to a single class "Prasinophyceae") forms the most basal divergences and that four classes, the Chlorodendrophyceae (prasinophyte green algae), Trebouxiophyceae, Chlorophyceae, and Ulvophyceae form the core of Chlorophyta (Turmel et al. 2007; Marin and Melkonian 2010). The current view, which appears to become more and more substantiated particularly from chloroplast phylogenomics (Turmel et al. 2009a, b), supports the assumption that the Trebouxiophyceae emerged before Chlorophyceae and Ulvophyceae (Fig. 1). Therefore, this particular subdivision into three major chlorophytean classes may be called the "TUC crown group" of Chlorophyta (Pombert et al. 2005; Fig. 1). While the single origin of Chlorophyceae is not disputed, the monophyly of Ulvophyceae has only recently been robustly assessed (Cocquyt et al. 2010), but a sufficient evidence for the monophyly of Trebouxiophyceae is still lacking, and its circumscription not fully understood.

It should be noted that our current view of green algal systematics has been shaped by the intriguing results of molecular phylogenetic analyses which became feasible and widely employed in the 1990s following "traditional" algal systematics based on morphology and ultrastructure. Aspects of the taxonomic history of green algal systematics and evolution have recently been reviewed by Lewis and McCourt (2004) and Pröschold and Leliaert (2007), including a discussion of current knowledge. However, green algal systematics is progressing fast, particularly by analyses of novel data sets consisting of concatenated sequences from many loci of both nuclear and chloroplast genomes, as well as organelle-based phylogenomics. A comprehensive state-of-the-art review of green algal phylogenetics has been presented by Leliaert et al. (2011). Therefore, the intention of the present review is to give a brief introduction into current views of green algal systematics.

There is an important trend to focus on the systematics of green algal genera, i.e., their phylogenetic structures comprising the species. Species descriptions become

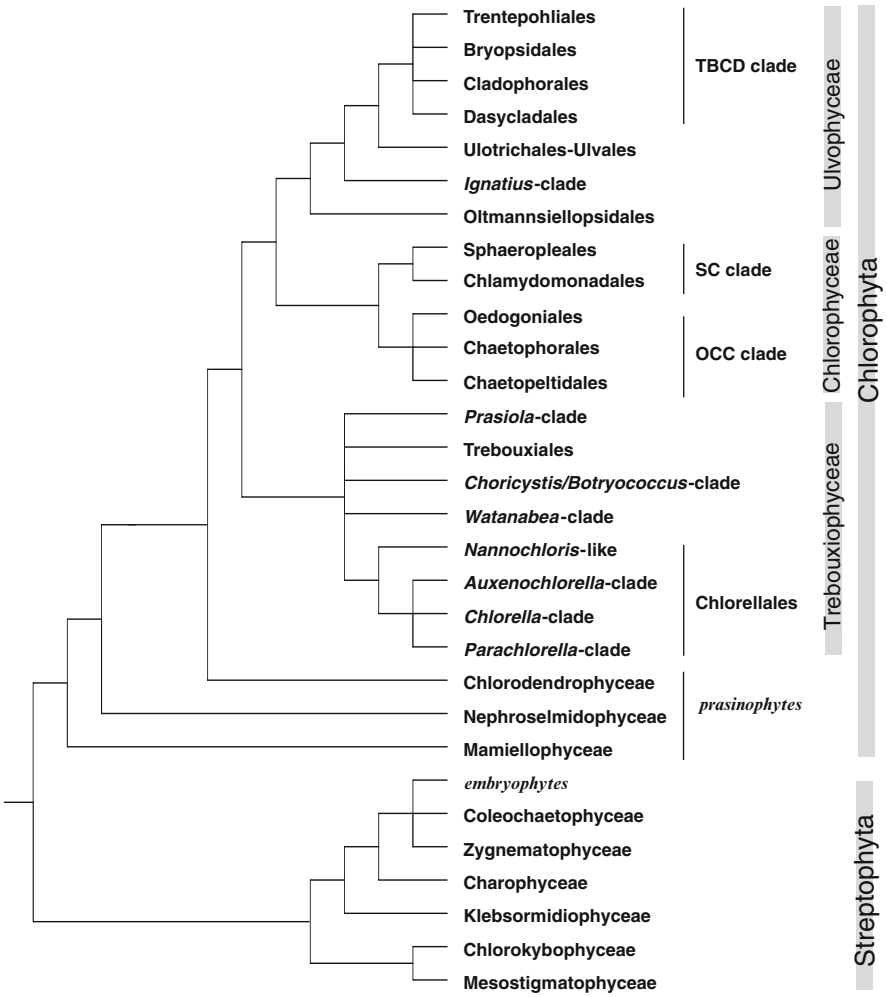

Fig. 1 Simplified schematic consensus phylogeny of the Green Algae. Details of the various clades are discussed in the text. Several lineages of prasinophyte green algae have been omitted for clarity. For the Trebouxiophyceae only those clades that are robustly resolved in 18S rDNA phylogenies and which include several genera are shown

more and more based on several markers and not only on a single gene, 18S, or rbcL. Most species-based works in green algae agree on a common molecular marker, i.e., ITS2 rDNA to unequivocally discriminate species (e.g., Müller et al. 2007; Coleman 2009). Certain nucleotide changes in ITS2 (CBCs), when mapped on the secondary structure model, which contains segments largely conserved over phylogenetic distances exceeding genera and families, help to guide decisions of what to include or exclude in a green algal species (Coleman 2003, 2007, 2009; Schultz et al. 2005). ITS2 rDNA is often discussed as the ideal candidate for a DNA

barcode within green algae because there are many examples for its successful application in different major lineages of Chlorophyta (e.g., Pröschold et al. 2005, 2011; Luo et al. 2010) as well as the streptophyte green algae Klebsormidiophyceae (e.g. Rindi et al. 2011). ITS2 rDNA has even been suggested as a "double-edged tool" which is valuable from species to order levels or even higher (Coleman 2003). ITS2 sequence analyses have successfully been employed to assess the monophyly of Sphaeropleales within the Chlorophyceae, even more robustly than 18S rDNA. An automated data analysis approach based on a large (>1,500 sequences) ITS2 data set with a secondary structure-based alignment demonstrated the power to reconstruct evolutionary patterns for highly divergent lineages at the levels of orders and classes (Buchheim et al. 2011).

2 Prasinophyte Green Algae

Prasinophytes represent the earliest branches of the Chlorophyta and are also a morphologically heterogeneous assemblage of green algae (for reviews, see Nakayama et al. 1998, 2007; Turmel et al. 2009a; Marin and Melkonian 2010). Most prasinophycean algae are unicellular flagellates possessing a scaly covering and having an asymmetrical cell architecture. Some species lack flagella, others lack scales, and in some cases, both flagella and scales are absent, e.g., in *Ostreococcus tauri*, which is the smallest known eukaryote (Turmel et al. 2009a; Yamaguchi et al. 2011). Coccoid forms may have evolved in several lineages of prasinophytes (Guillou et al. 2004). Most prasinophytes occur in marine habitats where they can be important components of coastal phytoplankton communities (Yamaguchi et al. 2011). Earlier analyses based on 18S rDNA already revealed that there is no monophyletic class "Prasinophyceae" (e.g., Nakayama et al. 1998, 2007). Instead, a paraphyletic assemblage of seven monophyletic groups of prasinophytes have been identified at the base of the Chlorophyta (e.g., Guillou et al. 2004) of which only three have been formally described as independent classes until now, i.e., the Chlorodendrophyceae, Nephroselmidophyceae, and Mamiellophyceae (Marin and Melkonian 2010; Yamaguchi et al. 2011, and references therein). The position of the Chlorodendrophyceae appears to be basal to, or even within, the radiation of the TUC crown group (Guillou et al. 2004), whereas Nephroselmidophyceae and Mamiellophyceae occupy rather basal positions within the Chlorophyta (Marin and Melkonian 2010). Prasinophytes are regarded as central to understand evolutionary patterns that accompanied radiation within the Chlorophyta and reduction of cells in some lineages (Turmel et al. 2009a). The whole genomes of two species of *Ostreococcus* (Mamelliophyceae; Palenik et al. 2007; Derelle et al. 2008), the smallest eukaryote, and two strains of *Micromonas* (Mamelliophyceae; Worden et al. 2009) have been sequenced. Evidence for sexual reproduction has been found by genome analyses (Grimsley et al. 2010), despite not being observed in these algae (cyrptic sex), which may indicate that the ancestor of Chlorophyta already exhibited sexual reproduction (Archibald

2009a). Full genome analyses also showed that the observed reduction of gene content occured in conjunction with important changes in cell organization (Turmel et al. 2009a). To date, chloroplast genome data are available for *Nephroselmis, Pyramimonas, Pycnococcus, Monomastix*, and *Ostreococcus* (Turmel et al. 2009a and references therein), and revealed that the ancestor of euglenoid chloroplasts may have been a close relative of the Pyramimonadales lineage (Turmel et al. 2009a), which is probably one of the most basal chlorophytean lineages (Marin and Melkonian 2010). For the green algal origin of plastids of the Chlorarachniophyta, also a prasinophyte, *Tetraselmis* (Chlorodendrophyceae) seems to be a likely candidate (Takahashi et al. 2007). Consequently, prasinophytes may have been involved in two independent secondary endosymbioses (Rogers et al. 2007).

3 Trebouxiophyceae

The majority of presently known members of the class are coccoid unicells, in some lineages also colonial coccoids occur. A few lineages may also form filaments, which, however, easily disintegrate (e.g., *Leptosira, Stichococcus*). The only known trebouxiophyte genera with firm, often even multiseriate filaments are *Prasiola* and *Rosenvingiella*. Flagellated vegetative forms are not known for the class. Members of Trebouxiophyceae are mostly found in dryer habitats, e.g., in soil, or are aerophytic algae. Many lineages include minute freshwater phytoplankton (e.g., Chlorellales). There are numerous examples for symbioses in ciliates, metazoa (e.g., various species of *Chlorella, Micractinium, Coccomyxa,* and *Elliptochloris*), and lichens (e.g., *Trebouxia* and *Asterochloris*).

At present, there is consensus that 18S rDNA phylogenies resolve five well-supported clades which comprise multiple genera, i.e., *Prasiola*-clade, Trebouxiales, *Watanabea*-clade, *Choricystis*/*Botryococcus*-clade, and Chlorellales. There are several other lineages for which only a single species (e.g., *Xylochloris*; Neustupa et al. 2011) or genus (e.g., *Leptosira, Lobosphaera*) are known so far. The relationship between these lineages, however, still remains ambiguous.

The *Prasiola*-clade comprises numerous examples of morphological plasticity, which seems to be a characteristic feature of the whole class. Morphologically defined species (morphotypes, morphospecies) may be distributed over several phylogenetic lineages and morphologically rather distinct algae may appear closely related in phylogenetic analyses. For example, *Stichococcus bacillaris*, which forms short filaments that easily disintegrate into cylindrical cells, are distributed over multiple lineages within the clade (Neustupa et al. 2007 and references therein). In 18S rDNA as well as rbcL-based phylogenies, species and strains of the microscopic genus *Stichococcus* are the closest relatives of *Prasiola* and *Rosenvingiella*, which are macroscopic filamentous or blade-shaped marine algae, often with tissue-like cell layers (Rindi et al. 2004, 2007). Also for the uniseriate filamentous forms of *Prasiola* and *Rosenvingiella*, a higher genetic diversity has been found than their simple morphology may indicate (Rindi et al. 2004). *Prasiola* species are involved

in a symbiotic relationship with the fungal genus *Mastodia*, which is regarded as a model to study interactions and processes in fungal–algal symbioses (Pérez-Ortega et al. 2010). Phylogenies based on rbcL gene sequences show that the algal partner is most closely related to *Prasiola borealis*. While most relationships within the *Prasiola*-clade remain ambiguous due to a relative poor resolution of the 18S rDNA sequences (Rindi et al. 2007), one lineage-including species of *Koliella*, *Raphidonema*, *Pabia*, and *Pseudochlorella*, is well supported as distinct from other members of the *Prasiola*-clade (Darienko et al. 2010; Neustupa et al. 2011).

The Trebouxiales (with three currently known genera, *Trebouxia, Asterochloris,* and *Myrmecia*) are common photobionts in lichen symbiosis (Friedl and Büdel 2008). *Trebouxia* is the most common lichen photobiont, and there is still a growing number of works which focus to reveal the algal species diversity within certain groups of lichens. Most often, algal-specific primers to directly amplify the *Trebouxia* ITS rDNA from lichen specimens without culturing are employed. Species diversity of *Trebouxia* appears larger than reflected by morphology (cryptic speciation) and has still not been fully explored. Intriguing examples have been presented by Blaha et al. (2006) and Skaloud and Peksa (2010). The 18S rDNA phylogenies showed that *Trebouxia* as previously defined was paraphyletic with *Myrmecia* and therefore, in a polyphasic approach using ITS and actin intron sequences as well as morphology, the genus *Asterochloris* has recently been segregated from *Trebouxia* (Skaloud and Peksa 2010).

Within the *Choricystis/Botryococcus*-clade, several genera thrive in symbioses as well as free living. *Elliptochloris* has been identified as symbiotic in lichens (Friedl and Büdel 2008) and marine invertebrates (Letsch et al. 2009), but species of *Elliptochloris* were also found free living on tree bark (Eliáš et al. 2008). *Coccomyxa,* the sister-group to *Elliptochloris* in the 18S rDNA phylogenies, includes symbionts of lichens and ciliates (previously summarized under "*Zoochlorella*") (Pröschold et al. 2011) as well as free-living species (Zoller and Lutzoni 2003; Friedl and Büdel 2008). Species of *Choricystis* species are known as phytoplankton members (Fawley et al. 2005) and as symbionts in sponges (Handa et al. 2006; Pröschold et al. 2011). The oil-producing *Botryococcus* is a prominent member of this clade (Senousy et al. 2004; Weiss et al. 2010).

Most of the currently known members of the *Watanabea*-clade were traditionally assigned to *Chlorella*, but appeared distinct from the type of *Chlorella*, *C. vulgaris*, in molecular phylogenies as well as morphological features (Darienko et al. 2010). A new genus, *Chloroidium*, has been established to encompass the previous species *C. ellipsoidea, C. trebouxioides*, and *C. saccharophila*. The *Watanabea*-clade also includes *Heterochlorella*, a newly established genus to encompass previous *"Chlorella" luteoviridis* (Neustupa et al. 2009). Two more lineages of *Chlorella*-like algae which, however, are distinct from the two aforementioned lineages, are *Kalinella* (Neustupa et al. 2009) and *Heveochlorella* (Zhang et al. 2008). So far, only a single symbiotic member of the *Watanabea*-clade has been reported, i.e., as a still unidentified strain from the lichen *Psoroglaena epiphylla* (Nyati et al. 2007).

The Chlorellales have been the focus of intensive taxonomic research during recent years. This group unites the Chlorellaceae, which is subdivided into the

Chlorella- and *Parachlorella* clades (Krienitz et al. 2004), with the "*Auxenochlorella*-clade" (Pröschold et al. 2011). While the latter comprises peculiar coccoid green algae without chlorophylls (Aslam et al. 2007), the order Chlorellales comprises mostly unicellular coccoids of minute size which thrive in an extremely broad range of habitats. For example, species of *Chlorella* (close relatives of the type species, *C. vulgaris*) have been described from terrestrial habitats (e.g., rock surfaces in Antarctica; Hu et al. 2008), symbioses with ciliates and sponges (*C. vulgaris, C. variabilis, C. heliozoae;* Hoshina et al. 2010; Pröschold et al. 2011), as well as freshwater phytoplankton (Henley et al. 2004; Krienitz et al. 2004). An overview of the "true" *Chlorella* species and genera most closely related with *Chlorella*, i.e., members of the *Chlorella*-clade of Chlorellales, has been presented by Luo et al. 2010. Several new genera which belong to the *Chlorella*-clade have been described from freshwater phytoplankton, e.g., *Meyerella* (Fawley et al. 2005), *Hegewaldia* (Pröschold et al. 2010), *Heynigia*, and *Hindakia* (Bock et al. 2010). Members of the same genus, *Micractinium*, are known as common components of freshwater phytoplankton (with cell wall appendices, bristles, inducible by grazers, Luo et al. 2006) as well as symbionts in the ciliate *Paramecium bursaria* (Pröschold et al. 2011). Many genera of the Chlorellales are common picoplanktonic green algae in freshwater, e.g., *Picochlorum, Nannochloris,* and *Marvania* (Henley et al. 2004). A new genus of marine phytoplankton, *Marinichlorella*, which is a member of the *Parachlorella*-clade, has recently been described on the basis of ultrastructural characters and 18S rDNA phylogenies (Aslam et al. 2007). A new genus of *Chlorella*-like phytoplankton, *Chloroparva*, a close relative of "*Chlorella" minutissima* within the "*Nannochloris*-like" clade of Chlorellales (Fig. 1), has also recently been described on the basis of 18S rDNA phylogenetic analyses, ultrastructure, and oleic fatty acids (Somogyi et al. 2011). Extensive ultrastructural studies in Trebouxiophyceae have become rare. However, an excellent ultrastructural study that focuses on the life cycle and reproduction by autospores in *Chlorella* and *Parachlorella* revealed ultrastructural features which support the separation of *Parachlorella* from *Chlorella*, independent of molecular phylogenetic analyses (Yamamoto et al. 2005). For the *Auxenochlorella*-clade, three genera of heterotrophic algae are currently known. *Auxenochlorella* has been described as a free-living aerophytic alga (Kalina and Punčochárová 1987), reported from symbioses with *Hydra* (Pröschold et al. 2011) as well as from a lichen symbiosis (*Psoroglaena stigonemoides*, Nyati et al. 2007). *Prototheca* species are well known as pathogens in vertebrates, e.g., cattle and humans, and the genus *Helicosporidium* in insects (Tartar et al. 2002; Tartar and Boucias 2004; de Koning and Keeling 2006). The close phylogenetic relationships of both parasitic genera have been resolved by 18S rDNA (Tartar et al. 2002) and phylogenomic analyses of mitochondrial (Pombert and Keeling 2010) and chloroplast DNA (de Cambiaire et al. 2007). As expected for parasitic algae not performing photosynthesis, the chloroplast genomes were rather reduced compared to other green algae (de Cambiaire et al. 2007). In 18S rDNA phylogenies, the three genera form a rather long branch, which almost certainly is due to systematic errors in phylogenetic inferences (long branch attraction, Verbruggen and Theriot 2008). Therefore,

deciphering whether or not they form a single monophyletic lineage, and their position within the Chlorellales is still a challenging task.

The circumscription of Trebouxiophyceae is not fully understood yet. The Oocystaceae, a monophyletic group of coccoid green algae, may form another multigenera group within the Trebouxiophyceae. First, Hepperle et al. (2000) showed affinities of the Oocystaceae to the Trebouxiophyceae, despite it exhibiting rather long branches, but its relationship within the class could not be resolved. Later, an affiliation of the Oocystaceae with Chlorellales had been found (Krienitz et al. 2003; Pažoutová et al. 2010; Neustupa et al. 2011). Oocystaceae was even used as outgroup taxa in 18S rDNA phylogenies to address relationships within the Trebouxiophyceae (Pröschold et al. 2011). Species of *Geminella* were also resolved, forming a clade of the Trebouxiophyceae (Mikhailyuk et al. 2008), but appeared rather distant from other members of the class. The phylogenetic positions of Oocystaceae and the *Geminella*-clade are in urgent need of reevaluation; both clades may even represent two additional independent clades diverging within the TUC crown group.

Sexual reproduction in Trebouxiophyceae has so far been observed only in two genera, *Micractinium* (references in Pröschold et al. 2010) and *Prasiola* (references in Rindi et al. 2004). Analysis of the whole genome of *Chlorella variabilis*, symbiont of the ciliate *P. bursaria*, however, revealed evidence for the presence of sexual reproduction in which flagellated stages may have been involved or at least genes involved in these processes may have been retained (Blanc et al. 2010). Therefore, it is tempting to conclude that sexual reproduction is more widespread in Trebouxiophyceae than assumed from observations (cryptic sexuality). Absence of flagellated stages is very widespread in lineages of the Trebouxiophyceae (e.g. *Watanabea*- and *Choricystis*/*Botryococcus*-clades), but genomic evidence for flagellar motion in *C. variabilis,* a species for which only reproduction by nonmotile autospores is known, has been found (Blanc et al. 2010). This may indicate that the many autosporic lineages in Trebouxiophyceae may have ancestors with flagellated reproductive stages as well. Chloroplast phylogenomics could not resolve the monophyletic origin of the Trebouxiophyceae, but revealed an affiliation of the green flagellate *Pedinomonas* (whose phylogenetic position has been unclear so far) with the Trebouxiophyceae, particularly with the Chlorellales (Turmel et al. 2009b). Phylogenomic analyses based on mitochondrial DNA, however, did not support a relationship of *Pedinomonas* with Trebouxiophyceae, but both phylogenomic analyses certainly suffer from poor taxon sampling, which is a problem inherent to all phylogenomic analyses so far (Rodríguez-Ezpeleta et al. 2007).

4 Chlorophyceae

In contrast to the Trebouxiophyceae, the Chlorophyceae includes a variety of unicellular or colonial flagellates (e.g., *Chlamydomonas*, *Volvox*; Nakada et al. 2008) and many members with firm unbranched (e.g., *Oedogonium*; Alberghina

et al. 2006) or branched filaments (e.g., *Stigeoclonium*, Michetti et al. 2010). Members of Chlorophyceae thrive mostly in freshwater or terrestrial habitats, and there are only very few symbiotic genera, e.g., *Scenedesmus* in ciliates (Pröschold et al. 2011). Chlorophyceae are not known yet from lichen symbioses (Friedl and Büdel 2008). Recent reviews of the Chlorophyceae are based on 18S rDNA (e.g., Müller et al. 2004) or chloroplast phylogenomics (e.g., Turmel et al. 2008). Consensus has been reached that the Chlorophyceae may be divided into five clades, Volvocales, Sphaeropleales, Oedogoniales, Chaetopeltidales, and Chaetophorales, which are robustly resolved in most phylogenetic analyses (Fig. 1). Monophyletic origin and distinction of Sphaeropleales has been substantiated by ITS2 secondary structure-based analyses (Keller et al. 2008). The actual conception of the group and its subdivision into clades has recently been reviewed by Krienitz et al. (2011). A recent overview of Volvocales [sometimes also called Chlamydomonadales, e.g., Müller et al. (2004)] can be found in Gerloff-Elias et al. (2005). Using a comprehensive large-scale data set of more than 400 18S rDNA sequences, the overall phylogenetic structure of the group has been revealed and phylogenetically-defined names have been applied to the monophyletic clades that constitute the Volvocales using PhyloCode (Nakada et al. 2008). Recent studies with expanded taxon samplings that would allow testing generic concepts or species delineations are still lacking for the Chaetopeltidales and Oedogoniales. A preliminary 18S rDNA analysis already indicated a polyphyletic origin for *Oedogonium* within the Oedogoniales (Alberghina et al. 2006). A most recent 18S rDNA-based overview of the Chaetophorales with its subdivisions can be found in Caisová et al. (2011).

Chloroplast phylogenomic analyses support a dichotomy of Chlorophyceae, i.e., a CS clade uniting the orders Chlamydomonadales (= Volvocales) and Sphaeropleales versus the OCC clade which comprises the orders Oedogoniales, Chaetopeltidales, and Chaetophorales (Turmel et al. 2008; Fig. 1). Until now, a total of eight chloroplast genomes has been sequenced for the six orders of both clades together (Brouard et al. 2011 and references therein). Recent analyses of 18S rDNAs alone have favored the dichotomy as seen in cpDNA phylogenetic analyses (Caisová et al. 2011), whereas analyses of concatenated 18S and LSU (28S) rDNA sequences (Buchheim et al. 2001) have supported only the CS clade (Shoup and Lewis 2003; Müller et al. 2004). As outlined by Lewis and McCourt (2004) and Leliaert et al. (2011), each of the two clades is defined by a unique absolute basal body configuration. Members of the CS clade produce biflagellated motile cells, while members of the OCC clade exhibit quadriflagellated stages or, in Oedogoniales, have multiple flagella (stephanokont zoids) (Alberghina et al. 2006). From chloroplast genomic data, it has been assumed that the ancestral state in Chlorophyceae may have been quadriflagellate motile cells with the DO + DO orientation, and the CW condition of flagellar basal bodies developed later convergently in the CS and OCC clades (Brouard et al. 2008; Turmel et al. 2008). Important conclusions for the evolution of green algae and the Chlorophyceae became possible by comparisons of genomes, i.e., that of the model organism *Chlamydomonas reinhardtii* with those of the multicellular *Volvox*

(Prochnik et al. 2010) and the more ancestral prasinophytes *Ostreococcus* spp. (Peers and Niyogi 2008).

Many studies over the last 5 years focused more on taxa of the CS clade, less on members of the OCC clade. For both clades, phylogenetic analyses concluded that traditional species delineation based on morphology is not congruent with the molecular distinction. This general finding holds true from minute phytoplankton species (Krienitz et al. 2011), prominent examples of colonial coccoids [e.g., *Pediastrum* and *Hydrodictyon*; McManus and Lewis (2005, 2011)], to more complex branched filamentous forms [e.g., *Chaetophora* and *Stigeoclonium*; Caisová et al. (2011)]. In minute common phytoplankton species of the Sphaeropleales, the morphological difference in solitary versus colonial forms was found in disagreement with phylogenetic groupings and therefore the species were assigned to a single genus *Mychonastes* with the species distinguished primarily by differences in their ITS2 rDNAs (Krienitz et al. 2011). Sequence analyses of ITS2 rDNA became almost a standard marker to delineate species within *Scenedesmus*, *Acutodesmus*, and *Desmodesmus* and differences in ITS2 were found congruent with morphological features as seen by scanning-electron-microscopy (Hegewald et al. 2010). ITS2 sequence comparisons have also been successful in the unambiguous identification of *Scenedesmus* and *Desmodesmus* isolates from lake phytoplankton (Johnson et al. 2007). Chloroplast genes have frequently been used as molecular markers to test species and genus assignments in the Volvocales, but not in the Sphaeropleales. For example, a data set of three concatenated chloroplast genes revealed a new genus, *Parallela*, within the Volvocales of the CS clade in combination with light and electron microscopy (Novis et al. 2010). New species of the coccoid *Asterococcus* and the colonial flagellate *Gonium* have been recovered using plastid-encoded rbcL and nuclear-encoded ITS rDNA, combined with ultrastructure (Nakazawa et al. 2004; Hayama et al. 2010).

5 Ulvophyceae

The Ulvophyceae is probably morphologically the most diverse group of the Chlorophyta (Cocquyt et al. 2010; Leliaert et al. 2011). Morphologies range from microscopic unicells to multicellular thalli and giant cells with unique types of cytomorphology. The class may be regarded as predominantly marine; it includes all macroscopic marine representatives of the Chlorophyta. Only in recent years, monophyly of the class as well as its internal phylogenetic structure have been assessed using multigene analyses, i.e., concatenated 18S rDNA and a number of other nuclear and plastid genes. The Oltmannsiellopsidales, consisting of quadriflagellate unicells or coccoids arranged in disks or packets, may be the most basal lineage of Ulvophyceae (Friedl and O'Kelly 2002; Watanabe and Nakayama 2007; Fig. 1). Its basal position has been confirmed by mitochondrial genome sequence comparisons (Pombert et al. 2006). The *Ignatius* clade, comprising unicellular

coccoids, is another more basal lineage of Ulvophyceae, which is independent of any other groups of the class (Watanabe and Nakayama 2007; Fig. 1). The Ulvales–Ulotrichales is a morphologically rather diverse clade, comprising most microscopic forms of Ulvophyceae. It includes two cytomorphological types: unicellular algae with a single nucleus and chloroplast, e.g., the unicellular or sarcinoid *Pseudendoclonium*, *Planophila*, and *Desmochloris* (Friedl and O'Kelly 2002; Darienko et al. 2009), and those with multicellular bodies composed of uninucleate cells, forming branched or unbranched filaments (e.g., *Acrosiphonia, Ulothrix, Uronema*; O'Kelly et al. 2004), blades or tubular forms (e.g., *Ulva* which is known to form "green tides," e.g., the Qindao nuisance alga, Leliaert et al. 2009; O'Kelly et al. 2010). So far, there has been no support from phylogenetic analyses to separate the Ulvales–Ulotrichales clade into orders (Leliaert et al. 2011). The single origin for a rather large assemblage of lineages within the Ulvophyceae, termed the "Trentepohliales–Bryopsidales–Cladophorales–Dasycladales (TBCD)" clade (Fig. 1), has been revealed by multigene analyses (Cocquyt et al. 2010). The TBCD clade unites lineages of various cytomorphological organization (see Cocquyt et al. (2010) and references therein). The Trentepohliales is a unique lineage of exclusively terrestrial members forming filaments of uninucleate cells. The Trentepohliales includes a variety of important photobionts in lichen symbiosis (for review see Nelsen et al. 2011). For a comprehensive treatment of genus boundaries and a phylogenetic assessment of the Trentepohliales based on 18S rDNA and rbcL genes, see López-Bautista et al. (2006) and Rindi et al. (2009). The Bryopsidales (also referred to as Caulerpales) comprise mainly marine macroscopic lineages with siphonous forms consisting of a single giant cell with millions of nuclei or a single macronucleus. The order Cladophorales consists of mostly branched filaments of the siphonocladous type (Cocquyt et al. (2010) and references therein) and the marine tropical Dasycladales of siphonous forms. A large number of works have contributed to this present-day view of the phylogenetic structure of Ulvophyceae; they have been reviewed and summarized by Cocquyt et al. (2010) and Leliaert et al. (2011).

6 Streptophyte Green Algae

The streptophyte green algae comprise not more than four or five morphologically rather simple lineages and two advanced lineages with true multicellular organization (McCourt et al. 2004; Becker and Marin 2009): the scaly flagellate Mesostigmatophyceae (Marin and Melkonian 1999), the sarcinoid Chlorokybophyceae (McCourt et al. 2004), the unbranched filamentous Klebsormidiophyceae (for overview see Mikhailyuk et al. 2008; Sluiman et al. 2008), and the Zygnematophyceae which are characterized by conjugation as the method of sexual reproduction and the absence of flagellated cells (Fig. 1). The unicellular genus *Spirotaenia*, traditionally classified as a member of the Zygnematophyceae, likely

represents another independent lineage of streptophyte green algae (Gontcharov and Melkonian 2004), confirming the earlier notion of Mollenhauer (1986). Multicellular organization and oogamous sexual reproduction exhibit the Coleochaetophyceae which form parenchymatous tissue (*Coleochaete* and *Chaetosphaeridium*; for review see Delwiche et al. 2002), and the Charophyceae (stoneworts) which form branched filaments with apical growth (McCourt et al. 2004; Becker and Marin 2009). A lineage uniting the flagellate *Mesostigma* and the sarcinoid *Chlorokybus* may represent the earliest diverging lineage of the Streptophyta as it was indicated by plastid phylogenomic analyses (Lemieux et al. 2007). Earlier studies suggested *Mesostigma* emerged even before the divergence of the Streptophyta and Chlorophyta (Lewis and McCourt 2004). Its basal position within the Streptophyta, however, is not debated anymore since a multigene family (BIP) and a GapA/GapB gene duplication shared with *Mesostigma* and other Streptophyta to the exclusion of Chlorophyta have been found (Nedelcu et al. 2006; Petersen et al. 2006). The Klebsormidiophyceae diverged after the *Mesostigma*/*Chlorokybus* lineage and is sister to a clade uniting the Zygnematophyceae, Charophyceae, and Coleochaetophyceae (Becker and Marin 2009; Wodniok et al. 2011). Which streptophyte algal group may be the closest living relatives of embryophytes has been the subject of a hot debate (see Chapman and Waters 2002 for comments), which began soon after an early multigene analysis suggested the Charophyceae as sister group to embryophytes (Karol et al. 2001). Most recent analyses of nuclear genes as well as chloroplast phylogenomic analyses, however, revealed either the Zygnematophyceae or a group uniting the Zygnematophyceae with Coleochaetophyceae is the sister group to embryophytes (Turmel et al. 2007; Wodniok et al. 2011). The Charophyceae is now seen as an earlier divergence and not as the closest relatives of land plants.

The Zygnematophyceae, in particular, the family Desmidiaceae, are the most species rich and taxonomically complex streptophyte green algae (Gontcharov and Melkonian 2011). However, due to a problematic species concept, the real taxonomic diversity of the Desmidiaceae family is not known with certainty (Kouwets 2008; Gontcharov and Melkonian 2011). The polyphyletic nature of almost all genera in Desmidiaceae has been recovered using nuclear and chloroplast-encoded molecular markers (e.g., Gontcharov and Melkonian 2008, 2011; Hall et al. 2008, and references therein), and there is still more work required to identify structural synapomorphies for the newly identified clades (Hall et al. 2008).

Recently, an increased interest in the common terrestrial Klebsormidiophyceae led to the discovery of a new member of the class, *Interfilum* (Mikhailyuk et al. 2008). Phylogenetic structures as well as species delineations within *Klebsormidium* have been analyzed using 18S, ITS, and rbcL sequences (Rindi et al. 2011), and new species have been described (Novis 2006). Concurrently, with the progress in *Klebsormidium* systematics, there have been further ecophysiological studies on the genus (Karsten et al. 2010; Karsten and Rindi 2010; Holzinger et al. 2011).

7 Conclusions

Phylogenies based on a single marker gene (mostly 18S rDNA) have already revealed most phylogenetic structures within Chlorophyta and Streptophyta; not much has changed since an already decent taxon sampling was achieved for most green algal groups in the early 2000s. The 18S rDNA is still the "universal marker" to uncover green algal diversity, e.g., in phytoplankton (Piganeau et al. 2011). Multigene sequencing and/or chloroplast phylogenomics not only confirmed previously resolved major lineages in the Chlorophyta, but revealed several novel robust groupings, e.g., in the Ulvophyceae or the assessment of the closest living relative of the embryophytes. There are still many "weak" areas in the phylogeny of Chlorophyta which continue to be ambiguous or unresolved. In particular, the circumscription and internal phylogenetic structure of the Trebouxiophyceae are still challenging. More analyses based on sequences from many single gene loci of at least the nuclear and chloroplast genomes are required, but systematic errors need to be overcome by increased taxon sampling and improved phylogeny inferences methods (Rodríguez-Ezpeleta et al. 2007; Verbruggen and Theriot 2008; Cocquyt et al. 2010). Transcriptome analyses using next generation sequencing (e.g., Timme and Delwiche 2010) is another promising and powerful tool for testing phylogenetic hypotheses in the green algae. Systematics, in general, attempts to recover the actual phylogenetic relationships as close as possible by using as many characters as possible, and, therefore, more than just phylogenies are needed. In many groups we now are left with a molecular grouping that cannot be explained satisfactorily by morphology or other phenotypical characters. The situation may be best exemplified in the Desmidiaceae (Zygnematophyceae) where the traditional generic concepts almost constantly fail. Morphology needs to be revisited and much work is needed to find structural or other phenotypical synapomorphies to define the molecular groupings. There are several good examples (e.g., in the Chlorellales, Trebouxiophyceae) where a reevaluation of morphology resulted in congruence with the molecular groupings and features whose phylogenetic significance has not been understood so far. Taxon sampling will become even more important for future studies in green algal systematics, not only in terms of numbers of taxa to be included, but also which taxa to select. Previous sequence analyses may guide us well to find the right critical taxa, but to consider traditional gathered expert knowledge from morphology, ultrastructure and ecology of a certain group of green algae will be pivotal.

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Part V
Ecology

Secondary Lichen Compounds as Protection Against Excess Solar Radiation and Herbivores

Knut Asbjørn Solhaug and Yngvar Gauslaa

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Abstract The functional roles of secondary lichen compounds are reviewed with focus on sun-screening and herbivore-deterring functions. Hypotheses on ecological functions can be tested because lichen compounds can nondestructively be extracted from air-dry lichens with 100% acetone. Substantial evidence supports a sun-screening function of cortical compounds. They screen solar radiation by absorptance (parietin, melanins) or by reflectance (atranorin). Their concentration correlates with light exposure and they protect the photobiont against excessive visible light. UV-B induces the formation of parietin, usnic acid, and melanins;

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the synthesis of the two first compounds has been shown to be boosted by photosynthates. The numerous extractable medullary lichen compounds hardly function as sun-screens. Some of these carbon-based compounds deter generalist herbivores, particularly in lichens in oligotrophic sites. Lichens in nitrogen-rich sites often deter grazing animals as efficient as those from oligotrophic sites despite low contents of lichen compounds. Acetone rinsing of nitrophytic lichens does not lead to increased grazing, meaning that their defense remains to be described. Thanks to grazing experiments using acetone rinsing, there is now solid support for the optimal defense theory in lichen–herbivore interactions. Recent studies have shown that lichen-feeding gastropods can shape epiphytic lichen communities.

1 Introduction

Lichens are symbiotic associations between a mycobiont and one or more photobionts (green algae and/or cyanobacteria). They often contain high amounts of secondary compounds produced by the fungal partner. About 1,050 different secondary lichen compounds have so far been isolated and identified (Molnár and Farkas 2010; Huneck and Yoshimura 1996). Concentrations as high as 30% of dry weight has been measured (Huneck 1973); the widespread foliose lichen *Hypogymnia physodes* may contain 22% secondary compounds of dry weight (Solhaug et al. 2009). The lichen compounds are normally deposited as small crystals on the outer surfaces of fungal hyphae and photobiont cells (Honegger 1991). Many hypotheses on their biological and ecological functions have been proposed [as reviewed by e.g. Fahselt (1994); Lawrey (1986); Huneck (1999); Favero-Longo and Piervittori (2010); Molnár and Farkas (2010)]. The secondary lichen compounds may deter herbivors (Slansky 1979; Lawrey 1983; Gauslaa 2005; Pöykkö et al. 2005) and/or screen excess solar radiation (Solhaug and Gauslaa 1996; Gauslaa and Ustvedt 2003; McEvoy et al. 2006, 2007b).

Other functions of lichen compounds will briefly be mentioned in the following paragraphs. For example, Hauck (2008) suggested that lichen compounds play important roles in metal homeostasis in lichens. He showed that the lichen compounds in *H. physodes* increased the tolerance to Cu^{2+} and Mn^{2+} by selectively inhibiting the uptake of these toxic elements.

Lichen compounds have also been ascribed allelopathic functions and may strengthen the competitive power of lichens against plants (Pyatt 1967; Brown and Mikola 1974; Fisher 1979). The widespread usnic acid effectively reduces photosynthesis in artificial systems (Vavasseur et al. 1991; Solhaug et al. 1995), but adverse effects under natural conditions remain to be shown.

Antimicrobial activity of lichen compounds has strong experimental support in lab studies (e.g., Ingólfsdóttir 2002; Ranković et al. 2008). However, Stark et al. (2007) failed to document antimicrobial effects of lichen compounds in *Cladina stellaris* on underlying natural soil microorganisms. Thus, available evidence for

antimicrobial and allelopatic effects of lichen compounds in natural systems is not strong.

Lichen compounds may also protect against parasite infestations. The specificity of lichenicolous fungi seems to be regulated by lichen compounds. Specific lichen compounds strongly inhibit some, but far from all lichenicolous fungi (Lawrey 2000).

It has been hypothesized that hydrophobic lichen compounds may prevent water from filling extracellular medullary air spaces and thus inhibit CO_2 diffusion at high water contents. Lange et al. (1997) found that net photosynthesis was not reduced at high water contents in *Diploschistes muscorum* after extraction of secondary compounds, whereas Souza-Egipsy et al. (2000) found that lichen compounds played a role in providing a water-free diffusion pathway in *Neofuscelia pokornyi*, *N. pulli*, and *Xanthoria parietina*, consistent with species-specific responses.

In the following, we will focus on the many recent experimental studies testing the solar radiation screening hypothesis of cortical lichen compounds and afterward discuss hypotheses on herbivore-deterrent functions of lichen compounds. As studies of these functions to a large extent used the acetone-rinsing method, we will first describe this technique.

1.1 Acetone-Rinsing Technique

Secondary compounds in lichens can be extracted from living air-dry thalli with 100% acetone without adverse effects on lichen bionts (Fig. 1) (Solhaug and Gauslaa 1996, 2001; Lange et al. 1997). A high-acetone tolerance seems to be a general feature of desiccation-resistant organisms, such as dry seeds (Milborrow 1963; Tao and Khan 1974), desiccation-tolerant bryophytes, and ferns (Solhaug and Gauslaa 2001). It is the unique location of most secondary compounds outside the living part of the cells that enables us to extract these compounds, since the acetone does not pass or destroy the membranes of desiccated cells. By cutting a lichen thallus in two pieces, extract compounds from one piece and use the other piece as a control; we have a powerful experimental setup to test proposed hypotheses on the roles of secondary compounds. In some cases, the secondary compounds cannot be

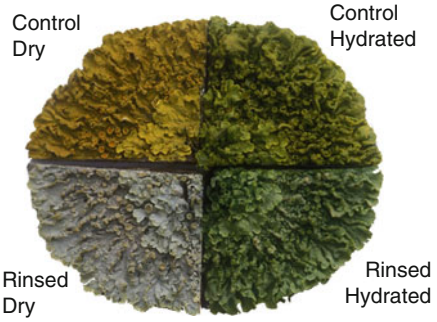

Fig. 1 A *Xanthoria parietina* thallus cut in four pieces. The *two upper pieces* are controls; the *two lower pieces* are acetone rinsed. The *two pieces to the left* are air dry; the *two other pieces* are hydrated. After Solhaug and Gauslaa (1996). Photo: Knut Asbjørn Solhaug

fully extracted from intact thalli without grinding (McEvoy et al. 2006). However, it is often enough to reduce the level of secondary compounds to test their ecological function. All compounds are normally extracted, but the extraction efficiency often varies between individual compounds. Chemical identification and analysis of the extracted and remaining compounds are often necessary. Selection of lichen species with one or two compounds only facilitates the testing of individual compound functions.

2 Lichen Compounds as Solar Radiation Screens

The most common cortical lichen compounds are:

- Parietin (an orange anthraquinone)
- Usnic acid (a faintly yellow dibenzofurane)
- Atranorin (a colorless depside forming white crystals)
- Melanic compounds

The first three compounds are reviewed chemically by Huneck and Yoshimura (1996), whereas melanic compounds are not well characterized. Among lichens, parietin is restricted to the fungal order Teloschistales, whereas the three other cortical compounds are widely distributed across various taxonomic lichen groups (Smith et al. 2009) Most lichens in sun-exposed sites produce only one of these cortical compounds. Lichens with parietin, usnic acid, atranorin, or melanic compounds often coexist in open habitats. Some lichens with atranorin or usnic acid can additionally produce melanic compounds under very sun-exposed conditions. Cortical pigments are presumably necessary in sun-exposed habitats since specimens with low pigment concentrations are often susceptible to high light (Demmig-Adams et al. 1990; Gauslaa and Solhaug 1996, 1999, 2000). Cortical compounds often occur in substantially lower concentrations than medullary compounds (e.g., Gauslaa and Ustvedt 2003; Hill and Woolhouse 1966; McEvoy et al. 2006; Pöykkö et al. 2005; Solhaug et al. 2009).

2.1 Screening Compounds Vary Along Spatial and Temporal Light Gradients

Variation in the concentration of colored pigments such as parietin and melanins can be seen with the naked eye. They are most clearly seen in the desiccated state, in which the longest and strongest sun exposures take place. For example, the melanin content in *Lobaria pulmonaria* varied around a *Quercus* stem with darkly brown thalli on the southern side and pale gray melanin-deficient thalli on the northern side (Gauslaa and Solhaug 2001). The browning of the upper cortex strongly increased after transplanting pale, shade-adapted thalli from forests to exposed clear-cut areas

(Gauslaa et al. 2006). *Xanthoria parietina* thalli on exposed roofs and sea cliffs had a stronger orange color due to much higher parietin content than those growing on trees (Hill and Woolhouse 1966). There was a linear relationship between the openness of the habitat and the parietin content, ranking from pale shade-adapted low-parietin thalli of *X. parietina* in closed forests to orange parietin-rich and high-light exposed thalli on sea cliffs (Gauslaa and Ustvedt 2003). In *H. physodes,* the concentration of the cortical compounds atranorin and the closely related chloroatranorin increased with the openness of their natural growing sites. In *Cladonia subtenuis*, the usnic acid showed a clear increase in concentration with increasing sunlight (Rundel 1969), and *Nephroma arcticum* thalli from a closed spruce forest contained much less usnic acid than thalli from an open subalpine birch forest or from an open alpine heath (McEvoy et al. 2007a).

Cortical compounds do not only vary on spatial scales. In *X. parietina*, the parietin content varied seasonally with twice as high contents per thallus area and per weight in midsummer than in winter (Gauslaa and McEvoy 2005). During the period from December to May, the rise in solar radiation was faster than the rise in parietin content. Although parietin screens visible light, the screening efficiency did not fully compensate for increased light levels during spring, leading to a decrease in maximal quantum yields of PSII (Fv/Fm). However, from May to June the parietin content and Fv/Fm increased simultaneously. This is consistent with the hypothesis inferring faster parietin synthesis in periods with higher metabolism due to higher temperatures. Thereby, the photobionts experienced less excess irradiance energy resulting in reduced level of photoinhibition (Vráblíková et al. 2006). Similar seasonal variations in usnic acid concentration occurred in the alpine lichen *Flavocetraria nivalis* (Bjerke et al. 2005).

2.2 UV-B Induction of Secondary Lichen Compounds

UV-radiation screening has long been assumed to be an important function of lichen compounds. Ultraviolet radiation consists of UV-C (200–280 nm), UV-B (280–315 nm), and UV-A (315–400 nm). As the ozone layer absorbs wavelengths below 300 nm, only UV-A and UV-B can reach the earth surface (Björn and McKenzie 2002). By extracting compounds in acetone and exposing rinsed thalli to treatments in the field and/or in growth chambers, one can test the power of environmental factors like UV-B to induce compound synthesis. Acetone-rinsed compound-deficient thalli can be cultivated under screening filters excluding specific UV-ranges. Such methods have shown that parietin synthesis in *X. parietina* (Solhaug et al. 2003) and *Xanthoria elegans* (Nybakken et al. 2004), melanin formation in *L. pulmonaria* (Solhaug et al. 2003), and usnic acid synthesis in *Xanthoparmelia somloensis* (McEvoy et al. 2006) all require UV-B. The UV-B is a much stronger inductive agent for the parietin synthesis than UV-A. Parietin synthesis in *X. parietina* thalli exposed to visible light and UV-A was marginally higher than in those exposed to visible light only (Solhaug et al. 2003). The parietin

synthesis increased linearly with log-transformed UV-B levels, and the content increased with UV-B exposure time (Solhaug and Gauslaa 2004). Therefore, intermediate levels of UV-B may over time result in high parietin synthesis. However, the correlation between parietin content and openness in the field may also be the result from the need for assimilates in compound synthesis. This hypothesis was tested by a factorial experiment with ± PAR, ±UV, and three levels of ribitol, which is the carbohydrate delivered from the photobiont to the mycobiont in *Xanthoria* (Fig. 2). We cultivated acetone-rinsed thalli for 7 days and quantified the resynthesized parietin. The photosynthate ribitol increased parietin resynthesis, especially in the UV-B treatment without light (Solhaug and Gauslaa 2004). The parietin resynthesis clearly requires UV-B, but a high parietin resynthesis also depends on sufficient supplies of photosynthates. We have not identified the steps in the parietin synthesis pathway triggered by UV-B. In higher plants, some responses to UV-B are mediated through nonspecific pathways involving DNA damage or reactive oxygen species that require high UV-B levels, whereas other responses are mediated through UV-B-induced metabolic pathways responding to low levels of UV-B (Jenkins 2009).

2.3 Do Cortical Compounds Protect Against PAR and/or UV-B?

Cortical lichen compounds strongly absorb UV-B when dissolved in an organic solvent. The absorbance spectra of parietin, usnic acid, and atranorin are shown in Fig. 3. However, these spectra for compounds in solution may not represent the

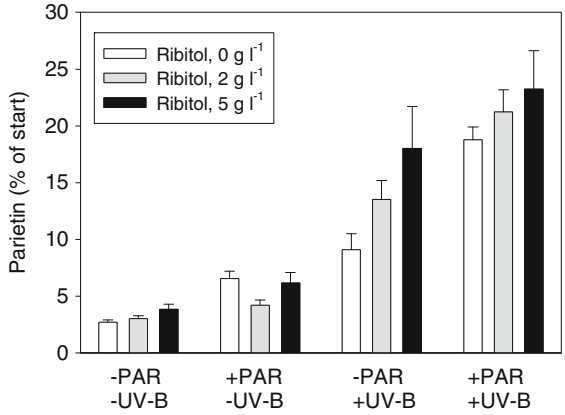

Fig. 2 Parietin resynthesis (percentage of original concentration) in *Xanthoria parietina* cultivated for 7 days in a factorial experiment with two UV-B levels (0 and 0.75 W m^{-2} 6 h per day), two visible light (PAR) levels (0 and 220 μmol m^{-2} s^{-1} PAR, 18 h photoperiod), and three ribitol levels (0, 2 or 5 mg L^{-1}). The thalli were soaked in water or ribitol solutions once every day. All thalli were extracted with acetone before start of the experiment. Error bars show 1 SE ($n = 10$). After Solhaug and Gauslaa (2004)

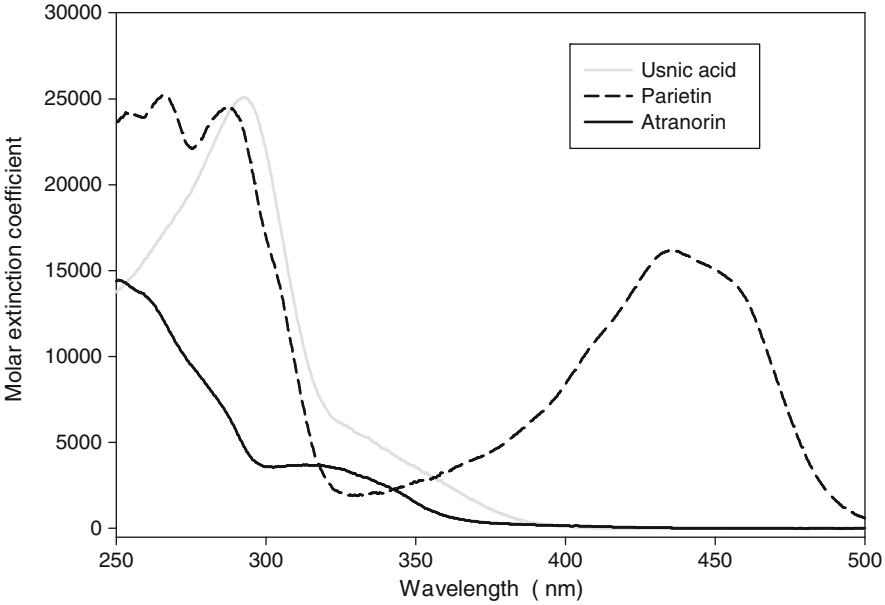

Fig. 3 Absorbance spectra of pure parietin, usnic acid, and atranorin dissolved in methanol. All spectra are normalized to 1 M concentration using the molar extinction coefficients in Huneck and Yoshimura (1996) to illustrate the absorptance efficiency of the three compounds. Unpublished data

screening efficiency for compounds in situ occurring as crystals outside fungal hyphae and photobiont cells. Usnic acid in solution transmits nearly all the visible light, whereas even low concentrations absorb most radiation below 400 nm. A layer of usnic acid crystals screens UV-B less efficiently because the radiation partly passes in between the crystals, but it screens visible light by reflectance from crystal surfaces (Fig. 4; McEvoy et al. 2007a). Absorption of pigments unevenly distributed in a leaf compared with isolated chloroplast is discussed in Merzlyak et al. (2009) from a theroretical point of view. They show a flattening of the absorption spectra in leaves due to scattering of light. Likewise, the colorless atranorin screens light by reflectance throughout the visible spectrum (Solhaug et al. 2010), as is presumably the case for all colorless or faintly colored lichen compounds. As crystals of colorless UV-B-absorbing compounds screen visible light, it is necessary to quantify the screening efficiency of cortical compounds in vivo for UV-B and PAR separately. To ascribe a protection function of UV-B and/or PAR, we must document that a given compound secures viability and survival under naturally high levels of UV-B or PAR (Cockell and Knowland 1999).

The crystalline structure of lichen compounds implies that absorption spectra of extracted pigments dissolved in an organic solvent have low ecological relevance for the in situ screening efficiency. Various methods can estimate the in vivo screening efficiency of secondary lichen compounds. In lichens with firm cortices, cortex fragments can be prepared for measuring cortical light transmittance directly

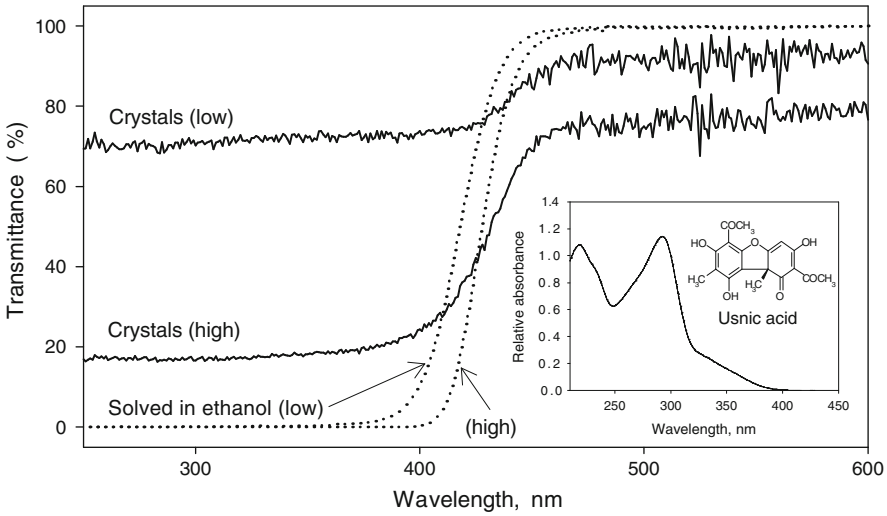

Fig. 4 Transmittance through a cover of few large (low) and many small (high) crystals of usnic acid formed on a plexiglass (*solid lines*). *Dotted lines* show the transmittance for the same two amounts of crystals dissolved in methanol. The *insert* shows the UV absorbance spectrum of dissolved usnic acid in methanol and the molecular structure of usnic acid. After McEvoy et al. (2007a)

(Ertl 1951; Dietz et al. 2000; Gauslaa and Solhaug 2001; McEvoy et al. 2007a; Nybakken et al. 2004). However, the use of acetone rinsing to quantify the screening efficiency of lichen compounds is difficult as acetone often incompletely extracts cortical compounds (McEvoy et al. 2007a). In *X. parietina*, the quantum yield of photosynthetic O_2 evolution in blue light was lower in control thalli than in parietin-free thalli, whereas quantum yield did not differ between control and acetone-rinsed thalli exposed to red light (Solhaug and Gauslaa 1996). These results were later confirmed with chlorophyll fluorescence measurements of effective quantum yield of PSII (Φ_{PSII}) by estimating the reduction in irradiance required to reach the same Φ_{PSII} after removal of parietin (Solhaug et al. 2010). These methods showed that the orange compound parietin screens blue light, but not red light. Furthermore, this chlorophyll fluorescence method, combined with spectral reflectance measurements, showed that the colorless compound atranorin screens light throughout the visible spectrum by increased reflectance (Solhaug et al. 2010).

The UV-B susceptibility of the *X. parietina*/*aureola* complex was tested for thalli sampled along a natural shade to light gradient from evergreen spruce forests to open sea cliffs. Irreversible depression of Fv/Fm occurred in the most shade-adapted thalli after 10 days continuous lab exposure to UV-B levels three times higher than natural levels during a clear summer day at 60°N. No damage occurred in the thalli from the sea cliffs (Gauslaa and Ustvedt 2003). Furthermore, parietin-free thalli of *X. aureola* showed no reduction in Fv/Fm after 3 weeks of exposure to natural UV-B levels in the field in summer, suggesting that parietin naturally occurs in

much higher concentrations than required for UV-B protection (Solhaug et al. 2003). Some lichens are remarkably resistant to high irradiation, and the lichens *Rhizocarpon geographicum* and *X. elegans* survived several days under outer space conditions with just minor reductions in viability (Sancho et al. 2007). Also, parietin-deficient thalli of *X. elegans* survived outer space condition with minor depression of Fv/Fm (de la Torre et al. 2010). Such experiments suggest that the primary role of parietin is not UV-B protection.

Maximal PSII efficiency (Fv/Fm) is often used as an indicator for measuring UV-B damage in photosynthetic organisms. However, UV-B may cause other types of damage involving DNA and other parts of the photosynthetic apparatus (Jansen et al. 1998). If the target of UV-B damage is PSII, Fv/Fm is a good indicator of damage. If the primary target of damage is DNA, the damage will not necessarily be measured as a decrease in Fv/Fm. In addition, Fv/Fm measures damage in the photobiont only. The mycobionts may be the most susceptible partner. In *Cladonia mitis* ssp. *mitis*, damage to mixed DNA from the whole thallus was measured as an increase in thymine dimers after UV-B exposure (Buffoni Hall et al. 2003). However, in *X. elegans*, the viability of both partners after exposure to high UV doses (simulated outer space conditions) was tested separately by using vital staining showing metabolic activity. The mycobiont cells were shown to be more resistant to high UV doses than photobiont cells (de Vera et al. 2008).

Cockell and Knowland (1999) list four criteria (see Table 1, first column) that should be fulfilled in order to ascribe a screening function of a given pigment. As discussed above, the PAR-protecting hypothesis of cortical compounds has stronger supporting evidence than the UV-B-protecting hypothesis because evidence for a UV-B-protecting role against natural UV-B levels is weak (Table 1).

3 Lichen Compounds Deter Herbivores

Most lichens have one cortical compound and one or more medullary compounds. For example, *L. pulmonaria* produces cortical melanins in response to UV-B in solar radiation (Solhaug et al. 2003). In addition, this species produces a number of medullary depsidones that do not respond to external factors, at least not over a 3-month period with high growth rates in a transplantation experiment to sites with highly different light exposure (McEvoy et al. 2007b). The location of medullary compounds inside and partly below the photobiont layer indicates that they hardly protect the photobiont against UV-B radiation, despite their strong UV-B absorptance. Several of these compounds are believed to serve as antifungal, antimicrobial (Ingólfsdóttir 2002), or antiherbivore agents (Lawrey 1980, 1983).

Lichens are long-lived organisms that tend to dominate nutrient-poor, cold, and/or dry areas where vascular plants hardly can grow, like high latitudes, high altitudes, on rocks, and in tree canopies. These sites often have few and low-quality fodder plants for herbivores. Even in such places, herbivores rarely consume lichens. The best-known exception is the reindeer that depends on a lichen diet during winter (Richardson and Young 1977). Few other mammals feed on lichens;

Table 1 Screening criteria [after Cockell and Knowland (1999)] of the four cortical compounds discussed in this review

Screening criteria:	Cortical compound	UV-B-protecting hypothesis	PAR-protecting hypothesis
The compound must absorb (or reflect) the respective radiation	Parietin	Solhaug et al. (2003)	Solhaug and Gauslaa (1996)
	Usnic acid	McEvoy et al. (2006)	McEvoy et al. (2007a)
	Atranorin	Huneck and Yoshimura (1996)	Solhaug et al. (2010)
	Melanins	Melanins cannot be extracted	Melanins cannot be extracted
The compounds should be induced or boosted by the relevant radiation	Parietin	Solhaug et al. (2003)	Solhaug and Gauslaa (2004)
	Usnic acid	McEvoy et al. (2006), Nybakken and Julkunen-Tiitto (2006)	McEvoy et al. (2006)
	Atranorin	–	–
	Melanins	Solhaug et al. (2003), Nybakken and Julkunen-Tiitto (2006)	–
Screening efficiency should be demonstrated *in vivo*	Parietin	Gauslaa and Ustvedt (2003)	Vráblíková et al. (2006), Solhaug and Gauslaa (1996), Solhaug et al. (2010)
	Usnic acid	Buffoni Hall et al. (2003), McEvoy et al. (2007a)	McEvoy et al. (2007a)
	Atranorin		Solhaug et al. (2010)
	Melanins	Gauslaa and Solhaug (2001)	Gauslaa and Solhaug (2001)
The compounds should protect against ecologically relevant radiation levels	Parietin	–	Solhaug and Gauslaa (1996)
	Usnic acid	–	Solhaug et al. (2010)
	Atranorin	–	Solhaug et al. (2010)
	Melanins	–	McEvoy et al. (2007b)

The evidence for the UV-B versus the PAR-protecting hypotheses is shown for each of the compounds by citations

examples are some rodents and flying squirrel (Dubay et al. 2008). A number of invertebrates like some oribatide mites, some gastropods, and insects feed on lichens (Gerson and Seaward 1977). Grazing marks from invertebrates can often be seen on lichens (Fig. 5). The frequency of grazing marks varies between habitats. *L. pulmonaria* growing on stems of broad-leaved deciduous trees with a rich gastropod fauna often have many grazing marks (Vatne et al. 2010), whereas thalli growing on spruce (*Picea abies*) twigs are rarely grazed (Fig. 6; Gauslaa et al. 2006). Extensive grazing by gastropods has been found on the globally endangered *Erioderma pedicellatum* in Nova Scotia where 80% of the gastropods were nonnative species (Cameron 2009).

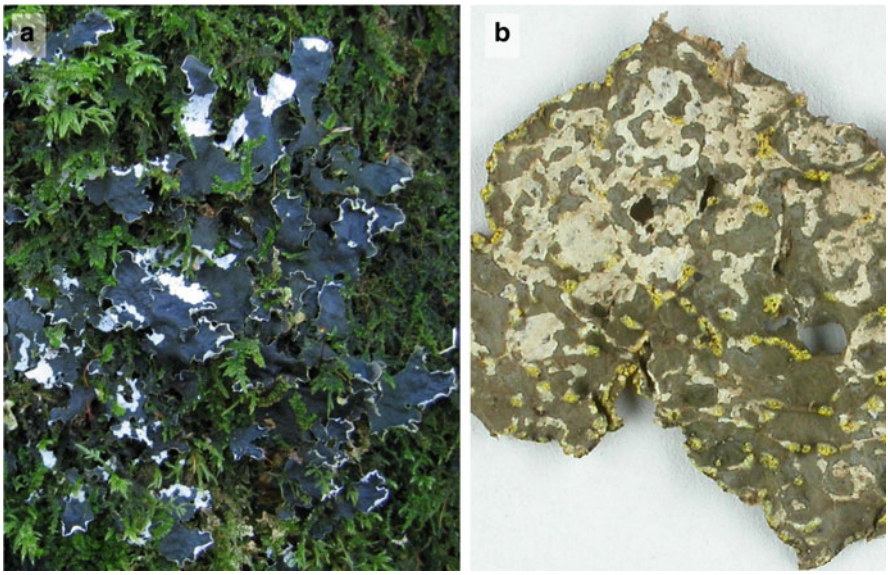

Fig. 5 Grazed *Peltigera polydactyla* (**a**; in the hydrated state) and *Pseudocypellaria crocata* (**b**; air dry state). The gastropods have grazed the upper cortex and the photobiont layer whereas the medulla is not grazed. The gastropods avoided the yellow soralia in *P. crocata*. Unpublished photos: Yngvar Gauslaa (**a**) and Knut Asbjørn Solhaug (**b**)

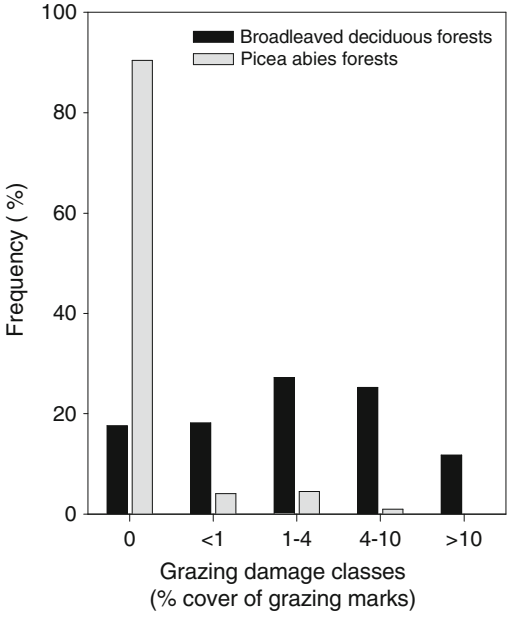

Fig. 6 The frequency of *Lobaria pulmonaria* thalli in five classes of grazing damage from gastropods. Grazing classes (abscissa) were based on estimated percent cover of grazing marks. The lichen material was collected in temperate broadleaved deciduous forests (*filled bars*; $n = 600$) and in boreal *Picea abies* forests (*open bars*; $n = 540$). After Gauslaa et al. (2006a)

3.1 Experimental Approaches Testing the Herbivore Defense

Removal of secondary compounds from lichen thalli with the acetone-rinsing technique allows testing of herbivore-deterrent functions for these compounds (Gauslaa 2005; Pöykkö et al. 2005). Gauslaa (2005) gave common garden snails (*Cepaea hortensis*) the choice to graze on two halves of one lichen thallus, one was acetone rinsed and one served as control. Grazing after one night was quantified as loss in dry weight. Such a protocol works well with a number of lichens such as *H. physodes* and *Evernia prunastri*. The experiment is simple and useful in ecology laboratory classes for students, and visualizes how powerful secondary compounds can be in deterring herbivores. Other lichen-feeding gastropods can also be used like *Arion fuscus*, *Cochlodina laminata*, and *Helicigona lapicida*, and the method functions also for woodlices (Isopoda) (Wolfgang Bilger, pers. comm.). The method has been used with the lichenivorous larvae of the moth *Eilema depressum* (Pöykkö et al. 2005), and a modified method has successfully been used with bank voles (Nybakken et al. 2010). Some snails also feed on filter paper and digest cellulose. By adding the acetone extracts with compounds to filter paper, the deterring role of the extracted compounds can be quantified. However, tests of pure compounds individually are necessary to elucidate which compounds are responsible for the deterrent effect (see Table 2).

3.2 Herbivore Defense in Lichens from N-Rich and N-Poor Habitats

Many lichens are protected against grazing regardless of their total extractable compound concentration (Table 3). Lichens from N-rich habitats and cyanolichens with N fixation are, with a few exceptions, deficient in lichen compounds or have low concentrations. For example, the nitrophytic lichens, *X. parietina*, *Anaptychia runcinata*, and *Physconia distotra*, have low amounts of acetone-extractable compounds, and the acetone rinsing did not lead to significant increase in snail grazing (Table 3). Acetone rinsing even reduced grazing of *X. parietina* by moth larvae (*E. depressum*), showing that parietin has no grazing-deterrent effect (Pöykkö et al. 2005). These nitrophytic lichens seem to be avoided by grazers despite the fact that they are low in secondary compounds. On the other hand, most lichens in nitrogen-poor environments, like most members of the Parmeliaceae, have high compound concentrations. In these species, a reduction in secondary compounds by acetone rinsing leads to substantially increased grazing (Table 3, lower part). These results suggest that the type of herbivore defense differs between N-rich and N-poor lichens. As N-content is one of the best predictors of good fodder quality for herbivores (White 1993; Mattson 1980), more grazing could have been expected on N-rich lichens. The combination of high N-content and low C-based extractable compound content is consistent with a hypothesis inferring that lichens from N-rich sites and N-fixing cyanolichens do not use acetone-extractable

Table 2 Herbivory deterrent and toxic effects of lichen compounds

Lichen compound	Lichen species	Herbivore species	Herbivory deterrence	Toxicity	Reference
Experiments with pure substances					
Usnic acid (dibenzofuran)	*Xanthoparmelia chlorochoa*	Domestic sheep	−	+	Dailey et al. (2008)
		Insect larvae (*Spodoptera littoralis*)	+	+	Emmerich et al. (1993)
	Cladonia stellaris	Bank vole (*Myodes glareolus*)	+		Nybakken et al. (2010)
Stictic acid (depsidone)		Insect larvae (*Spodoptera littoralis*)	−	(+)	Emmerich et al. (1993)
Atranorin (depside)	*Letharia vulpina*	Insect larvae (*Spodoptera ornithogalli*)		+	Slansky (1979)
Vulpinic acid (pulvinic acid derivate)		Insect larvae (*Spodoptera littoralis*)	+	+	Emmerich et al. (1993)
	Letharia vulpina	Insect larvae (*Spodoptera ornithogalli*)		+	Slansky (1979)
m-Scrobiculin (depside)	*Lobaria scrobiculata*	Snail (*Cochlodina laminate*)	+		Asplund et al. (2010c)
Experiments with lichen thalli or crude lichen extracts					
Stictic acid (depsidone)	*Lobelia pulmonaria*	Bank vole (*Myodes glareolus*)	+		Nybakken et al. (2010)
Fumarprotocetraric acid (depsidone)	*Cladonia pyxidata*	Moth larvae (*Eilema complana*)	+		Hesbacher et al. (1995)
Atranorin (depside)	*Parmelia sulcata*	Moth larvae (*Eilema depressum*)	+	−	Pöykkö et al. (2005)
Physodic acids (depsidones)	*Hypogymnia physodes*	Moth larvae (*Eilema depressum*)	+	+	Pöykkö et al. (2005)
Unspecified lichen compounds	Several lichen species	Bank vole (*Myodes glareolus*)	+		Nybakken et al. (2010)
Unspecified lichen compounds	Several lichen species	Snail (*Cepaea hortensis*)	+		Gauslaa (2005)
Several lichen compounds	Several lichen species	Slug (*Pallifera varia*)	+		Lawrey (1980)

A few studies are based on pure compounds, whereas other studies are based on which compounds are removed by acetone rinsing. Positive effect: +, negative effect: −, uncertain positive effect: (+), no symbol: not investigated

Table 3 Percentage content of acetone-extractable compounds and consumption (%) of acetone-rinsed and control thalli ($n = 10$) fed to *Cepea hortensis* during one night

Studied lichens	Lichen compounds (% of DM)	Percent herbivory		*p*-level pair-wise *t*-test
		Acetone rinsed	Control	
Anaptychia runcinata	0.21	4.2	4.2	ns
Physconia distorta	0.25	20.0	17.2	ns
Xanthoria parietina	0.83	33.1	23.7	ns
Lobaria pulmonaria	2.22	18.6	4.4	0.011
Parmelia saxatilis	4.27	39.3	13.9	0.008
Parmotrema chinensis	4.78	55.2	9.8	0.002
Vulpicida pinastri	5.42	89.5	9.5	0.002
Evernia prunastri	6.42	98.7	7.6	0.000
Hypogymnia physodes	7.49	95.5	18.2	0.000
Parmelia tiliacea	11.4	100	25.8	0.000

Adapted from Gauslaa (2005) where the total dataset with 17 lichen species is given

carbon-based lichen compounds to defend themselves. We hypothesize that these lichens may have N-based herbivore defense systems. N-based compounds cannot be extracted with acetone because they probably are located in living compartments inside cell membranes. An N-based defense may be a more qualitative defense system based on highly toxic substances such as alkaloids functioning in low amounts, whereas a carbon-based defense needs high amounts of less toxic substances to reduce digestibility or palatability of the fodder (Stamp 2003).

Addition of N to lichens in a long-term field experiment did not change the concentration of C-based secondary compounds in *Alectoria sarmentosa*, *Lobaria scrobiculata*, and *X. aureola*, whereas compound concentration decreased with N in *Platismatia glauca* (Nybakken et al. 2009). These results suggest a constitutive defense in three of four studied lichen species. Snails consistently preferred the unfertilized thalli of the three foliose species, but preferred the N-enriched pendulous *A. sarmentosa* that unlike the three other species grows inaccessibly to gastropods on thin twigs in the canopy of conifers (Asplund et al. 2010a). The production of N-based defense compounds when excess nitrogen is added needs to be tested in future experiments, as the N-based herbivore defense hypothesis has not yet been investigated for lichens. Possible N-based defense compounds in lichens are lectins found in a number of lichen species including the nitrophytic *X. parietina* (Legaz et al. 2004) and alkaloids that have been detected in the N-fixing lichen *Collema* spp. (Temina et al. 2010) and in *X. parietina* (Solberg 1971). The significance of both lectins and alkaloids in plant defense is well documented (see e.g., Peumans and Van Damme 1995; Karban and Baldwin 1997).

3.3 Induced Versus Constitutive Defense

Asplund and Gauslaa (2007) have shown that juvenile *L. pulmonaria* thalli have lower defense compound concentration than adult thalli. These data imply that

grazing invertebrates may be particularly detrimental for young, developing thalli (Scheidegger 1995; Scheidegger et al. 1995). Increased compound content with age could result from an induction due to exposure to grazing animals, but may also be considered a function of natural developmental processes. Thus far, grazing induction experiments of herbivore-deterrent C-based lichen compounds have been negative. In simulated grazing experiments (Nybakken and Julkunen-Tiitto 2006) and field experiments with real gastropod grazing (Asplund et al. 2009), no induction of C-based lichen compounds occurred. These experiments are consistent with a constitutive defense of C-based medullary lichen compounds. As one gastropod can consume a fairly large lichen thallus within a short period of time, an inducible defense would hardly respond fast enough to stop grazing in time.

3.4 Grazing-Deterrent Effect and Toxicity of Various Lichen Compounds

There are few studies quantifying the herbivory-deterrent effect and/or toxicity of specific lichen compounds for herbivores. Table 2 summarizes documented antiherbivore and toxic effects of lichen compounds. Removal of secondary compounds from *Vulpicida pinastri* and from *H. physodes* greatly increased survival of larvae of the moth *E. depressum*, whereas acetone rinsing of *Parmelia sulcata* had no long-term impacts on the larvae (Pöykkö et al. 2005). These data indicate that vulpinic and pinastric acids from *V. pinastri*, and, e.g., physodic acid and/or physodalic acid from *H. physodes* are more toxic for the larvae than atranorin that is present in both *H. physodes* and *P. sulcata*. For all these three lichen species, the moth larvae preferred acetone-rinsed thalli. In total, such results indicate that some substances such as pinastric and physodic acid are both toxic and deterrent to moth larvae, whereas atranorin and/or salazinic acid (present in *P. sulcata*) mainly is deterrent (Pöykkö et al. 2005). The deterring or toxic effects may differ for various herbivores. Reindeers tolerate usnic acid because they host symbiotic bacteria in their stomach that can detoxify this compound (Sundset et al. 2008), whereas it is toxic to sheep (Dailey et al. 2008). Usnic acid is also toxic to other mammals and insect larvae (Dailey et al. 2008; Emmerich et al. 1993) and deters grazing of bank voles (Nybakken et al. 2010). However, usnic acid did not deter grazing of the snail *C. laminata,* whereas the lichen substance *m*-scrobiculin had a strong grazing deterrent effect (Asplund et al. 2010c). Some invertebrates may use lichen compounds in their own defense, for example, some Lepidopteran larvae may sequestrate these compounds in their body (Hesbacher et al. 1995). More experiments testing the antiherbivore and toxic effects of quantified amounts of specific lichen compounds on various herbivores are needed.

3.5 Spatial Distribution of Secondary Compounds Within a Lichen Thallus and Optimal Defense Theory

Reproductive structures like soralia often produce lichen compounds other than the remaining thallus (e.g. Imshaug and Brodo 1966; Tønsberg 1992; Nybakken et al. 2007). Snails avoid soralia in *Pseudocyphellaria crocata* (Gauslaa 2008) and *L. scrobiculata* (Asplund et al. 2010c). In the latter species, the snail *C. laminata* avoided the soralia and grazed on vegetative tissues of control thalli. However, after acetone rinsing, *C. laminata* instead preferred the soralia (Fig. 7; Asplund et al. 2010c). The soralia contained five times more *m*-scrobiculin than the vegetative thallus parts, but soralia had almost no usnic acid compared with the vegetative parts. By adding *m*-scrobiculin and usnic acid to filter paper fed to the snails, it was shown that *m*-scrobiculin efficiently deterred grazing, whereas usnic acid had almost no deterring effect (Asplund et al. 2010c). Therefore, *L. scrobiculata* allocates its most efficient grazing-deterrent compound to reproductive parts that likely are most valuable for the lichen fitness, consistent with the optimal defense theory (McKey 1974; Rhoades 1979). Spatial variation in compound content occurs in many lichens, like in *Umbilicaria hyperborea*, where the upper regions of the medulla contain much more secondary compound crystals than the middle and lower regions (Fahselt and Alstrup 1997). The upper medulla with the photobiont layer is probably most attractive to herbivores because the photobiont has the highest nitrogen content. The photobiont is also important for the lichen because it provides photosynthates. Higher secondary compound content in this layer

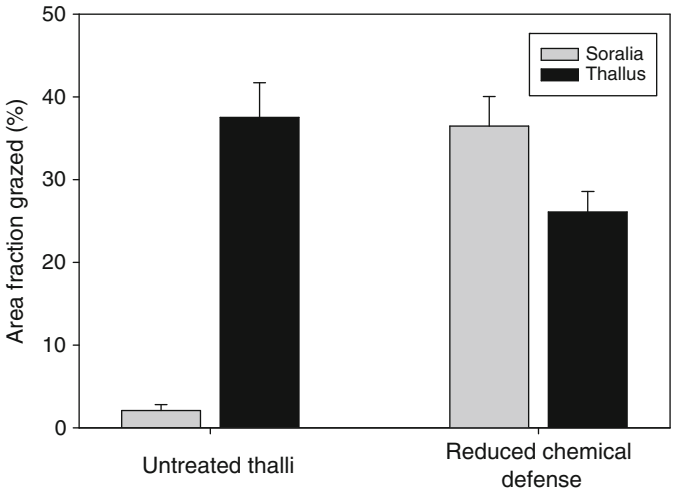

Fig. 7 Grazing on reproductive (soralia) and somatic (thallus) parts of *Lobaria scrobiculata* with natural (control) and reduced chemical defense. Values are mean and SE. Significant differences are denoted by *asterisks* (paired *t* test, $n = 10$ trials). *$P < 0.05$; ***$P < 0.001$. After Asplund et al. (2010c)

therefore supports the optimal defense theory. However, the defense is not sufficient in all lichens as snails sometimes graze the upper cortex and the photobiont layer and leave the less nutritious medulla untouched (Fig. 5).

3.6 Gastropod Grazing Can Shape Epiphytic Lichen Communities

There is increasing evidence showing that lichen-feeding gastropods can shape epiphytic lichen communities. Asplund and Gauslaa (2008) transplanted *L. pulmonaria* on vertical boards in two broadleaved deciduous forests replicated across shady and partly open gap positions in both stands. All boards were paired; gastropods had free access to the transplants in one board, in the other, a copper tape below the transplants excluded the gastropods from climbing the lichen sites. One forest had a species-rich lichen flora including various *Lobaria* species; the other forest with similar structure was poor in lichens and lacked *Lobaria* species. Grazing of *L. pulmonaria* was significantly more severe in (1) the lichen-poor forest, (2) in shady compared with sunny positions, (3) in positions with no gastropod exclusion, and (4) in acetone-rinsed thalli (Asplund and Gauslaa 2008). The first three responses were caused by spatial variation in natural populations of climbing lichen-feeding gastropods.

In another lichen study, indigenous gastropods had the choice between transplanted *L. pulmonaria* and *P. crocata* in boreal rainforests. The gastropods, mainly the slug *Arion fuscus*, consistently preferred *P. crocata* (Gauslaa 2008), despite the fact that the total amount of lichen compounds was 7.1% in the preferred *P. crocata* versus 3.1% in the rejected *L. pulmonaria* thalli (Nybakken et al. 2007). However, the cover of grazing marks in *P. crocata* decreased with increasing content of the medullary compounds stictic and constictic acids. Hardly any grazing occurred in *L. pulmonaria*. Therefore, the dominant lichen compounds in *P. crocata* (tenuiorin and its derivates) hardly function as gastropod-defense compounds. Stictic and constictic acids occur in both studied lichen species, but their concentration was only 0.6% in *P. crocata* compared with 2.5% in *L. pulmonaria*. Thus, stictic and constictic acids deter gastropods in *L. pulmonaria* and *P. crocata*, but the two compounds often occur in too low concentration in *P. crocata* to deter gastropods (Gauslaa 2008). Also the American slug *Pallifera varia* avoided lichens with stictic acid (Lawrey 1980).

Gastropods also participate in forming the vertical zonation of epiphytic lichens in temperate forest canopies. Transplantation of four *Lobaria* species to three heights on *Fraxinus excelsior* trunks showed that grazing decreased with height above the ground, and that *L. amplissima* and particularly *L. scrobiculata* were consistently more grazed than *L. virens* and *L. pulmonaria* (Fig. 8). There was a relationship between the natural lower distribution limit on tree trunks and the species-specific level of grazing damage (Asplund et al. 2010b). The highly preferred forage lichen *L. scrobiculata* partly avoids grazing by its ability to grow on bark with lower pH (Gauslaa 1985) where gastropods are less common (Vatne et al. 2010).

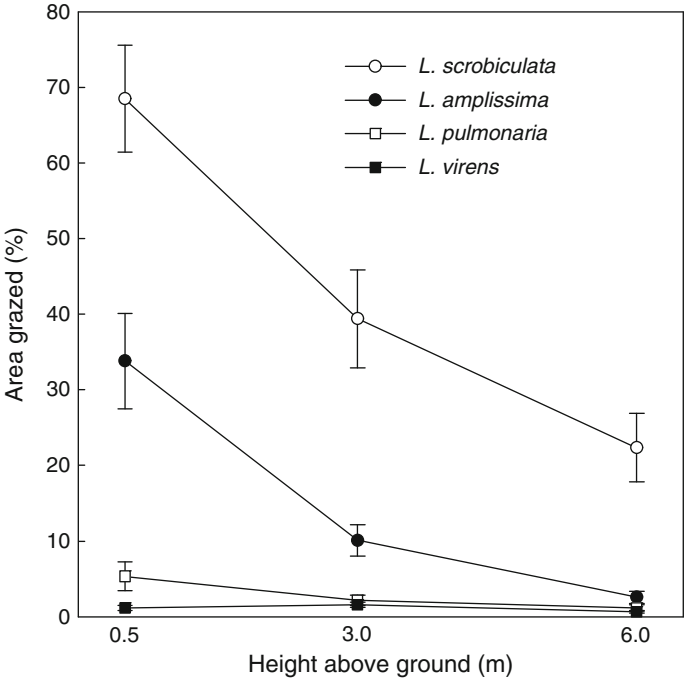

Fig. 8 Grazing on four *Lobaria* species transplanted for 137–139 days to trunks of *Fraxinus excelsior* at three different heights above ground. Mean ± 1 SE, $n = 25$. After Asplund et al. (2010b)

Such transplantation experiments emphasize the importance of biotic interactions in shaping lichen-dominated communities. This opposes a common view that lichen distribution is mainly driven by physical and chemical factors like climate, pollution, and ionic environment.

4 Conclusion

Cortical lichen compounds protect against excess solar radiation. Some cortical compounds like parietin do not deter herbivores. Other cortical compounds (as e.g., usnic acid) may protect against excessive radiation as well as deter herbivores, but the latter function is not yet sufficiently tested for cortical compounds. Herbivore defense is a well-documented role for some of the lichen compounds located in the medulla.

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