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The Plant Plasma Membrane

Springer

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Editors

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Preface

The spontaneous formation of lipid-bounded compartments is one of the preconditions for the genesis of the earliest living cells. Such membranous structures retain essential components, serve as a barrier to intrusion of external contaminants, and, via differential diffusion of ions, generate transient electrochemical gradients that can energize selective exchange processes. In plant cells, the outer barrier plasmalemma, or plasma membrane, is a highly elaborated structure that functions as the point of exchange with adjoining cells, cell walls, and the external environment. Transactions at the plasma membrane include uptake of water and essential mineral nutrients, gas exchange, movement of metabolites, transport and perception of signaling molecules, and initial responses to external biota. Central to all of these processes is the formation of a chemiosmotic gradient across the plasma membrane that results from ATP-driven proton extrusion. This gradient generates a net negative charge on the inner surface of the membrane and a delta pH of 1.5–2. Selective channels and carriers harness this electromotive force to control the rates and direction of movement of small molecules across the membrane barrier and manipulate the turgor that maintains plant form and drives plant cell expansion. Where required, ATP-dependent transporters mobilize the movement of essential molecules against the gradient.

However, it is erroneous to view the plasma membrane as just a diffusion barrier studded with transport proteins. Like other cellular membranes, the plasma membrane provides an environment in which molecular and macromolecular interactions can occur more efficiently. This is primarily a result of the enhanced efficiency of diffusional interactions taking place in two dimensions, the clustering of proteins in oligomeric complexes via protein–protein or protein–lipid interactions for more efficient retention of biosynthetic intermediates, and the anchoring of protein complexes to enhance regulatory interactions. Coupling of signal perception at the membrane surface with intracellular second messengers also necessarily involves transduction across the plasma membrane. Finally, the generation and ordering of the external cell walls involve processes mediated at the plant cell surface by the plasma membrane.

This volume is divided into three parts. Part I, consisting of five chapters, describes the basic mechanisms that regulate all plasma membrane functions. Chapter "Lipids of the Plant Plasma Membrane" by Furt et al. describes the most fundamental aspect of the plasma membrane – its lipid composition and the ordering of membrane lipids into leaflets and domains. The chapter "Plasma Membrane Protein Trafficking" by Peer describes the mechanisms by which proteins are trafficked to and from the plasma membrane. The chapter "The Plasma Membrane and the Cell Wall" by Sampathkumar et al. describes the role of the plasma membrane in cell wall production as well as the interactions between the plasma membrane surface and the cell walls during development. The chapter "Plasmodesmata and non-cell autonomous signaling in plants" by Lee et al. describes the plasmodesmal structures that provide unique regulated conduits that can partially bridge cell wall barriers to provide direct intercellular interactions. The chapter "Post-translational Modifications of Plasma Membrane Proteins and Their Implications for Plant Growth and Development" by Luschnig and Seifert details the regulatory posttranslational modifications made to many plasma membrane proteins.

Part II describes plasma membrane transport activity. Chapter "Functional Classification of Plant Plasma Membrane Transporters" by Schulz provides an overview of the structure and classification of plasma membrane transporters and uses structural characteristics to classify these proteins into groups. In the chapter "Plasma Membrane ATPases" by Palmgren et al., a similar structural analysis is combined with functional analyses derived from experimental results to describe the ATPases that export protons and calcium at the plasma membrane. Chapter "Physiological Roles for the PIP Family of Plant Aquaporins" by Vera-Estrella and Bohnert uses a similar approach to characterize the aquaporin intrinsic membrane protein channels that transport water and other small molecules in and out of the cell. In chapters "The Role of Plasma Membrane Nitrogen Transporters in Nitrogen Acquisition and Utilization" by Tsay and Hsu, "Plant Plasma Membrane and Phosphate Deprivation" by Nussaume et al., "Biology of Plant Potassium Channels" by Hedrich et al., "Mechanism and Evolution of Calcium Transport Across the Plant Plasma Membrane" by Connorton et al., "Sulfate Transport" by Hawkesford, "Metal Transport" by Atkinson, and "Organic Carbon and Nitrogen Transporters" by Tegeder et al., the regulated transport of nitrogen, phosphorus, potassium, calcium, sulfur, metals, and cellular metabolites across the plasma membrane are described. Chapter "ABC Transporters and Their Function at the Plasma Membrane" by Knöller and Murphy returns to a more structural approach to describe what is currently know about the plasma membrane ATP-binding cassette transporters of the ABCB and ABCG subfamilies. The transporter part of the book is rounded out by a description of hormone transport in chapter "Hormone Transport" by Kerr et al.

Part III of the book describes signaling interactions at the plasma membrane, with chapters describing hormone signaling (chapter "Plant Hormone Perception at the Plasma Membrane" by Pandey), light sensing (chapter "Light Sensing at the Plasma Membrane" by Christie et al.), lipid signaling (chapter "The Hall of Fame:

Lipid Signaling in the Plasma Membrane" by Im et al.), abiotic stress responses (chapter "Plasma Membrane and Abiotic Stress" by Barkla and Pantoja), and biotic interactions (chapter "The Role of the Plant Plasma Membrane in Microbial Sensing and Innate Immunity" by Nürnberger and Küfner).

Although these topics have been the subject of many current and past reviews, they are given a unique treatment in this volume, as we have made an effort to concentrate on events and mechanisms that occur at the plasma membrane rather than discuss mechanisms that occur throughout plant cells. It is hoped that this effort will provide the reader with a strong sense of the unique role that the plasma membrane plays in plant physiology and development. Further, the authors of the individual chapters have made an effort to identify areas where there are substantial gaps in our understanding of mechanisms sited on this critical cellular structure. Finally, we hope to convince the reader that a more complete knowledge of plasma membrane structure and function is essential to current efforts to increase the sustainability of agricultural production of food, fiber, and fuel crops.

Lafayette, USA A. Murphy
1 May 2010

Contents

Section I
Plasma Membrane Structure and Basic Functions

Lipids of the Plant Plasma Membrane

Fabienne Furt, Françoise Simon-Plas, and Sébastien Mongrand

Abstract The plasma membrane (PM) is arguably the most diverse membrane of the plant cell. Furthermore, the protein and lipid composition of the PM varies with cell type, developmental stage, and environment. Physical properties of lipids and associate proteins allow the formation of a barrier that is selectively permeable to macromolecules and solutes. As the plasma membrane delineates the interface between the cell and the environment, it is the primary part of signal recognition and transduction into intracellular responses for nutritional uptake/distribution, environmental responses, and developmental signaling. Many essential PM functions are carried out by proteinaceous components. However, PM lipids play a crucial role in determining cell structures regulating membrane fluidity and transducing signals. The composition and physical state of the lipid bilayer influence lipid–protein and protein–protein associations, membrane-bound enzyme activities, and transport capacity of membranes. Analyses of membrane function require highly selective and efficient purification methods. In this chapter, we first briefly review the methods to isolate PM from plant tissue and describe the lipid content of purified membranes. We further examine the involvement of different lipid species on signaling events that allow the plant cell to cope with environmental fluctuations. Finally, we discuss how regulated segregation of lipids inside the PM is of crucial importance to understand signaling mechanisms.

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1 Biochemical Analysis of Plant Plasma Membrane

1.1 Isolation of Highly Purified Plasma Membrane Fractions from Plant Tissues

Isolating highly purified fractions of organelles or membranes from other cellular compartments is a key requirement for in-depth identification and characterization of membrane proteins and lipids. PM fractions were first purified from microsomal membranes (a mix of various cellular membranes) by their high density on flotation gradient after high-speed ultracentrifugation. This approach has been shown to inefficiently fractionate the PM from other membranes, particularly the tonoplast. Higher efficiency partial separations of PM vesicles by free flow electrophoresis have also been reported (for review Canut et al. 1999).

In the early 1980s, Larsson developed an effective tool for preparative isolation of PM fractions by partitioning microsomal fractions in aqueous polymer two-phase systems using aqueous solutions of polyethylene glycol (PEG) and dextran (Widell et al. 1982). The method is rapid and uses only standard laboratory equipment. The separation is continued on after stirring, the system spontaneously forms two phases, and microsomal membranes separate according to differences in surface properties rather than in size and density. As PM vesicles are more negatively charged than most other cellular membranes, they are recovered into the upper phase. Up to 98% purity can be reached with this method. This technique therefore represents an attractive alternative to conventional fractionation protocols and has been shown to be effective for multiple plant tissues (root, leaves, etc.). The respective proportions of the two polymers, pH, and the ionic strength of the aqueous phase are the crucial parameters to ensure PM purity (Larsson et al. 1987).

The outer (apoplastic) side of the PM bilayer is negatively charged. Consequently, PM vesicles purified by two-phase partition are mostly sealed in a right-side-out topology. Morphological studies of highly purified PM fractions pelleted by centrifugation, fixed by chemical or high-pressure freeze substitution, and embedded in resin showed that these fractions contained mostly membrane vesicles ranging from 50 to 500 nm in diameter, should the majority of which exhibited a diameter between 200 and 300 nm. Higher magnifications showed that the membrane leaflets were highly contrasted and 8 nm thick, which corresponds to in situ observations of PM in intact tissues (Fig. 1).

1.2 Lipid Content of Plant Plasma Membrane

The lipid-to-protein mass ratio in the plant PM is ca. 1. However, considering that the average lipid molecular mass is far below than the average molecular mass of protein, the lipid-to-protein molar ratios in the PM range from 50:1 to 100:1. Analyses of highly purified PM lipid extracts are performed by thin layer

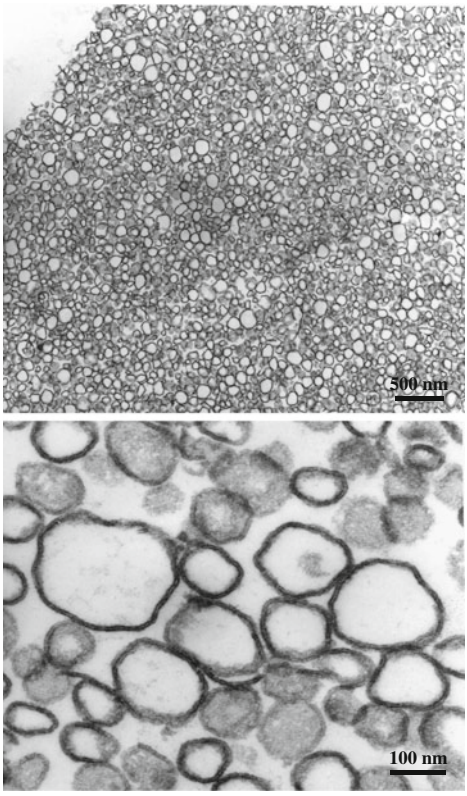

Fig. 1 Electron microscopy observation of a two-phase partition highly purified plasma membrane fraction isolated from tobacco leaves

chromatography (TLC), gas chromatography (GC), high-pressure liquid chromatography (HPLC), and GC/HPLC coupled to mass spectrometer. More recently, mass spectrometry approaches have been adapted to "lipidomic" analysis. For instance, tandem mass spectrometry (MS/MS) or MS^3 strategies that can simultaneously identify multiple lipid species are required because they provide structural information regarding polar head groups, length, unsaturation of fatty acid chains, and presence of glycosyl units in lipid molecules. However, as there are often close to 1,000 lipid species in a single cell (Van Meer 2005), even MS/MS methods are not sufficient to fully resolve the complexity of lipid mixtures. Therefore, although the results of lipid analyses from several plant species have been available for many years, a complete characterization of the plasma membrane is still lacking.

Three main classes of lipids exist in the PM: glycerolipids (mainly phospholipids), sterols, and sphingolipids (Fig. 2). Except for complex sphingolipids, which are synthesized in the *trans*-Golgi network, most lipids are assembled in the endoplasmic reticulum and are transported through the secretory pathway to the PM (Van Meer and Sprong 2004). Briefly, fatty acids are synthesized in plastids and mainly exported to the ER S-acylated to coenzyme A to enter the Kennedy pathway for phospholipids (see for review Bessoule and Moreau 2004) and sphingolipids pathway (see for review Pata et al. 2010).

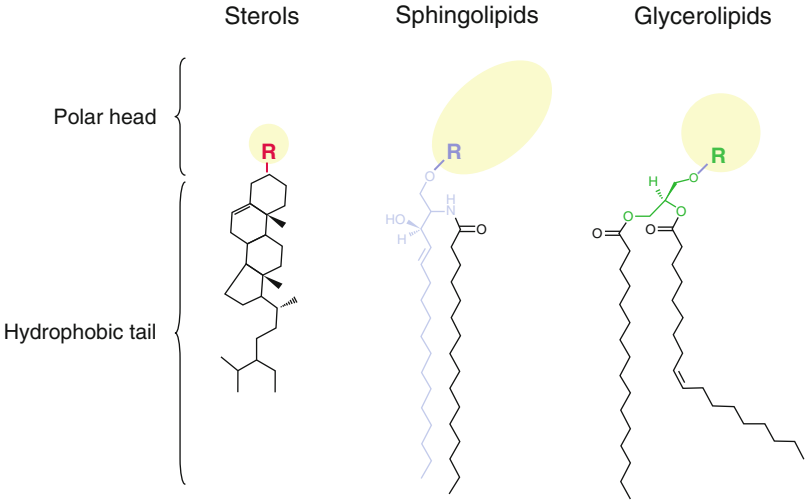

Fig. 2 Chemical features of the three major classes of plant plasma membrane lipids. The various polar heads are represented in *gray*

A great diversity is observed in PM lipid composition across plant species (e.g., Uemura and Steponkus 1994; Uemura et al. 1995) and within the different organs of a given plant species (e.g., Sandstrom and Cleland 1989). However, compared with other cellular membranes, the PM is always strongly enriched in sterols and sphingolipids with a sterol-to-phospholipid ratio ranging from 0.6 to 1.5 (Table 1). PM lipids are generally classified by abundance as well as by structure: the most abundant are often referred to as "structural lipids" and less abundant as "signaling lipids." These two categories are somewhat artificial as several lipids referred to as examples of abundant lipids may exhibit signal-transducing function. This chapter focuses on the biosynthesis of signaling lipids rather than on the synthesis of structural lipids (see for review Bessoule and Moreau 2004; Pata et al. 2010), and clustering of lipid and protein in PM microdomains.

1.2.1 Glycerolipids

Glycerolipids are tripartite molecules made up of a head group nucleated by a glycerol moiety to which two fatty acyl chains are esterified at positions *sn1* and *sn2* as shown in Fig. 2. The third position consists of a hydroxyl group to form diacylglycerol (DAG) and is further modified to form the different classes, namely, glycolipids and phospholipids (Fig. 3). With the notable exception of PM-localized digalactosyl diacylglycerol (DGDG), glycoglycerolipids are mostly present in plastids. DGDG replaces phospholipids in the PM bilayer during plant phosphate deprivation to preserve the integrity of the membrane and remobilize the phosphate pool (e.g., Andersson et al. 2005; Tjellstrom et al. 2008).

Table 1 Lipid content of plant plasma membrane, expressed as percent of total lipids

	Vigna radiata hypocotyl	*Secale cereale* leaves	*Secale cereale* leaves	*Avena sativa* coleoptyle	*Avena sativa* roots	Spring oat leaves	Winter oat leaves	*Hordeum vulgare* roots	*Zea mays* roots	*Arabidopsis thaliana* leaves	*Solanum tuberosum* leaves	*Solanum commersonii* leaves	*Nicotiana tabacum* BY-2 cells	*Nicotiana tabacum* leaves
PL	48.9	31.7	36.6	41.7	50.1	28.2	28.9	45	43.9	46.8	46.4	48.3	40.3	38.5
SL	6.8	16.2	16.4	26.1	10.1	27.2	30.4	8	6.8	7.3	6.5	6.1	11.3	20.6
St	43.6	52.1	46.6	32.2	39.7	41.3	39.1	43	49.3	46	45	41.6	26.7	22.6
Other	0.7	–	0.4	–	–	2.7	1.7	4	–	–	2.1	4	6.6	5.8
St/PL	0.9	1.6	1.3	0.8	0.8	1.5	1.3	0.9	1.1	1	1	0.9	0.7	0.6
References	Yoshida and Uemura (1986)	Lynch and Steponkus (1987)	Uemura and Steponkus (1994)	Sandstrom and Cleland (1989)	Sandstrom and Cleland (1989)	Uemura and Steponkus (1994)	Uemura and Steponkus (1994)	Brown and Dupont (1989)	Bohn et al. (2007)	Uemura et al. (1995)	Palta et al. (1993)	Palta et al. (1993)	Mongrand et al. (2004)	Mongrand et al. (2004)

PL phospholipid, *SL* sphingolipids, *St* sterols

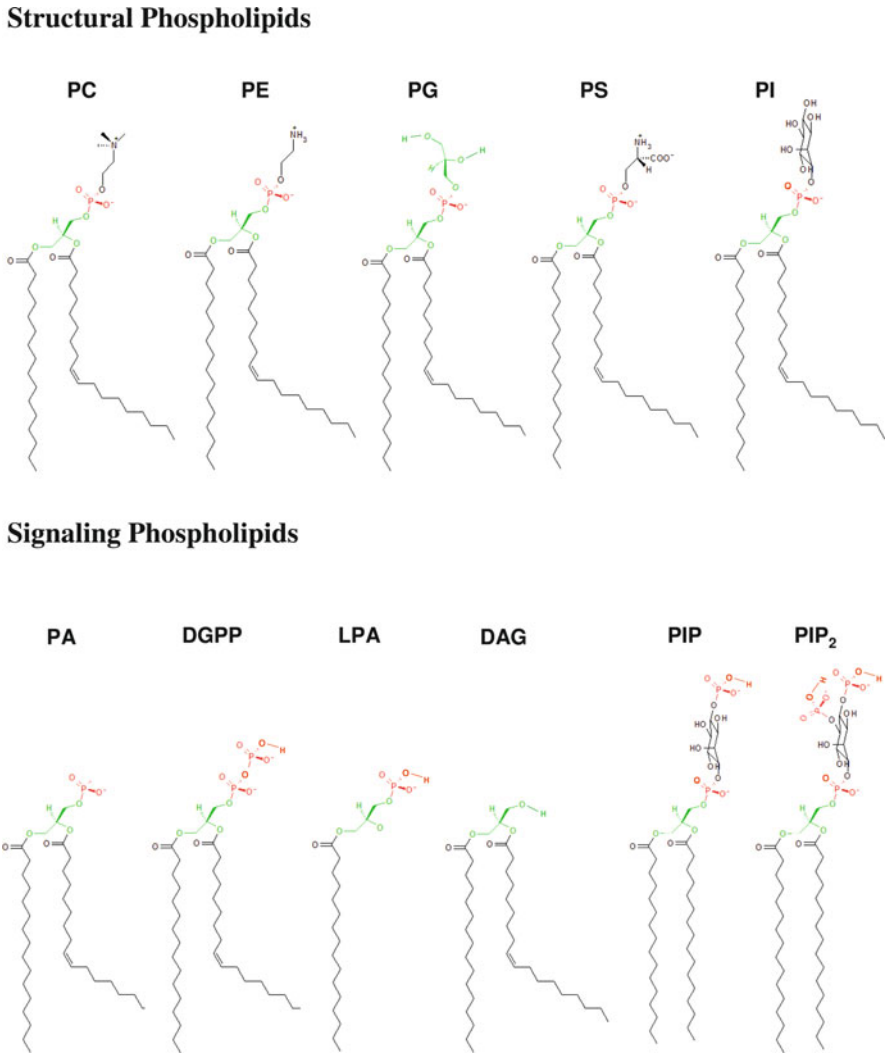

Fig. 3 Chemical structure of the *structural* and *signaling* phospholipids. Abbreviations: *PC* phosphatidylcholine, *PE* phosphatidylethanolamine, *PG* phosphatidylglycerol, *PS* phosphatidylserine, *PI* phosphatidylinositol, *PA* phosphatidic acid, *DGPP* diacylglycerolpyrrophosphate, *LPA* lysophosphatidic acid, *PIP* phosphatidylinositol monophosphate, *PIP2* phosphatidylinositol bisphosphate

Structural Glycerolipids

Phosphatidylcholine (PC) and phosphatidylethanolamine (PE) represent up to 68–80% of structural phospholipids. The remainder consists of phosphatidylglycerol (PG), phosphatidylinositol (PI), phosphatidylserine (PS), and phosphatidic acid (PA) (Fig. 3; Table 2). Phospholipid fatty acid composition is well conserved

Table 2 Structural phospholipid content of plant plasma membrane, expressed as percent of total lipids

	Vigna radiata hypocotyle	*Secale cereale* leaves	*Secale cereale* leaves	Oat roots	Spring oat leaves	*Hordeum vulgare* roots	*Zea mays* roots	*Zea mays* roots	*Arabidopsis thaliana* leaves	*Solanum tuberosum* leaves	*Solanum commersonii* leaves	Cauliflower bud
PC	32.7	46.7	36.3	33.8	35.1	36.9	39	36	35.5	38.4	42.6	39
PE	38	34.4	33.9	31.2	29.2	32.1	35	32.3	38.9	35.7	36.2	35
PG	4.5	5.7	8.2	13.9	6.6	3	14	12.1	9	3.9	3.7	14
PI	5.3	2.2	7.4	4.8	9.4	7.6	9	10.5	10.3	7.5	5.5	9
PS	3.1	4.7	–	–	–	7.1	–	9.1	6.4			
PA	16.4	5.4	10.4	16.3	15.3	6.3	4	–	–	10.2	9.5	4
Other	–	<0.9	3.8	–	4.5	7	–	–	–	4.3	2.6	
References	Yoshida and Uemura (1986)	Lynch and Steponkus (1987)	Uemura and Steponkus (1994)	Norberg and Liljenberg (1991)	Uemura and Steponkus (1994)	Brown and Dupont (1989)	Grandmougin et al. (1989)	Bohn et al. (2007)	Uemura et al. (1995)	Palta et al. (1993)	Palta et al. (1993)	Wright et al. (1982)

PC phosphatidylcholine, *PE* phosphatidylethanolamine, *PG* phosphatidylglycerol, *PS* phosphatidylserine, *PI* phosphatidylinositol, *PA* phosphatidic acid, *DGPP* diacylglycerolpyrophosphate, *LPA* lysophosphatidic acid

among plant species, with palmitic (16:0) and linoleic (18:2) acids being the primary species (20–60%), and linolenic (18:3) acid representing another 7–26% (Table 3). Mass spectrometry analyses have shown that palmitic acid and linoleic acid are often associated (35–50% of total phospholipids), followed by 18:1/18:3 and 18:2/18:2 associations. Several lines of evidence suggest that temperature and osmotic stress produce changes in the fluidity of plant cell membranes that initiate cellular responses to modify the extent of unsaturation of fatty acids, mostly of phospholipids (for review Mikami and Murata 2003).

Phosphoinositides

Phosphoinositides (PIs) or inositolphospholipids constitute a minor fraction of total cellular lipids in all eukaryotic cells and play an important regulatory role in cell physiology. The repertoire of cellular processes known to be directly or indirectly controlled by this class of lipids continues to expand. Through interactions mediated by their headgroups, which can be reversibly phosphorylated to generate up to seven species, PIs play a fundamental part in controlling membrane interfaces (van Leeuwen et al. 2004). HPLC analysis of plants cells identify six different isoforms: PI(4)P, PI(4,5)P_2, PI(3)P, P(5)P, PI(3,4)P_2, and PI(3,5)P_2 (Irvine et al. 1989; Brearley and Hanke 1992; Meijer et al. 2001) (Fig. 4a). The enzymes that catalyze PI synthesis and hydrolysis, as well as the genes that encode them, have been characterized plants and are briefly described below.

PI(3)P and PI 3-Kinase

Depending on plant species, PI(3)P represents 2–15% of monophosphate phosphoinositides (Munnik et al. 1998, 2000). In animals, PI 3-kinases are classified into three distinct families according to their biochemical properties. Plant enzymes only belong to the third group containing a C2 domain, a helical domain, and a catalytic domain, but no RAS-binding domain. Three genes have been cloned in *Arabidopsis thaliana* (Welters et al. 1994). Moreover, only PI is phosphorylated to give PI(3)P (for review Mueller-Roeber and Pical 2002). PI (3)P can bind to FYVE protein domain, and chimeric GFP translational fusions indicate that PI(3)P localizes to the pre-vacuolar compartment and endosomes, suggesting a role for this lipid in vacuolar trafficking (Vermeer et al. 2006). PI(3)P is also likely to be involved in stress-related signaling as its levels increase after NaCl treatment in *Chlamydomonas* (Meijer et al. 2001), and inhibitors of PI 3-kinase prevent reactive oxygen species production and stomatal closure in guard cells (Park et al. 2003; Jung et al. 2002).

Table 3 Fatty acid composition of plasma membrane phospholipids from various plants and tissue

	Vigna Unguiculata hypocotyles						Oat roots		Maize roots					*Solanum tuberosum* leaves		*Solanum comersonii* leaves	
	PE	PC	PG	PI	PS	PA	PC	PE	PC	PE	PG	PI	PS	PC	PE	PC	PE
16:0	42	26.1	67.1	43.2	18.5	35.3	27.2	32.3	30.8	35	47.8	50.2	23.7	36.5	39.9	28.2	31.7
18:0	4.2	8.7	5.7	7.3	6.9	4.6	0.3	0.4	1	0.5	2.8	5.8	3	4.3	3.6	4.2	3.2
20:0	–	–	–	–	–	–	–	–	–	–	–	–	–	0.7	2.4	1.7	1.5
16:1	–	–	–	–	–	–	–	–	–	–	–	–	–	0.6	0.5	0.7	0.5
18:1	8	11.6	5.6	8.7	5.9	9	1.7	1.3	5.3	2.5	5.4	4.2	2.3	4.7	2.2	12.3	5.2
20:1	9	1.2	–	–	14.4	0.7	–	–	–	–	–	–	–	–	–	–	–
22:1	4	1.1	0.5	–	8.3	0.5	–	–	–	–	–	–	–	–	–	–	–
18:2	23.4	24.4	8.5	16	19.3	25.4	54.1	50.4	61.2	60.2	42.3	38.2	36	34.4	35.9	33.3	40.5
20:2	0.3	0.4	4.2	2.6	0.8	0.7	–	–	–	–	–	–	–	–	–	–	–
22:2	–	–	–	–	–	–	–	–	–	–	–	–	2	–	–	–	–
16:3	–	–	–	–	–	–	1.6	1.3	–	–	–	–	–	–	–	–	–
18:3	19.3	23.3	7.3	22.2	26.2	24.2	15.9	12.9	1.8	1.7	1.7	1.1	1.1	18.4	15.1	19	17
20:3	1.5	3.5	2	–	0.4	0.1	–	–	–	–	–	–	–	–	–	–	–
Others	–	–	–	–	–	–	1.8	3	–	–	–	–	27.8	–	–	–	–
References	Yoshida and Uemura (1986)						Norberg and Liljenberg (1991)		Bohn et al. (2007)					Palta et al. (1993)			

PC phosphatidylcholine, *PE* phosphatidylethanolamine, *PG* phosphatidylglycerol, *PS* phosphatidylserine, *PI* phosphatidylinositol, *PA* phosphatidic acid, *DGPP* diacylglycerolpyrophosphate, *LPA* lysophosphatidic acid

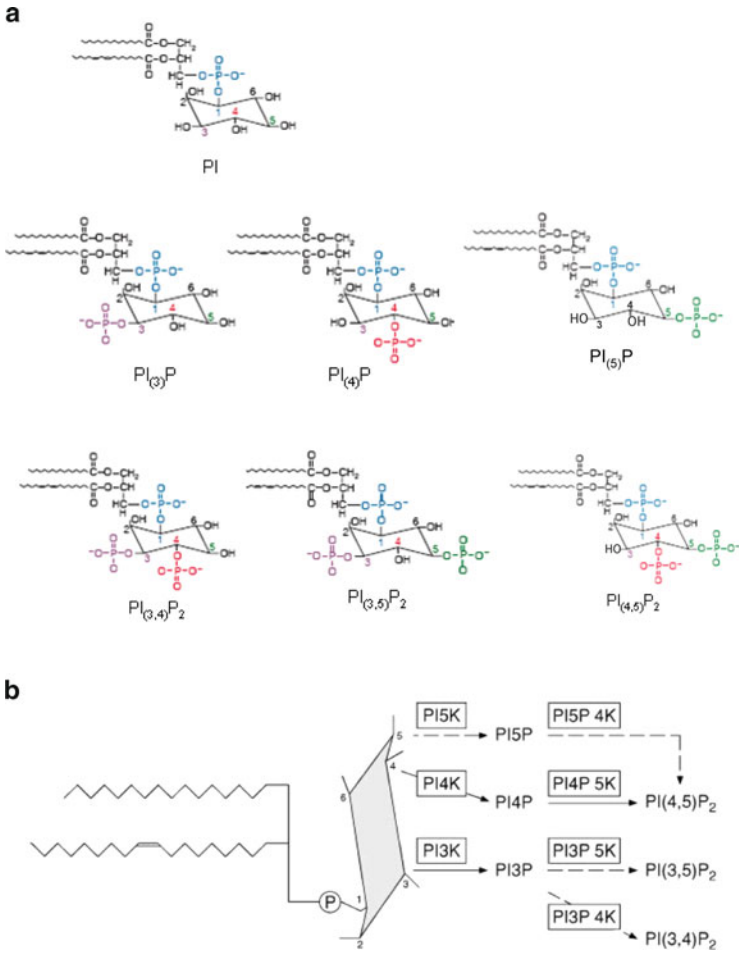

Fig. 4 (**a**) Chemical structure of the different isomers of polyphosphoinositides. (**b**) Different phosphoinositide kinases responsible for the phosphorylation of the inositol ring

PI(4)P and PI 4-Kinase

PI(4)P is the major isomer of monophosphate phosphoinositides, with up to 80% of them in *Chlamydomonas* (Meijer et al. 2001). All PI 4-kinase isoforms display a conserved catalytic domain in the C-terminus of protein. The *Arabidopsis* genome contains eight genes encoding type II PI 4-kinase (MW 55–70 kDa, membrane associated, and inhibited by calcium and adenosine) and four genes encoding putative enzymes of type III [MW 68–230 kDa, Pleckstrin domain (PH) or novel homology (NH), and an α-helix]. PI(4)P levels vary in response to elicitors, cold, sorbitol (Van deer Luit et al. 2000; Ruelland et al. 2002; Pical et al. 1999; Cho et al. 1993) and generally correlate with PI(4,5)P_2 levels, suggesting a tight control of PI pools.

PI(5)P

PI(5)P is a minor isomer (3–18% in *Chlamydomonas*), and no PI 5-kinase has been isolated to date in plants. Few data are available concerning its cellular role, albeit a transient increase is observed after an NaCl treatment of *Chlamydomonas* (Meijer et al. 2001).

PI(4,5)P_2 and PIP Kinase

Three isomers of PIP_2 are detected in plants, with PI(4,5)P_2 being by far the most abundant except in *Commelina communis*, where PI(3,4)P_2 is the major isoform (Parmar and Brearley 1993). PIP_2 is synthesized by a large set of PIP kinases which all share a conserved catalytic domain. Fifteen genes encode putative PIP kinases in the *Arabidopsis* genome. Among them, four are more similar to animal class III PI(3)P 5-kinases with a PI(3)P-binding FYVE domain and a catalytic domain at the C-terminus of proteins. The others can be classified into two subgroups, which are distinct from animal PIP kinases. Subgroup A contains an incomplete dimerization domain and a catalytic kinase domain at the C-terminus. Subgroup B displays a complete dimerization domain and eight repetitive MORN (membrane occupation and recognition nexus) motifs. To date, only *Arabidopsis* PIPK10 and PIP5K1 have been thoroughly characterized (Elge et al. 2001; Perera et al. 2005; Mikami et al. 1998), although kinase activity has been detected in PM fractions (Sandelius and Sommarin 1986). Recent evidence suggests that PI(4,5)P_2 resides in cellular pools associated with various fatty acid compositions. These pools may compartmentalize along organellar borders or in microdomains within one membrane (König et al. 2007).

Phosphatidic Acid/Diacylglycerol Pyrophosphate

Phosphatidic acid (PA) has only recently been identified as an important signaling molecule in plants and animals. In plants, its formation is transient and results from the activation of phospholipases C and D (for review Testering and Munnik 2000, 2005). More recently, a novel phospholipid, diacylglycerol pyrophosphate (DGPP), produced through phosphorylation of PA by the novel enzyme PA kinase (PAK), has been found in plants and yeast, but not in higher animals (Zalejski et al. 2005, 2006). No gene encoding a PA kinase has been yet characterized in any organism. PA and DGPP have emerged as second messengers in plant signaling (see the chapter “Plasma Membrane and Abiotic Stress” by Barkla and Pantoja).

1.2.2 Sphingolipids

Sphingolipids consist of a long-chain base (LCB) amidated by a fatty acid and a polar head attached to the alcohol residue of the LCB. There are over 500 different

molecular species of sphingolipids in plant cells (Pata et al. 2010). Greater LCB diversity is seen in plants than in yeast and animals: up to eight different molecular moieties can be formed from the most basic LCB sphinganine which has an acyl chain of 18 carbon atoms with two alcohol residues at C1 and C3 and an amine residue at C2 (Fig. 5). Sphinganine is further modified by hydroxylation at carbon 4 and/or desaturation at C4 or C8. In the latter case, *cis* (*Z*) and *trans* (*E*) isomers are formed. In plants, four LCBs are used to form complex sphingolipids, namely, (*E*)-sphing-8-enine (d18:18*E*), 4*E*,8*E*-sphinga-4,8-dienine (d18:24*E*,8*E*), (4*E*,8*Z*)-sphinga-4,8-dienine (d18:24*E*,8*Z*), and (*E*)-4-hydroxysphing-8-enine (t18:18*E*) (reviewed in Sperling and Heinz 2003; Pata et al. 2010) (Fig. 5), whereas animals and yeast used (*E*)-sphing-4-enine (d18:14*E*) and 4-hydroxysphinganine (t18:0),

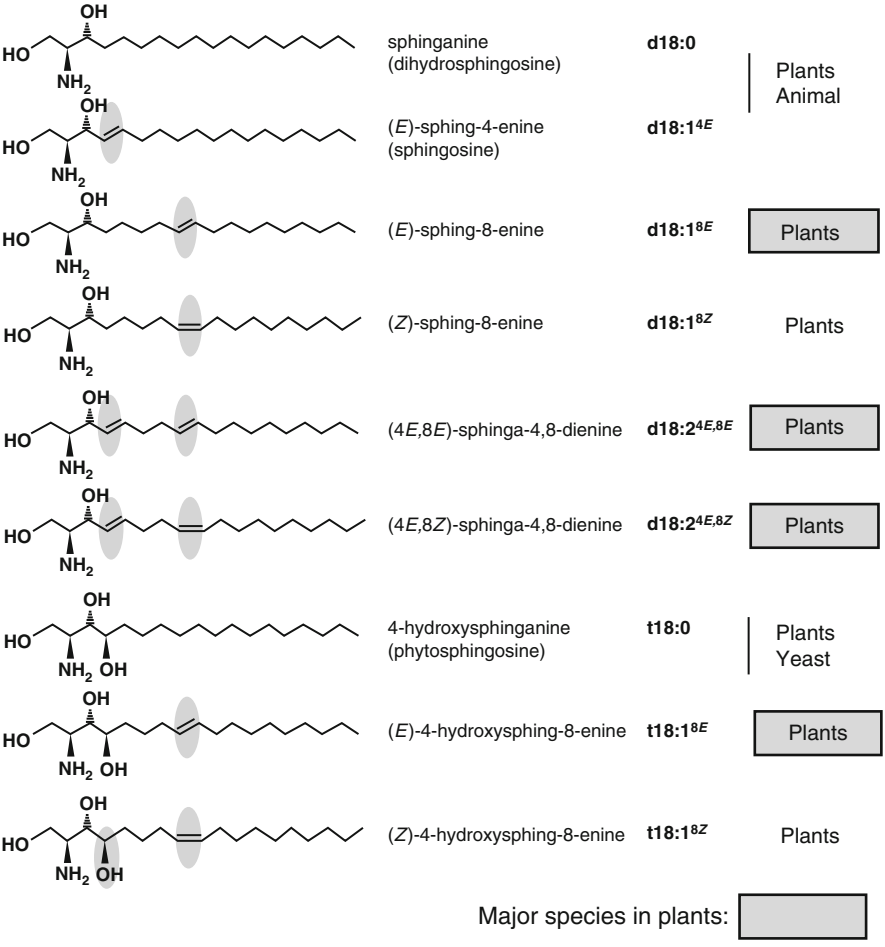

Fig. 5 Structures of long-chain bases (LCB) from animal, yeast, and plants (trivial names are given in *parenthesis*)

respectively. Acyl chains amidated to plant LCBs vary from 16 to 26 carbon atoms. They can be α-hydroxylated and are generally saturated.

Depending on the chemical nature of the polar head at C1 position of the LCB, two types of complex sphingolipids can be obtained: the cerebrosides with one or more glycosyl residues and the inositol phosphorylceramides (IPCs), which are formed by the addition of an inositol monophosphate group. IPC can be further polyglycosylated to form glycosyl inositol phosphorylceramides (GIPCs), previously called "phytoglycolipid" (Carter et al. 1958). One must emphasize that GIPCs are atypical lipids because they are insoluble in chloroform/methanol, a solvent system generally used to extract a broad spectrum of biological lipids. For this reason, GIPCs were long considered as minor plant sphingolipids. However, by using a single-solvent system with reversed-phase high-performance liquid chromatography coupled to electrospray ionization tandem mass spectrometry detection, the complex mixture of plant sphingolipids has been recently deciphered (Markham and Jaworski 2007; Markham et al. 2006), and it now appears that GIPCs are the most abundant sphingolipids in *Arabidopsis thaliana* leaves. Indeed, hexosehexuronic-inositolphosphoceramides (GIPCs), monohexosylceramides (GluCers), and ceramides accounted for ca. 64%, 34%, and 2% of the total sphingolipids, respectively. Extraction and separation of sphingolipids from soybean and tomato showed that the neutral sphingolipids consisted of ceramide and monohexosylceramides; however, the major polar sphingolipid was found to be *N*-acetyl-hexosaminehexuronic-inositolphosphoceramide (Markham et al. 2006; Markham and Jaworski 2007). GIPC family can reach a great complexity and up to 13 different polar heads have been described (for review Pata et al. 2010). Sphingolipids are mainly associated with the PM (Sperling and Heinz 2003) although there is no report of the exact sphingolipid composition of the plant PM per se. Other minor sphingolipids, such as free long-chain bases and their phosphorylated derivates, have been detected and are likely to be involved in signaling (see the chapter "Plasma Membrane and Abiotic Stress" by Barkla and Pantoja).

1.2.3 Plant Sterols

Sterols are isoprenoids formed by a cylcopentaperhydrophenantren moiety, made up of four rigid rings, that is hydroxylated at position 3 (Fig. 2). Animal and yeast membranes incorporate only one sterol (cholesterol and ergosterol, respectively), but plant membranes contain multiple sterol species. Phytosterols differ by the number and the position of double bonds in the cycle, and by the nature (branched vs. linear, saturated vs. unsaturated) of the lateral chain inserted at the C17 position (reviewed in Hartmann and Benveniste 1987; Mongrand et al. 2004; Lefebvre et al. 2007; Palta et al. 1993). Phytosterols are derived from cycloartenol, a polycyclic sterol intermediate unique to plants, which is further alkylated at the C24 position to produce a triene. A C14 sterol reductase and an 8-7 isomerase act on the triene to yield 24-methylenelophenol, a branch point in the pathway. Parallel pathways lead to the synthesis of membrane sterols, sitosterol, and campesterol. Campesterol also

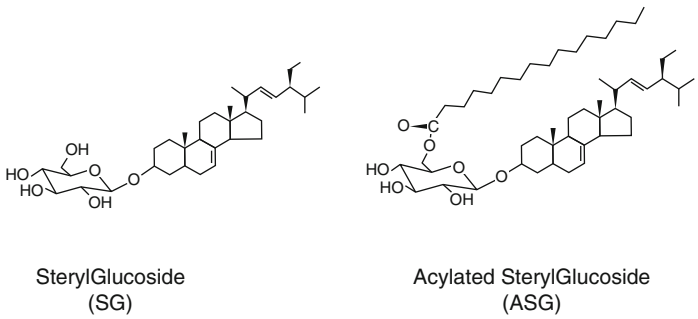

Fig. 6 Structure of conjugated phytosterols found in plant plasma membrane

serves as the progenitor of brassinosteroid hormones that are required for normal plant development.

Phytosterols can be acylated by a sugar (primarily glucose, but also mannose, xylose, and galactose) to form steryl glycosides (SGs), which can be further acylated to form acylated steryl glycosides (ASGs, Fig. 6). In the Solanaceae, the acyl chain is mainly palmitic acid (65%), but linoleic acid can also be detected. Steryl conjugates are also found in some bacteria such as *Helicobacter* (Haque et al. 1995) and *Spiroplasma* (Patel et al. 1978). Free sterols esterified with fatty acids are also detected in plant cells but are rarely constituents of the PM, except in cauliflower (Table 4). Esterified sterols are generally present in cytoplasmic oil bodies.

Free sterols generally represent 70–90% of total sterols in the PM of most plant species and across tissue types (Table 4). For example, in oat roots and coleoptyles, free sterols are predominant, whereas in leaves ASGs represent the major form (Table 4). Free sterols are synthesized in the ER and then transported to the PM by the secretory pathway. Sterol glycosylation to produce SG followed by acylation to form ASG occurs in the PM (Hartmann and Benveniste 1987).

Biochemical analysis coupled with forward and reverse genetic studies of sterol-deficient mutants in *Arabidopsis* revealed that sterols are essential for normal plant development, particularly embryogenesis, cell elongation, and vascular differentiation (reviewed in Clouse 2002). These developmental effects are independent of the conversion of sterol in the brasinosteroid phytohormones (Clouse 2002). The molecular mechanisms underlying these pleiotropic developmental effects are still poorly understood.

Sterols are crucial structural components of membranes because their insertion in the bilayer is known to regulate lipid chain order and modify the thermotropic phase transition between *liquid-disordered* and *solid-ordered* phases by inducing an intermediate *liquid-ordered* phase. This liquid-ordered phase combines a high rotational or translational mobility and a high conformational order in the lipid acyl chain. Sterols therefore increase the bilayer permeability and optimize the mechanical properties of the PM while maintaining its liquid form. The composition of sterol mixtures in the plant PM suggests that sterols regulate the structural and

Table 4 The sterol family in plant plasma membrane, expressed as total sterol content

	Vigna radiata hypocotyle	Seigle leaves	Seigle leaves	Oat coleoptyle	Oat roots	Oat roots	Spring oat leaves	Winter oat leaves	*Zea mays* coleoptyles	*Zea mays* roots	*Solanum tuberosum* leaves	*Solanum commersonii* leaves	Cauliflower bud
Free sterols	91.2	62.8	81.8	60.2	62	73.5	20.3	26.1	89	82.7	9.3	4.1	27
SG	5.3	29	12	22.7	26.7	1	13.6	9.5	3	11.8	19.7	17.9	18.9
ASG	3.5	8.2	6.2	17.1	11.3	26.6	66.1	64.4	8	5.5	71	78	32.5
Esterified sterol	–	–	–	–	–	1.5	–	–	–	–	–	–	21.6
References	Yoshida and Uemura (1986)	Lynch and Steponkus (1987)	Uemura and Steponkus (1994)	Sandstrom and Cleland (1989)	Sandstrom and Cleland (1989)	Norberg and Liljenberg (1991)	Uemura and Steponkus (1994)	Uemura and Steponkus (1994)	Hartmann and Benveniste (1987)	Bohn et al. (2007)	Palta et al. (1993)	Palta et al. (1993)	Wright et al. (1982)

SG steryl glycosides, *ASG* acylated steryl glycosides

functional properties of PM, particularly when adapting to large temperature variations (Beck et al. 2007). The role of free and conjugated sterols in structuring membrane microdomains is discussed below.

Concerning sterol conjugates, their role in plant biology is largely unknown. SG was proposed to participate in cellulose synthesis to produce cellobioside homologs with β-1,4-linked glucosyl residues. The resulting disaccharide is split off and used as primer for further elongation to cellulose (Peng et al. 2002). Recently, reverse genetic studies were carried out on two genes (*UGT80A2, UGT80B1*) that encode UDP-glucose:sterol glycosyltransferases, enzymes that catalyze the synthesis of SGs. Although mutant lines in ugt80A2 exhibit no phenotype, mutant in ugt80B1 displayed an array of pronounced phenotypes in embryo and seed, such as a transparent testa phenotype and a reduction in seed size. In addition, analysis of this mutant showed that the outer integument of the seed coat lost the dense cuticle layer at its surface and displayed altered cell morphology, which was confirmed by a decrease in suberin and cutin-like polymers. These findings suggest a membrane function for conjugated phytosterol in trafficking of lipid polyester precursors. One must note that the cellulose biosynthesis was unaffected in the double mutant, which is inconsistent with a predicted role for SG in priming cellulose synthesis (Peng et al. 2002). The exact function of SG and ASG in structuring the plant PM has not been elucidated.

1.3 Asymmetrical Distribution of Lipids Across the Plasma Membrane

In animal cells, it has been recognized that the asymmetrical distribution of PM lipids between the cytosolic and apoplastic leaflets also plays important roles for membrane functions. For instance, alteration of lipid asymmetry can affect cell fusion, activation of coagulation, and recognition and removal of apoptotic cell by macrophages (for review Fadeel and Xue 2009). Besides, asymmetric transversal distribution of lipids is required for maintaining a low permeability to solutes (Hill et al. 1999).

Only recently, researchers investigated the asymmetrical distribution of PM lipids in plants and focused their studies on the context of phosphate deprivation, where phospholipids are replaced by a galactolipid called DGDG (Tjellstrom et al. 2008). In normal culture condition, a clear asymmetry in PM phospholipids, free and conjugated sterols, and the glucosylceramide exists between the cytosolic and apoplastic leaflets with a molar ratio of 65:35, 30:70, and 30:70, respectively (Tjellstrom et al. 2010). The phospholipid-to-DGDG replacement after phosphate deprivation almost exclusively occurred in the cytosolic leaflet, where DGDG accounted for one-third of the lipids. In the apoplastic leaflet, phospholipids were not replaced by DGDG but by ASG.

2 Evidence for Membrane Domains in the Plant Plasma Membrane

2.1 Organization of Lipids Within the Plasma Membrane: The Concept of Membrane Rafts

Cells determine the bilayer characteristics of different membranes by tightly controlling their lipid and protein compositions. Local changes in the physical properties of lipid bilayers allow membrane deformation and facilitate vesicle budding and fusion. Moreover, localized lipid speciation results in recruitment of cytosolic proteins involved in structural functions and/or signal transduction. The molecular shape of lipids and their ability to interact with proteins and other lipids determine the physical properties of membranes. These interactions are the basis of lipid domain formation. Therefore, biological membranes are better regarded as composites of discrete, ordered islands rather than homogeneous bilayers composed of lipids and proteins. Such domains allow the segregation of active membrane components involved in signaling, cell adhesion, and membrane trafficking (reviewed in Rajendran and Simons 2005).

Over the last 10 years, characterization of membrane domains or "lipid rafts" has advanced rapidly as a result of cooperative efforts of biologists, biochemists, and biophysicists. Rafts are mainly composed of sphingolipids packed together with sterols (Fig. 7a), although the existence of other lipid-based microdomains has also been proposed (Simons and Ikonen 1997; Brown and London 1998; Kurzchalia and Parton 1999). Lipid-to-lipid interactions appear to be the primary force that enables raft formation. Models derived from biophysical studies suggest that sphingolipids are able to associate with each other through interactions between their polar head groups and their long and mostly saturated acyl chains, whereas sterols fill voids between the sphingolipids (Simons and Ikonen 1997). Sterols and sphingolipids containing saturated hydrocarbon chains are then thought to form tightly packed subdomains in a *liquid-ordered* phase. Rafts are thought to be surrounded by a more fluid *liquid-disordered* phase constituted of unsaturated phospholipids (Fig. 7b).

Biochemical characterization of lipid rafts has generally relied on their insolubility in nonionic detergents at cold temperature and has, thus, engendered the use of the terms detergent-insoluble membranes (DIMs) or detergent-resistant membranes (DRMs). Different nonionic detergents can be used to isolate these fractions, such as Tween 20, Brij 58, 96, or 98, Lubrol WX, CHAPS, and Triton X-100 (Schuck et al. 2003). Of these, Triton X-100 is by far the most common. Sonication of membrane preparations to purify rafts has also been described in plants (Peskan et al. 2000).

Rafts have been extensively studied because they can potentially support the specificity, selectivity, and rapidity of biochemical processes associated with membranes that cannot be readily explained by other models. A consensus view is that proteins and lipids contribute to the formation and stability of small

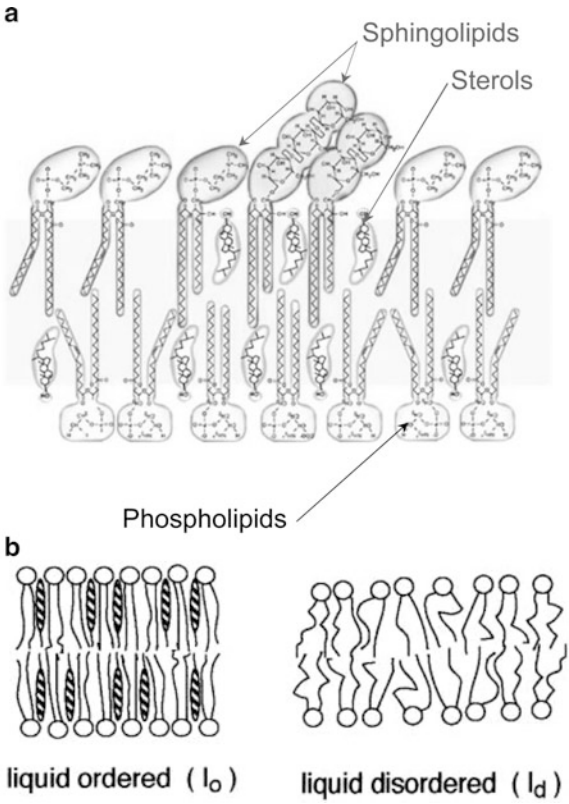

Fig. 7 (**a**) Model of organization of lipids in membrane rafts. (**b**) The fatty acid chains of sphingolipids within the rafts together with sterol (*hatched*), tend to be extended and so more tightly packed, creating domains with higher order, called liquid ordered (l_o). Other lipids – mostly phospholipids – with unsaturated acyl chains formed a liquid-disordered phase (l_d)

(10–200 nm), heterogeneous, highly dynamic, sterol- and sphingolipid-enriched membrane domains that compartmentalize cellular processes and that these domains should be referred to as "membrane rafts" (Pike 2006). Small rafts can sometimes be stabilized to form larger platforms through protein–protein and protein–lipid interactions (Simons and Toomre 2000). This organizational hierarchy of lipid and protein constituents in the PM mediates many life processes involving perception and early transduction of external stimuli. These processes allow the adaptation of the organism to cope with pathogen threats and adverse environmental conditions. Animal membrane rafts have been shown to recruit and/or exclude enzymes and lipids involved in the transduction of stimuli and function as part of the platform controlling the response (reviewed in Rajendran and Simons 2005). In animal, PM rafts have also been suggested to act as portals for pathogen entry and selective import of macromolecules into host cells and their subsequent translocation to various subcellular sites (Riethmüller et al. 2006).

2.2 *Characterization of Detergent-Insoluble Plasma Membranes from Plants*

Biochemical characterizations have led to the identification of lipid and protein components of plant DIMs. First, it is important to emphasize that the lipid-to-protein ratio of DIMs extracted from animal membrane is much higher than that of DIMs extracted from plant membranes (Mongrand et al. 2004; Borner et al. 2005). This can probably account for the higher density of plant DIMs after centrifugation. Free phytosterols, conjugated sterols, and sphingolipids were found to be enriched in plant DIMs and constitute up to 80% of total raft lipids. An exhaustive analysis of the lipid composition of plant membrane rafts is yet to be published.

2.2.1 Sterols

The sterol composition of DIMs has been thoroughly characterized (Mongrand et al. 2004; Borner et al. 2005; Lefebvre et al. 2007; Roche et al. 2008). The proportions of free sterols and sterol conjugates were similar to those of the PM, indicating that no selective sorting occurred. The presence of saturated fatty acyl chains (C16:0 and C18:0) in ASG (Lefebvre et al. 2007) is consistent with the hypothesis that lipid domains are closely packed with sterols and saturated lipids. SG molecules are likely oriented in membranes with the sterol moiety embedded in the hydrophobic phase of the bilayer and the sugar in the plane of the polar head groups of phospholipids. The sterol and the fatty acyl chain moieties of ASGs are probably inside the bilayer with the sugar group appressing the hydrophilic surface. The occurrence of SG and ASG in plant membrane rafts has been suggested to modulate the activity of raft-associated H^+-ATPases (Mongrand et al. 2004; Bhat and Panstruga 2005) as they do in the case of tonoplast ATPase (Yamaguchi and Kasamo 2001).

The role of phytosterols in the lateral structuring of the PM of higher plant cells has been recently reported (Roche et al. 2008). The cyclic oligosaccharide methyl-β-cyclodextrin (MCD), a tool commonly used in animal cells to decrease cholesterol levels, was used to treat PMs of tobacco BY-2 cells. A drastic reduction (50%) in the PM total free sterol content of the plant material was observed without modification in amounts of steryl conjugates. Fluorescence spectroscopy experiments using DPH, TMA-DPH, Laurdan, and di-4-ANEPPDHQ indicated that such a depletion in sterol content increased lipid acyl chain disorder and reduced the overall liquid-phase heterogeneity in correlation with the disruption of phytosterol-rich domains. MCD also prevented isolation of a PM fraction resistant to solubilization by nonionic detergents. These results suggest that free sterols are key compounds for the formation of plant PM microdomains (Roche et al. 2008). Physical tools to study the kinetics of ordering in biological membranes are still being developed.

Model membranes with compositions approaching those found in plant membrane rafts have been investigated using solid-state ^{2}H-NMR and deuterated

dipalmitoylphosphatidylcholine. A liquid-ordered phase, which may be an indicator of rigid sterol-sphingolipid domains, was detected, but the dynamics of ternary mixtures mimicking plant rafts showed a lesser sensitivity to thermal shocks when compared with rafts mimicking mammals or fungi (Beck et al. 2007).

Laloi et al. (2007) investigated the role of sterols in DIM formation along the plant secretory pathway. DIMs were detected in the PM and Golgi apparatus but not in the endoplasmic reticulum. After treatment of leek seedlings with fenpropimorph, a sterol biosynthesis inhibitor, sterols, and GluCer do not reach the PM, and most DIMs were recovered from the Golgi apparatus after treatment. These results suggest that sterols and sphingolipids play a crucial role in lipid microdomain formation and delivery to the PM. This study also combined DIM purification procedures with genetic approaches using *Arabidopsis* cell lines mutant fad2 and overexpresser FAD3, hyper-accumulating 18:1 and 18:3 fatty acids, respectively. The amount of DIM recovered from the mutant was 20% of the wild-type levels, underlining the expected importance of the degree of fatty acid unsaturation in the formation of plant rafts.

2.2.2 Sphingolipids

Because of the complex range of polarity existing in plant sphingolipids, authors adopted an indirect measurement of total sphingolipids based on the levels of sphingoid LCB. Analyses of LCB composition of DIM indicate a six- to sevenfold enrichment when compared with PM from which they originate (Borner et al. 2005; Lefebvre et al. 2007). In *Arabidopsis*, using the ratio of *cis*:*trans* t18:1 LCBs as an estimation of the relative levels of sphingolipids (Sperling et al. 2005), Borner et al. (2005) concluded that GIPCs are likely to be the primary sphingolipids present in DIM. In *Medicago* root PM, although the levels of sphingolipids were increased in the DIM fraction, no significant change in LCB profile was observed. In particular, the *cis*:*trans* ratio of t18:1 was unaltered, with the *trans*-form being the predominant stereoisomer. Clearly, a detailed characterization of sphingolipids at the level of the polar head in plant DIM is still needed.

2.2.3 Phospholipids

Phospho- and glycoglycerolipids of the PM were largely excluded from DIMs (Mongrand et al. 2004; Lefebvre et al. 2007). In good agreement with biophysical data, glycerolipids of DIM contained more saturated fatty acids than their PM counterpart, such as 16:0, 18:0, and 20:0, which would contribute to the higher rigidity of the liquid-ordered phase of membrane rafts.

Our group also analyzed the lipid composition of DIMs purified from tobacco PM focusing on polyphosphoinositides, lipids known to be involved in various signal transduction events. Polyphosphoinositides [mainly PI4P and PI(4, 5)P_2]

were enriched in DIMs compared with whole PM, and up to half of the polyphosphoinositides present in PM are located in DIM. Here again the fatty acid composition analyses suggest that enrichment of polyphosphoinositides in DIMs is accompanied by their association with more saturated fatty acids (Furt et al. 2010). Interestingly, the pattern of fatty acids associated with the precursor of polyphosphoinositides, PI, is less saturated, suggesting that a specificity of lipid kinase exists for the synthesis of polyphosphoinositide, according to acyl chain content of PI (König et al. 2007). The activities of various enzymes involved in polyphosphoinositide metabolism were measured in DIMs and PM. Data showed that these activities are present in the DIM fraction but not enriched, suggesting that polyphosphoinositides are synthesized outside the raft and distribute between rafts and non-rafts afterward, based on their acyl composition and the ensuing biophysical properties of the lipids themselves.

2.2.4 Protein Content

Proteomic studies have shown that plant DIMs are able to recruit a specific subset of plasma membrane proteins while excluding others (Shahollari et al. 2004; Morel et al. 2006; Borner et al. 2005; Lefebvre et al. 2007). In particular, the proteome of tobacco cell DIMs seems to be enriched in proteins involved in signaling and environmental stress response, suggesting that plant microdomains could function as signaling platforms as is the case in animals (Morel et al. 2006). Kierszniowska et al. (2008) used an N^{14}/N^{15} quantitative proteomic approach to distinguish between true sterol-dependent plant raft proteins, sterol-independent non-raft proteins, and co-purifying contaminants in DIMs. Predominantly, proteins with signaling functions, such as receptor kinases, G-proteins, and calcium signaling proteins were identified as variable members in plant lipid rafts depending on the physiological status of the cells (e.g., stimuli), whereas cell-wall-related proteins and specific proteins with unknown functions make up a core set of sterol-dependent plant plasma membrane proteins.

Differential posttranslational protein modification may function in partitioning of proteins within the different regions of the PM. Analyses of posttranslational modifications of plant DIM proteins showed that very few posses or are predicted to have GPI anchors, whereas many are predicted to be palmitoylated and/or myristoylated (Morel et al. 2006). When fluorescence resonance energy transfer (FRET) microscopy using lipidated green fluorescent protein (GFP)-fusion proteins coexpressed in cowpea protoplasts was used to study plant plasma membrane organization, the hypervariable region of the ROP7 small maize GTPase and the N-myristoylation motif of the calcium-dependent protein kinase 1 (CPK1) of tomato were both localized to DIMs (Vermeer et al. 2004). These results suggest the clustering of lipid-modified proteins in PM microdomains.

2.3 Visualization of Rafts in Plant Plasma Membrane

Correlations between detergent insolubility and clustering in membrane domains have been extensively studied in animal cells using imaging technologies ranging from atomic force microscopy to immunogold labeling (reviewed in Jacobson et al. 2007). Using an immunogold electron microscopy strategy coupled to statistical point pattern analysis, Raffaele et al. (2009) showed that a group of proteins specific to vascular plants, called remorins (REMs), almost exclusively present in DIM fraction, clustered into microdomains of approximately 70 nm in diameter in the PM in tobacco, providing a link between biochemistry (DIM purification) and imaging (membrane microdomain observation). Importantly, the cluster formation of REM was sensitive to sterol depletion induced by MCD, linking phytosterol with PM domain formation in plant PM as described in Roche et al. (2008) with biophysical tools.

Using the same kind of approach, Furt et al. 2010 were also able to visualize nanodomains of DIM-enriched lipid PI(4,5)P_2 in the plane of the PM, with 60% of the epitope clustered in 25-nm-diameter domain and 40% randomly distributed at the surface of the PM. Surprisingly, the lipid PI(4,5)P_2 cluster formation was not significantly sensitive to sterol depletion induced by MCD, likely reflecting another kind of interaction between polyphosphoinositides and raft lipids and proteins perhaps through the polar head. Existence of PI(4,5)P_2 domain in plant PM suggests that, in addition to their classical roles as second messengers in signal transduction, phosphorylated products of PI could play critical roles in the regulation of membrane trafficking as regulators for the recruitment or the activation of proteins essential for cytoskeletal dynamics or vesicular transport. Indeed, local changes in the concentration of polyphosphoinositides, and in particular of PI(4,5)P_2, within the PM could also represent cell signals.

2.4 Putative Roles of Plasma Membrane Raft in Plant Biology

In animal, membrane rafts are subdomains of the PM that are hypothesized to play important roles in a variety of biological functions by coordinating and compartmentalizing diverse sets of proteins to facilitate signal transduction mechanisms, regulation of cytoskeleton, and membrane trafficking (for review Rajendran and Simons 2005).

Only few studies addressed the question of rafts as putative lipid signaling platforms in plants. Mongrand et al. (2004) suggested the involvement of membrane rafts in signaling and plant–pathogen interactions by demonstrating the presence of NADPH oxidase, enzyme responsible for ROS formation, in tobacco cell membrane rafts after elicitation by cryptogein to mimic defense reaction. Recently, a global quantitative proteomics approach, using N^{14}/N^{15} metabolic labeling, was used to compare the protein content of tobacco DIM extracted from

tobacco cells treated or not with cryptogein. The results obtained indicate that, although the association of most proteins with DIM remained unchanged upon cryptogein treatment, five proteins had their relative abundance modified (Stanislas et al. 2009). Four dynamins related to cell trafficking were less abundant in the DIM fraction after cryptogein treatment, whereas one 14-3-3 protein was enriched. This analysis suggests that microdomains play a role in the early signaling process underlying the setup of defense reaction in plant PM which involved signaling per se and vesicular trafficking as already observed in similar studies performed in animal cells upon biological stimuli. The ways the dynamic association of proteins with microdomains could participate in the regulation of signaling process may be therefore conserved between plants and animals.

The auxin efflux transporter, PIN1, localized in the basal pole of plant cells, has also been found in DIMs characterized by the ABC auxin transporter ABCB19 (Titapiwatanakun et al. 2008), reinforcing the idea that a microsegregation at the level of the membrane may lead to the formation of macrodomains at one pole of the cell. Indeed, this study indicates that ABCB19 stably located in PM microdomains recruits PIN1 to raft, subsequently enhancing its auxin transport activity. These results suggest that rafts may not only recruit proteins but help constitute rafts, as it is apparently the case with ABCB19 (Titapiwatanakun et al. 2008) and some mammalian ABC transporters. The fact that MCD was required to release ABCB19 from *Arabidopsis* membranes suggests a crucial role of sterol in this process. Sterol mutant analyses such as sterol methyltransferase1 (smt1) and cpi1-1 mutant are interesting because the polar localization of some PIN proteins is altered. For example, the apical localization of PIN2 is altered in cpi1-1 root epidermal cells, where PIN2 can be found at both apical and basal membranes.

Analysis of the root hair morphogenesis transcriptome from *Arabidopsis* suggests that GPI-anchored proteins associated with membrane rafts may also function in polarized root hair growth (Jones et al. 2006). Several genes previously shown to be required for root hair development also encode proteins associated with membrane rafts (Jones et al. 2006).

Finally, it was recently reported that rafts are functional components of the PM within plasmodesmata (PD) (Raffaele et al. 2009). To overcome the cell wall barrier, plants have developed these unique channel-like structures that connect neighboring cells together allowing exchange of informational molecules. Despite 40 years of extensive research, the molecular composition of these membranous channels has remained rather elusive. The role of lipids in PD function has been so far largely under considered if considered at all. Strikingly though, recent data suggest that the PM lining PD has properties similar to membrane rafts. Experimental arguments are the following: (1) proteomic analyses have revealed the presence of GPI proteins (callose binding protein; β-1-3 glucanase) in the PD fraction, a type of lipid anchor known to have a high affinity for membrane rafts (Simpson et al. 2009; Levy et al. 2007); (2) remorin, the first identified marker of plant membrane rafts, also accumulates at PD, where it seems to counteract viral movement from cell to cell (Raffaele et al. 2009); (3) the transmembrane domain of PDLP1 alone is sufficient to drive the accumulation of YFP at PD (Thomas et al. 2008).

Lipid–protein interaction and thereby the raft lipid environment of PD is likely to be crucial for protein targeting. Altogether, these data open up new perspective about PD not only in terms of biogenesis but also for the role of raft in PD function in cell-to-cell communication and virus propagation.

3 Conclusions

A diverse range of PM-associated lipids contributes substantially to cellular mechanisms that depend on membrane signaling events. Meaningful coordination of these events requires exquisite spatial and temporal control of lipid metabolism and organization, as well as reliable mechanisms for specifically coupling these parameters to dedicated physiological processes. Lipids are not homogeneously distributed along the PM and other membrane systems. Rather they are dynamically organized into domains that impose spatial and temporal regulation on interfacial protein binding and/or enzymatic reactions. Moreover, it is becoming increasingly apparent that populations of lipid molecules are functionally segregated from others of the same species with respect to their involvement in specific activities. The next scientific challenge is the exploration of lipid fluctuations in response to physiological and genetic changes and their impact on protein localization and function. It will also be important to assess the impact of these changes on lipid-modifying enzymes and their segregation on the PM during the very early steps of the adaptative response of the cell. Altogether, these data give an integrative picture of the dynamic processes by which PM lipids control cell physiology.

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Plasma Membrane Protein Trafficking

Wendy Ann Peer

Abstract The plasma membrane is the interface between the cytosol and the external environment. The proteins that reside and function on the plasma membrane regulate the cellular entrance and exit of bioactive molecules, actuate signaling cascades in response to external stimuli, and potentiate interactions between cells. The presence and abundance of proteins on the plasma membrane is regulated by anterograde and retrograde intracellular vesicular trafficking, exocytosis, and endocytosis. The cytoskeleton is an integral component of cellular trafficking mechanisms, as the vesicles and endosomes move on actin filaments or microtubules. Selection and movement of the protein cargo to be trafficked to and from the plasma membrane depends to a great extent on signature organellar targeting motifs within the proteins themselves as well as interactions with various adaptor proteins. Endocytosis is essential not only to the recycling/turnover of plasma membrane proteins, but it also functions in dynamic processes that recycle proteins back to the plasma membrane. Some evidence suggests that transcytotic trafficking mechanisms function in plants, although these are distinct from basolateral – apical redirection mechanisms characterized in animal cells.

1 Types of Trafficking at the Plasma Membrane

Protein trafficking at the plasma membrane (PM) involves (1) secretion of proteins to the PM or to the apoplast (exocytosis, secretion, or anterograde trafficking), (2) uptake of proteins at the PM for recycling or regulation of their activity (endocytosis or retrograde trafficking), and (3) moving proteins from one location on the PM to another (transcytosis). The integration of these processes is required for dynamic

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maintenance of cellular homeostasis following biotic or abiotic stimuli, such as herbivory, fungal infection, drought stress, or gravitropic or phototropic stimuli.

Secretion is the process by which proteins, lipids, and other molecules are trafficked, usually though the endoplasmic reticulum and Golgi-apparatus, and targeted to the plasma membrane (PM), extracellular space, or other organelles in the cytosol (Fig. 1). Endocytosis is the process by which nutrients, sterols, lipoproteins, peptide hormones, growth factors, and receptor-binding toxins are taken into cells. Endocytosis regulates the abundance and distribution of PM transport and receptor proteins, and it is an important mechanistic component of degradative and recycling mechanisms resulting in reuse of expensive transmembrane proteins and organelle homeostasis (Samaj et al. 2004). Transcytosis is well documented in animal systems where proteins undergo transcystic mediation of apical to basolateral redirection. Animals have gap junctions, which define the polarity of cells. Although the structural/mechanical basis of cellular polarity has not been elucidated in plants, transcytosis has been documented in plants cells: during embryogenesis, the PIN1 auxin efflux carrier is translocated from opposite sides of the cell via a guanine-nucleotide exchange factor for ADP-ribosylation factor GTPase-dependent transcytosis-like mechanism (Kleine-Vehn et al. 2008a).

Proteins that are targeted to or function at the PM are integral or peripheral membrane proteins. Integral membrane proteins have a membrane spanning helix

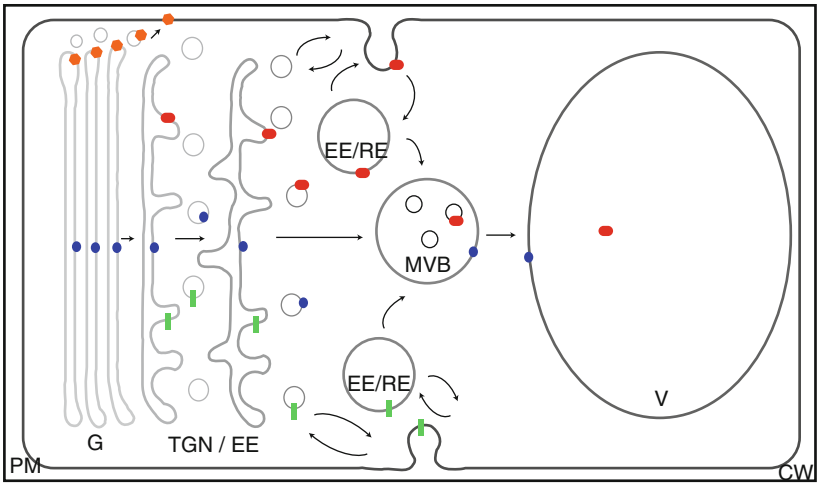

Fig. 1 Overview of trafficking pathway: ER to Golgi to TGN to PM; ER to PM. (**a**) simplified view of trafficking of proteins through the endomembrane system to and from the plasma membrane. *Orange polygons* show Golgi to PM traffic. *Blue circles* show Golgi to tonoplast (vacuolar membrane) traffic through the endomembrane system. *Red ovals* show a PM protein that is undergoing turnover in the vacuole. *Green rectangles* show a protein that is constitutively recycled. *G* Golgi, *TGN* trans-Golgi network, *PM* plasma membrane, *EE* early endosome, *RE* recycling endosome, *MVB* multivesicular body (prevacuolar compartment), *V* vacuole, *CW* cell wall. The nucleus, mitochondria, plastids, actin filaments, microtubules and endoplasmic reticulum have been omitted for clarity. *Arrows* indicate the direction of movement

or helices that anchor them to the PM, while peripheral membrane proteins are associated with the membrane through hydrophobic regions of the protein and may or may not be associated with integral membrane proteins. Initially, a protein that is targeted to the PM is synthesized on the rough endoplasmic reticulum (ER) (Fig. 1). From there, the protein may be trafficked to the *cis*-Golgi via COPII-coated vesicles (type I protein) or may be directly targeted to the PM (type II protein). From the *cis*-Golgi, the protein may be trafficked back to the rough ER via COPI-coated vesicles, or may continue through to the *medial*- and *trans*-Golgi compartments. If the protein requires modification for proper function, such as glycosylation by the glycosyl transferases resident in the Golgi stacks, then the protein may undergo multiple rounds of trafficking among the Golgi stacks via COPI-coated vesicles. From the *trans*-Golgi, the protein then traffics through the *trans*-Golgi network (TGN) via clathrin-coated vesicles (CCVs). From there, the TGN/early endosome compartment, the protein may traffic to another compartment or organelle or to the PM. In plants, the TGN and early endosomes (EE) appear to be one compartment in contrast to animals where discrete compartments (identified via markers) have been visualized (Lam et al. 2007, 2009). The TGN/EE compartment is the intersection of the secretory and endocytosis pathways, with proteins from the *trans*-Golgi, PM, prevacuolar compartment, and multivesicular bodies (Lam et al. 2007, 2009). Once at the PM, the protein may remain there or be trafficked back to the TGN/EE compartment through endocytosis via CCVs or receptor-mediated endocytosis. Once there, the protein may be sorted to return to the PM or be targeted to another compartment or organelle. The vesicles move on actin filaments or microtubules (composed of tubulin), which are protein scaffolds of cellular structure.

2 Exocytosis/Secretion/Anterograde Trafficking

Exocytosis has several synonyms including secretion and anterograde trafficking. Proteins that are targeted to the PM include peripheral membrane proteins, such as ADP-ribosylation factor Ras GTPases (ARFs), and integral membrane proteins such as transporters, symporters, and channels. The residence time of the proteins on the PM in living cells may be transitory (e.g., ARFs) to semipermanent for the functional lifetime of the protein (e.g., PM H^+ ATPase). Exocytosis is required for the formation of the cell plates in newly divided cells and also for the polar growth of the tips of root hairs and pollen tubes. Secreted proteins and peptides are destined for the apoplast, such as cell wall remodeling enzymes or rhizosphere (reviewed in Mathesius 2009).

In order for the protein to traffic to the PM, the protein must be associated with a vesicle. There are several types of vesicles, including CCVs and exocysts, which have unique protein coats. Vesicle formation usually requires the adaptor protein (AP) complex to form CCVs, while vesicle fusion requires the soluble *N*-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) complex. Both vesicle

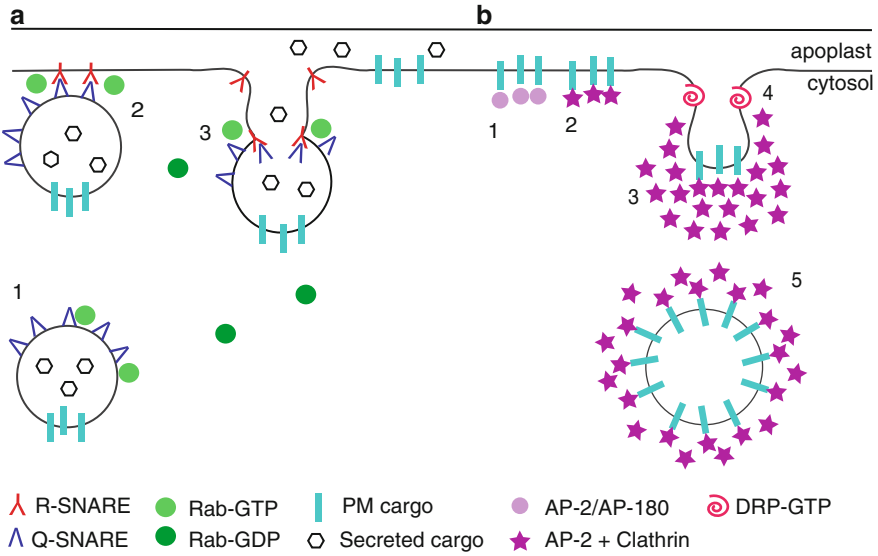

Fig. 2 Vesicle fusion and formation. (**a**) An example of vesicle fusion with the PM. Vesicle fusion requires the soluble *N*-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) complex. In this example, the Q-SNARE on the vesicle is recognized by the R-SNARE on the PM (1, 2). Then GTP is hydrolyzed by Rab GTPase, and the vesicle fuses with the plasma membrane (3). The GDP-bound Rab is then released from the membrane to be regenerated in the Rab GTP-bound form. After vesicle fusion with the PM, proteins are localized at the PM or cargo within the vesicle is secreted to the apoplast. (**b**) An example of vesicle formation from the PM. Vesicle formation usually requires the adaptor protein (AP) complex to form clathrin-coated vesicles. In the example, AP complex binds cargo on the PM (1). Then clathrin is recruited to the AP-cargo complex (2). More and more clathrin is recruited until a clathrin-coated pit is formed (3). DRP interacts with the clathrin light chain and DRP-clathrin-mediated endocytosis occurs in plants, although it is not known if the DRP-GTPase functions in the same way in plants. DRP hydrolyzes GTP, and that hydrolysis may provide the energy for vesicle scission (4). Then the clathrin-coated vesicle is released (5)

formation and fusion require energy. Figure 2 illustrates vesicle fusion and formation (budding/scission).

2.1 Clathrin-Coated Vesicles

Plant CCVs (50–90 nm) are smaller than their mammalian counterparts (120 nm), most likely because of the rigid cell wall and turgor pressure of plant cells (1–4.5 MPa). Three heavy (180–190 kDa) and three light (30–50 kDa) clathrin chains make up the basic unit of the clathrin coat in both plants and animals, but the chains are 10–15 kDa larger in plants (Holstein et al. 1994). Clathrin coats can spontaneously assemble in low ionic strength and low pH buffer, but clathrin

assembly requires adaptor proteins (AP) under physiological conditions to recruit cargo proteins. CCVs also function in secretion.

The mechanism of CCV assembly and uncoating has been studied in yeast and animal cells, but it has largely been inferred in plants. ADP-ribosylation factor Ras GTPases (ARFs) can stimulate phosphatidylinositol production in the membranes, particularly phosphatidylinositol 4-phosphate (PtdIns4P) in *trans*-Golgi and phosphatidylinositol 4,5 bisphosphate (PIP2) in the PM, where they function as coat protein-docking sites. In mammals, activated GTP-bound ARF1 recruits AP-1, GGA, and clathrin at the *trans*-Golgi, and ARF6 recruits AP-2, clathrin, and other components at the PM. In mammals, direct interactions between ARF6 and H^+ V-ATPase subunits are dependent on luminal pH, establishing a link between endosomal acidification, recruitment of coat proteins, and trafficking (Hurtado-Lorenzo et al. 2006; Recchi and Chavrier 2006; Marshansky 2007). Uncoating of CCVs occurs via the activity of Hsc70 and its cofactor auxilin before fusion with specific endosomes/organelles (Ungewickell et al. 1995). In mammalian cells, dissociation of clathrin coats and APs from early endosomes (pH 5.9–6) may be dependent on acidification of CCVs derived from the PM (pH 7.0) (Lemmon 2001; Schlossman et al. 1984). In plant cells, CCVs derived from the PM are thought to retain the extracellular pH of 5.0–5.5, which is already lower than acidified mammalian early endosomes, suggesting differences between AP/clathrin-mediated vesicle trafficking and sorting in plants and animals.

2.2 Adaptins and Adaptor Protein Complexes

AP complex function has been extensively studied in animals and yeast (Murphy et al. 2005). Adaptor proteins can be classified into two groups: monomeric and heterotetrameric. Animals have several monomeric adaptors like AP180, β-arrestin, GGA, and stonins, but plants have only one known monomeric adaptor, an ortholog of AP180 (Barth and Holstein 2004).

Four heterotetrameric adaptor complexes have been identified: AP-1, AP-2, AP-3, and AP-4. Each of the AP complexes is made up of two large adaptin subunits (one of γ/α/δ/ε and one β1-4, respectively, 90–130 kDa), one medium adaptin (μ1–4, 50 kDa), and one small adaptin (σ1–4, 20 kDa). AP-1, AP-2, and AP-3 are found in all eukaryotes, but AP-4 is found in mammals, plants, birds, and slime mold but not in insects or yeast (Robinson and Bonifacino 2001). All four AP complexes have been shown to associate with clathrin, although a clear clathrin-binding motif has not been identified in AP-4 (Barois and Bakke 2005). AP-1, AP-3, and AP-4 associate with the TGN and other vesicular compartments, and AP-2 associates with the PM and is responsible for rapid endocytosis from the membrane (Robinson and Bonifacino 2001). Essentially, AP-1 mediates secretion from TGN to endosomes. AP-2 mediates endocytosis from the PM in mammals and yeast. AP-3 mediates trafficking from *trans*-Golgi to the vacuole in yeast. AP-4 function is not well characterized.

Knockout mutations in the adaptins of the AP-1 and AP-2 complex are lethal in the embryos of mice and insects (reviewed in Ohno et al. 2006). Deletion of AP-3 adaptins results in pigmentation defects in humans, mice, and fruit flies, but it is lethal in the embryo of nematodes, and results in neurological defects in mice (Ohno et al. 2006). Deletions in AP-4 adaptins have not been recovered in animal model systems, but AP-4 appears to have some role in Golgi to lysosome trafficking (Barois and Bakke 2005) and basolateral redirection (Simmen et al. 2002), although its function has not been demonstrated.

The Arabidopsis genome encodes orthologs of animal clathrin-mediated vesicular trafficking proteins (Boehm and Bonifacino 2001). Adaptin orthologs in Arabidopsis identified by propeptide sequence alignments (Sanderfoot and Raikhel 2002; Boehm and Bonifacino 2001) indicate that there are four AP complexes and, therefore, four β, σ, and μ-adaptin isoforms. There are also three γ, two α, and one each δ and ε adaptin. Tentative annotations of the adaptins were based on similarity with animal adaptins. However, these assignments are based on differences in a small number of residues, and experimental evidence is needed for either validation or reassignment. Of the four β-adaptins, β1and β2 have 92% sequence identity (MatGAT, Campanella et al. 2003). High sequence identity of β1 and β2 suggests that they may be in AP-1 and/or AP-2 (Boehm and Bonifacino 2001). Recently, Dacks et al. (2008) concluded that β1 and β2 arose from a gene duplication event. Therefore, assignment of the β-adaptin isoforms to AP complexes based on experimental evidence is an outstanding question.

The AP180, μ, and σ-adaptins from Arabidopsis have been characterized (Happel et al. 2004; Barth and Holstein 2004; Holstein and Oliviusson 2005), and structural subunits α, δ, γ-adaptins have been partially characterized by inference (Song et al. 2009; Lee et al. 2007). β-adaptins in plants were first identified by Holstein et al. (1994) but have not been investigated further. These reports suggest that AP-1 mediates TGN to vacuole trafficking, while AP-3 mediates TGN to vacuole trafficking via a different vesicular population than AP-1. AP-2 functions in endocytosis. AP-4's function is unknown.

2.3 Vesicle Fusion with the Plasma Membrane

Once the vesicle traffics to the PM, the vesicle docks at the PM prior to fusion. The soluble *N*-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) complexes are composed of Q-SNAREs (Qa, Qb, Qc) and R-SNAREs, categorization based on sequence motifs (Fasshauer et al. 1998, Bock et al. 2001). Q-SNAREs are syntaxins of plants (SYPs) associated with synaptosome-associated proteins (SNAPs) at the PM, while R-SNAREs are vesicle-associated membrane proteins (VAMPs), which contain a longin domain required for subcellular sorting and vesicle targeting (Uemura et al. 2005). Q-SNAREs and R-SNAREs form complementary pairings that regulate specificity of docking for vesicle fusion and form a ternary complex with SNAPs to facilitate vesicle fusion (Kwon et al. 2008).

The Arabidopsis genome has 54 SNARE genes (18 Qa-SNAREs/Syntaxins, 11 Qb-SNAREs, 8 Qc-SNAREs, 14 R-SNAREs/VAMPs and 3 SNAP-25s) (Uemura et al. 2004), many of which are plant-specific and those associated with the PM have roles in cytokinesis, hormone responses, and pathogen resistance (Collins et al. 2003). Nine Qa-SNAREs are on the PM: SYP111/KNOLLE, SYP112, SYP121, SYP122, SYP123, SYP124, SYP125, SYP131, and SYP132 (Uemura et al. 2004). Some of these Qa-SNAREs show ubiquitous expression and uniform localization on the PM (SYP132), while others show polarized localization in the growing tips of root hairs (SYP123) or pollen tubes (SYP131) (Enami et al. 2009), suggesting diverse function and selective recognition of vesicles prior to fusion. Four Qb-SNAREs (VTI11, NPSN11, NPSN12 and NPSN13) are on the PM, while VTI12 regulates transport between TGN/EE compartment and the PM (Uemura et al. 2004). Thus far, one Qc-SNARE (SYP71) has been characterized as on the PM as well as the ER (Suwastika et al. 2008), suggesting that it regulates trafficking between the ER and PM that bypasses the Golgi/TGN pathway. SNAP-25s (SNAP29, SNAP30, SNAP33) are targeted to the PM by posttranslational modification (Gonzalo et al. 1999).

R-SNAREs have a longin domain, as mentioned above, and five are associated with the PM (VAMP721, VAPM722, VAPM724, VAPM725, VAMP726) (Uemura et al. 2004). However, a subclass of the VAMP72 group does not have a SNARE motif in the central region (Vedovato et al. 2009). These non-SNARE longin proteins are plant-specific (phytolongins), suggesting that additional components are involved in vesicle sorting, targeting, and subsequent fusion. Q-SNAREs and SNAP-25s can have polar localization and associations with lipid rafts, presumably for targeted R-SNARE delivery of cargo to these lipid domains.

Vesicle fusion requires energy, typically through the hydrolysis of GTP via the Rab family of GTPases, a subset of the Ras GTPase superfamily (Rutherford and Moore 2002). Rabs are small GTP-binding proteins that cycle between the active GTP-bound state and inactive in the GDP-bound state. GTP-bound Rab associates with the PM and also recruits other factors to the PM, while GDP-bound Rab dissociates from the PM (Rutherford and Moore 2002). The Arabidopsis genome encodes 57 Rab proteins. The Rab-A family functions in TGN/EE to PM trafficking, with the Rab-A2 and Rab-A3 GTPases also playing a role in cell plate formation (Chow et al. 2008), while the Rab-A4d GTPase is instrumental in regulating polarized growth, such as root hair tips and pollen tubes (Preuss et al. 2004; Szumlanski and Nielsen 2009). Rab-E is also involved in post-Golgi secretion to the PM and appears to have a role in plant defence (Speth et al. 2009). GTP-bound Rab-E interacts with phosphatidylinositol-4-phosphate (PtdIns4P) 5-kinase 2 (PIP5K2) on the PM and stimulates PIP5K2 kinase activity (Camacho et al. 2009). The interaction between active Rab-E GTPase and PIP5K2 may increase localized PtdIns(4,5)P2 production on the PM (Camacho et al. 2009). This may enhance endocytosis and thereby balance the rates of exocytosis and endocytosis (Zoncu et al. 2007; Camacho et al. 2009). After vesicle fusion, GTP-bound Rab is regenerated and can return to the TGN/EE, where it can participate in another round of vesicle fusion.

Acidification mechanisms regulate vesicle trafficking and fusion in yeast, animals, and plants. The vacuolar V-ATPase is required for secretion, endocytosis, Golgi organization, and vacuole function in embryogenesis (Dettmer et al. 2005, 2006; Strompen et al. 2005). V-PPase AVP1 has a role in secretion of the PM H^+ATPase (Li et al. 2005). In addition, the V-ATPase and V-PPase may physically interact (Fischer-Schliebs et al. 1997), which has further implications for regulatory mechanism of pH on trafficking.

2.4 Exocyst

The exocyst, sometimes referred to as the Sec6/8 complex, is a specialized complex that is involved in tethering vesicles to PM prior to SNARE docking and subsequent membrane fusion. It is a heteromeric complex composed of eight proteins in yeast and mammals with homologs in plants: Sec3p, Sec5p, Sec6p, Sec8p, Sec10p, Sec15p, Exo70p, and Exo84p (Hála et al. 2009; Samuel et al. 2009; Chong et al. 2009). Seven of these have been experimentally identified in the complex thus far, with Exo84p as the outlier (Hála et al. 2009).

SEC6 and SEC8 localize in the growing tips of tobacco pollen tubes (Hála et al. 2009). In yeast two-hybrid assays, EXO70A1/SEC3a, SEC15b/SEC10, and SEC6/SEC8 pairs showed strong interactions (Hála et al. 2009). The mutational data suggests that exocysts are involved in many cellular functions ranging from cell wall formation to polar auxin transport to self-incompatibility (reviewed in Hála et al. 2009; Samuel et al. 2009). Chong et al. (2009) demonstrated that exocyst subunits Sec15 and Exo70 colocalized with SNAREs in transient expression in BY-2 cells, and Exo70 can recruit Sec5, Sec8, Sec15, and Exo84 components to form the exocyst (Chong et al. 2009). Analysis of loss-of-function mutants, including *exo84p*, shows pleiotropic defects including pollen germination and pollen tube growth (Hála et al. 2009).

2.5 Secretory Vesicle Cluster

The secretory vesicle cluster (SVC) is a linked set of secretory vesicles distinct from the Golgi and TGN/EE, although it appears to originate from the TGN/EE in tobacco BY-2 cell cultures (Toyooka et al. 2009). The SVC is characterized by several markers: the secretory carrier membrane protein 2 (SCAMP2), the SNARE SYP41, and the small GTPase Rab11-D; SVCs are not associated with CCVs (Toyooka et al. 2009). Among the markers that characterize SVCs are JIM7, a monoclonal antibody against homogalacturonan of pectic polysaccharides (Clausen et al. 2003), a cell wall component. SCVs appear to be involved in mass secretion to the PM in nondividing cells and are targeted to the cell plate in dividing cells (Toyooka et al. 2009) consistent with a role in secreting pectins.

2.6 *The Microtubule-Associated Cellulose Synthase Compartment*

The plasma membrane is the interface between the cytosol/symplast and cell wall/apoplast. The proteins on the PM are among the regulators of the components that enter and exit the cell. The cell wall is comprised of carbohydrates (e.g., cellulose, hemicelluloses, pectins), polyphenols (e.g., ligins), minerals (e.g., boron, silica), and sometimes waxes (e.g., suberin, cutin) that are secreted into the extracellular space. Once in the apoplast, these cell wall components often undergo modification due to developmental programs (e.g., cell enlargement, cell loosening in maternal tissues for pollen tube extension) or in response to biotic or abiotic stressors. These changes can be achieved by secretion of cell wall remodeling proteins, which may function enzymatically (cellulases, pectinase) or nonenzymatically (expansions) (reviewed in Lebeda et al. 2001; Sampedro and Cosgrove 2005).

Cellulose is synthesized by rosette-shaped cellulose synthase complexes (CSCs), ~25–30 nm, composed of cellulose synthase proteins (CESAs) on the PM and by a cytosolic component, ~45–50 nm (Mueller and Brown 1980; Bowling and Brown 2008). CESA3 is found in four different compartments using functional fluorescent protein fusions and immunogold labeling techniques: Golgi bodies (Paredez et al. 2006), TGN/EE (Crowell et al. 2009), a unique microtubule associated cellulose synthase compartment (MASC) that originates from the medial- or *trans*-Golgi (Crowell et al. 2009), and a population of small CESA compartment (SmaCCs) (Paredez et al. 2006). Tethered SmaCCs colocalize with microtubules (Paredez et al. 2006), and this tethered population may be synonymous with MASCs. Microtublues have been shown to define the trajectory of the CSCs (Paredez et al. 2006; Gutierrez et al. 2009), and intact, functional microtubules are required for MASC trafficking (Crowell et al. 2009). As the Golgi apparatus moves along the cortical microtubules, the Golgi bodies and TGN/EE pause on discrete sites, which are in proximity to MASCs (Crowell et al. 2009). The pause in trafficking occurs when the Golgi body is beneath the PM and leads to localized increases of CSC density. Crowell et al. (2009) conducted fluorescence recovery after photobleaching (FRAP) experiments and showed that CSC was not inserted randomly into the PM but followed the linear tracks of the microtubules and CSCs were inserted in rows. They went on to hypothesize that CSCs were associated with the TGN/EE only during endocytosis, as has been shown for the auxin transporter PIN2 (Robert et al. 2008), and that MASCs are the result of CSC internalization as MASCs correspond with the decrease in CSCs at the PM.

3 Endocytosis/Retrograde Trafficking

Endocytosis results in the internalization of nutrients, sterols, lipoproteins, hormones, receptor-binding toxins, and transport and receptor proteins from the PM. The proteins are trafficked back to the TGN/EE where they are sorted and either

recycle back to the PM or are targeted to another organelle where the protein may be modified and then redirected back to the PM or to another organelle, such are the lytic vacuole or peroxisome. Therefore, endocytosis and exocytosis pathways partially overlap and converge in the TGN/EE (Fig. 1). Coordination of endocytosis and exocytosis is required for homeostasis of the PM, since the PM and its components are taken up into the cell and the PM must be replenished for the mature cell to maintain size and integrity, and therefore function.

Endocytosis can be classified by the types of cargo and molecular machinery driving its internalization: clathrin-mediated endocytosis, caveolae/lipid raft-mediated endocytosis, and fluid phase endocytosis. Fluid phase vesicles are 0.5–2 μm in diameter and larger than CCVs (~30–100 nm) and caveolin vesicles (~50 nm) (Johannes et al. 2002; Dhonukshe et al. 2007). In animals, fluid phase endocytosis is dependent on the concentration of endocytosed soluble molecules, but receptor-mediated endocytosis, including that involving CCVs, is saturable, and thus is consistent with membrane localized receptors. Caveolae are composed of cholesterol, sphingolipids, and GPI (glycosylphosphatidylinositol)-anchored protein- rich microdomains on PM (Brown and London 2000). Although there are no published data about caveolin in plants, structural sterols such as stigmasterol, sitosterol, and sphingolipid are thought to organize lipid rafts in plants instead of cholesterol (Mongrand et al. 2004).

Most endocytotic vesicles originate as CCVs (Brett and Traub 2006), and clathrin-mediated endocytosis is better characterized in mammals than plants. Despite the long-standing evidence of CCVs in plants (Holstein et al. 1994), clathrin-mediated endocytosis has only recently been demonstrated (Dhonukshe et al. 2007; Leborgne-Castel et al. 2008). There are at least two endocytosis pathways in plants: one is characterized by the styryl dye FM4-64, SCAMP1, and Rab-F2 (ARA7/RHA1), and the other is characterized by SCAMP2 (Toyooka et al. 2009).

3.1 Clathrin-Mediated Endocytosis

The adaptor proteins AP-2 and AP-180 mediate clathrin cage assembly. AP-2 is a heterotetramer like AP-1, AP-3, and AP-4, as described earlier, but AP-180 functions as a monomer and also appears to interact with αC-adaptin (AP-2 subunit) (Barth and Holstein 2004). The AP-2 subunit μ2 is responsible for cargo selection, and until recently was thought to be the only subunit involved in cargo selection. The β2 subunit has also been shown to participate in cargo selection independent from μ2 in mammals, and a point mutation in β2 cannot recruit specific proteins (e.g., β-arrestin or autosomal recessive hypercholesterolemia protein) to clathrin structures (Keyel et al. 2008).

Cargo selection and sorting signals for recruitment into the clathrin-mediated endocytotic pathway are the tertiary structures of SNAPs, ubiquitin-tagged proteins, and YxxØ (where Ø is a bulky hydrophobic residue) on the C terminus of

proteins recognized by the AP complexes and the acidic dileucine [DE]xxxL [LIM] motifs recognized by α, σ2, and GGAs (Golgi-localizing, γ-adaptin ear homology domain, ARF-interacting proteins) (Keyel et al. 2008; Kelly et al. 2008). The AP-2 complexes appear to have differential binding affinities for the various acidic dileucine motifs and therefore afford specify for internalization of proteins from the PM. It appears that the affinities of β2 or μ2 for [DE]xxxL [LIM] are based on competitive binding, binding pockets, and the structure of the cargo protein (Kelly et al. 2008).

While vesicle fusion requires energy to overcome the hydrophobic and electrostatic forces required for vesicle fusion, vesicle formation (or scission) can occur spontaneously without ATP or GTP hydrolysis, via chemical changes in the clathrin lattice (Mashl and Bruinsma 1998); the frequency of this occurrence is unknown. More commonly, dynamin and dynamin-related protein (DRP) GTPases are active participants in endocytosis and complete vesicle fission via GTP hydrolyses (Fig. 2).

Dynamin and DRPs are large GTPases with multiple roles in protein trafficking, and cell and organelle division (Konopka et al. 2006), and Konopka et al. (2008) showed that plant dynamins play a role in clathrin-mediated endocytosis. In Arabidopsis, 16 genes, divided into six families, are predicted to encode dynamins (Hong et al. 2003; Gao et al. 2006). DRP2 interacts with the γ subunit of AP-1 (Jin et al. 2001), which suggests a role for DRP2 in clathrin-mediated trafficking to the PM. The mammalian dynamin 1 is most similar to plant DRP2. In contrast, the plant-specific DRP1 subfamily is necessary for cell expansion and division (Kang et al. 2001; 2003) and plays an active role in endocytosis (Konopka et al. 2008). The DRP1 subfamily has five isoforms (A–E), and so far, only partial functional redundancy has been observed (Konopka and Bednarek 2008). Using a combination of approaches and fluorescently tagged DRP1C and the clathrin light chain, Konopka et al. (2008) showed that DRP1C and clathrin light chain are simultaneously recruited to sites on the PM that are active in protein trafficking about 70% of the time, in contrast to the stepwise recruitment observed in mammals. Fluorescence of the fused proteins was not observed following endocytosis, presumably due to dissociation of the proteins from the complexes. The DRP1-clathrin-mediated endocytosis is not directly linked to actin polymerization but is mediated by microtubules.

3.2 Receptor-Mediated Endocytosis

Receptor-mediated endocytosis, sometimes called ligand-mediated endocytosis, results in the internalization of diverse molecules, such as hormones or peptides. Ligand binding increases the rate of endocytosis of the receptor, which either results in attenuation of the signal transduction cascade initiated at the PM [e.g., G protein-coupled receptors (GPCRs)] or promotion of the signal transduction

cascade if it occurs in the endosome (e.g., FLS2 and BRI1), and therefore effecting changes in transcription, metabolism, or trafficking.

Comparison of the animal and plant models is complicated by a proliferation of mechanisms in mammals, especially those involving GPCRs (Wolfe and Trejo 2007). GPCRs are heterotrimeric proteins that regulate signal transduction, and they are composed of Gα, Gβ, and Gγ subunits. Recent evidence indicates that a GPCR regulates the signaling response of the plant hormone abscisic acid (Pandey et al. 2009). Interestingly, it appears that the GDP-bound GTGs (GPCR-type G proteins 1 and 2) are the active signaling forms and not the GTP-bound forms. Recently, a nonprototypical G-protein complex has been shown to play a role in disease resistance (Zhu et al. 2009).

The receptor-like kinases (RLK) family has 610 members in Arabidopsis and contains receptor kinases and nonreceptor kinases (receptor-like cytoplasmic kinases), and RLKs in plants have been divided into 15 families (Shiu and Bleecker 2003). RLKs are transmembrane proteins with a cytoplasmic kinase domain, while the extracellular domain is variable. Self-incompatibility in Brassicaceae is regulated by the S-locus cysteine-rich protein (SCR) in the pollen which binds to the S-locus receptor kinase (SRK) in the stigma resulting in inhibition of pollen tube growth (reviewed in Peer and Murphy 2005). Serendipitously, most of the research has been on what turned out to be leucine-rich repeat (LRR) receptors, which have great diversity and function in nearly every aspect of plant growth and development and defense against pathogens. In one instance, there appears to be an overlap in the endocytotic pathways of BRI and FLS2 via BAK1/SERK1 (BRI1-associated kinase 1/ serine and proline rich receptor kinase 1). BRI1 is the bassinosteroid receptor, and after brassinolide binding to BRI1, BRI1 forms a dimer and phosphorlyates BRI1 kinase inhibitor 1, which then dissociates from BRI1. Then BRI1 and BAK1 form a heterodimer complex that undergoes endocytosis (reviewed in Chinchilla et al. 2009). Brassinolide signaling then occurs from the endosomal compartment.

Similarly, flagellin, a bacterial protein that comprises flagella, binds to the FLS2 receptor (flagellin sensitive 2) on the plant PM. Then FLS2 and BAK1 form a heterodimer, which is internalized, and the defense signal in response to the pathogen then occurs from the endosomal compartment (Robatzek 2007; Robatzek et al. 2006; Kwon et al. 2008; reviewed in Chinchilla et al. 2009). It is also possible that FLS2 and BAK1 are associated with each other before ligand binding, and the conformational changes that follow ligand binding produce the stable heterodimer. Systemin, an 18 amino acid peptide derived from prosystemin, is another example of an endogenous peptide ligand to an LRR that is involved in defense responses after mechanical wounding (e.g., herbivory) (Ryan et al. 2002). While BRI1 is able to bind systemin, it appears that the defense response is not elicited via BRI1, and that the yet unidentified systemin receptor is specifically in the vascular tissue, as is prosystemin (Malinowski et al. 2009). Cryptogein is a secreted fungal protein that binds to an unidentified PM receptor and stimulates a signal transduction response of which one of the results is rapid clathrin-mediated endocytosis. Interestingly,

cryptogein induction of CCV endocytosis occurs via reactive oxygen species (Leborgne-Castel et al. 2008).

Phytosulfokine (PSK) is an endogenous five amino acid sulfonated peptide [H-Tyr(SO3H)-Ile-Tyr(SO3H)-Thr-Gln-OH] that is involved in cell proliferation and elongation (Matsubayashi et al. 2002). High affinity and low affinity PSK receptors were identified (Matsubayashi et al. 1997), and subsequently, an LRR PSK receptor was purified (Matsubayashi et al. 2006). CLV3 (CLAVATA 3) is a 79 amino acid signaling polypeptide along with the LRR's CLV1 and CLV2, which are important for meristem cell maintenance (reviewed in Wang and Fiers 2009). ACR4 (ARABIDOPSIS CRINKLY4) is an LRR required for cell layer organization, and its ligand is CLE40 (CLAVATA/ENDOSPERM SURROUNDING REGION40).

3.3 Sorting and the Return Trip to the PM

Since the TGN and early endosome (EE) compartments overlap (Lam et al. 2007), both outward- and inward-bound vesicles are present in this compartment. Therefore, there must be a mechanism to sort which cargo continues on to other compartments or returns to the PM. The recycling mechanisms result in the reuse of expensive transmembrane proteins and degradation of the damaged or unneeded proteins. Therefore, there are several trafficking pathways that converge in endosomes: endosome to PM trafficking, endosome to TGN retrieval, and endosome to vacuole targeting.

In addition to the sorting signals involved in cargo selection described above, the ADP-ribosylation factor (ARF)-GTPase and Rho-GTPase of plants (ROP-GTPase) proteins and the proteins regulating their function are essential components regulating traffic to and from the PM. ARF-GEFs (ARF-guanine nucleotide exchange factors) catalyze the GTP-bound form of ARF, while ARF-GAPs (ARF-GTPase-activating proteins) catalyze the GDP-bound form of ARF GTPases, and therefore regulate the activity of the ARFs. Similarly, the ROP-GEFs and ROP-GAPs recycle the active and inactive forms of the ROP-GTPases (reviewed in Yalovsky et al. 2008; Payne and Grierson 2009).

ADP-ribosylation factor-guanine-nucleotide exchange factors (ARF-GEF) such as GNOM and GNOM-LIKE 1 are instrumental in recruiting the protein coats for vesicle formation and also for cargo selection (Donaldson and Jackson 2000; Richter et al. 2007; Teh and Moore 2007). GNOM is involved in recycling of proteins, like the PIN auxin transporters, from endosomes to the PM (Geldner et al. 2003), while GNOM-LIKE 1 is important for trafficking of the ABCB auxin transporters (Titapiwatanakun et al. 2009). The ADP-ribosylation factor-GTPase-activating protein (ARF-GAP) VAN3/SCARFACE (Koizumi et al. 2005; Sieburth et al. 2006) is involved in leaf vein patterning and is required for correct trafficking of auxin efflux carriers and auxin signaling. The ligand for VAN3 appears to be specific phosphoinositides generated by the polyphosphate 5′-phosphatases

COTYLEDON VASCULAR PATTERN2 (CVP2) and CVP2 LIKE1 (CVL1) (Carland and Nelson 2009).

Rho-GTPases of plants (ROPS) are also important in developmental patterning. For example, SCN1, a RhoGTPase GDP dissociation inhibitor (RhoGDI), restricts ROP activity to one focus on the PM to produce a single root hair (Carol et al. 2005), while overexpression of constitutively active ROP2 produces two root hairs in one cell (Jones et al. 2002), indicating that two foci develop on the PM instead one. ROP2 is also light-regulated, and expression of constitutively active ROP2 is observed on the PM and inhibits stomatal opening, while constitutively inactive ROP2 is cytosolic (Jeon et al. 2008). In animals, GTPases with both ARF-GAP and Rho-GAP domains have been identified, suggesting cross-talk between the pathways (Miura et al. 2002). Although these have not yet been described in plants, mutational analyses of ARF1 indicates genetic interactions with ROP2 and trafficking of PIN2, which is mediated by the ARF-GEF GNOM (Xu and Scheres 2005; Kleine-Vehn et al. 2008b).

There are also sorting signals like the retromer protein complex, characterized by VPS cargo recognition heterotrimer [VPS35 (a–c), VPS26 (a,b), VPS29], and SNX1 (sorting nexin 1) (reviewed in Otegui and Spitzer 2008), which return receptors and other proteins from the MVB to the TGN/early endosome. SNX1 signals are also observed in GNOM-containing endosomes, consistent with SNX1 involvement in recycling of PM proteins. SNX1 signals overlap with the prevacuolar compartment/multivesicular body, and markers for the secretory (BP80) and endocytotic (BR1, PIP2a) pathways show that both overlap in SNX1-containing endosomes (Jaillais et al. 2008), and these data suggest a role in recycling proteins from the MVB back to the PM. However, trafficking inhibitors, such as the fungal inhibitor wortmannin, resulted in mistargeting of the PM proteins to the lytic vacuole (Jaillais et al. 2008).

3.4 Endosomes and Multivesicular Bodies

Following endocytosis, the vesicles fuse with endosomes. The endosomal compartment is comprised of a gradient of endosomal populations, which can be loosely defined as early, late, and recycling endosomes. As discussed above, in contrast to animal cells, and the early endosome and TGN compartments overlap in plants (Lam et al. 2007); therefore, discrete assignation of the membrane populations is not possible. Spatially or functionally discrete endosomes are identified by markers (often ARF-GEFs, RABs or SYPs), which also have overlapping or partial localizations in the endosomal populations. Endosomes are also unique, multifunctional organelles as both the BR1 and FLS2 receptors appear to signal from the endosomes and not from the PM. Recycling endosomes, as discussed above, have been shown to have at least two pathways, GNOM-dependent and GNOM-independent (reviewed in Otegui and Spitzer 2008).

The late endosomes are often synonymous with multivesicular bodies (MVBs), also known as the prevacuolar compartment. MVBs may be the most complex endosome, containing many intraluminal vesicles of proteins and macromolecules resulting from endocytosis. The intraluminal vesicles form when an endosome invaginates and buds into its own lumen. Although the signal(s) for this are complex and not well understood, many of the proteins in the intraluminal vesicles are ubiquintinated. The intraluminal proteins are usually targeted to the lytic vacuole for degradation, although this is yet to be shown for plant PM proteins. Sorting of cargo in MVBs involves the ESCRT (endosomal sorting complexes required for transport) and ESCRT-related CHMP1A and B (charged MVB protein/chromatin modifying protein 1A and B) proteins (Spitzer et al. 2009). Recently, through molecular genetic and cell biology techniques, Spitzer et al. showed that the auxin carriers PIN1 and 2 and AUX1 are MVB cargo sorted by the ESCRT machinery, and this function is lost in *chmp1a chmp1b* double mutants.

4 Role of the Cytoskeleton in Plasma Membrane Protein Trafficking

The cytoskeleton provides the scaffolding or framework for the shape for the cell, and protein trafficking to the PM requires motorized movement of the vesicles to and from the PM. This is hypothesized to occur on either the actin cytoskeleton or on microtubules. Experimental evidence exists for microtubule involvement in secretion to the PM, and microtubule and actin participation in endocytosis from the PM. Although the role of actin in secretion to other organelles has been demonstrated, an active role for actin in secretion to the PM remains an outstanding question (Staiger et al. 2009). The cytoskeleton also provides the framework for new cell plate formation. The cytoskeleton establishes the preprophase band where the new cell plate will form and coordinates vesicle trafficking from the center of the cell plate towards the periphery resulting in two daughter cells.

4.1 Actin

A role of actin in secretion to the PM has been hypothesized, as a treatment with actin inhibitors reduces mucilage production (Hawes et al. 2003). However, ROP1 assembly and disassembly occurs via RICs (ROP-interactive CRIB-containing proteins): actin disassembly via RIC3 is required for exocytosis, while RIC4-mediated actin assembly resulted in polar vesicle accumulation during tip growth (Gu et al. 2005; Lee et al. 2008). A role for actin has also been demonstrated for clathrin-mediated endocytosis. The motive force for endocytosis not only involves the clathrin triskelia, and the energy released from GTP hydrolysis via the large and

small GTPases, but also the force generated by actin ploymerization (Conner and Schmid 2003). Actin may organize endocytotic "hotspots," and accessory proteins have been shown to bind to actin (Qualman et al. 2000; Conner and Schmid 2003; Samaj et al. 2004). Use of chemical inhibitors of actin polymerization, such as the fungal toxin latrunculin B, also points to the importance of actin as part of the endocytotic machinery (Blancaflor et al. 2006).

4.2 Microtubules

However, protein trafficking in plants utilizes both actin and microtubules for secretion and endocytosis. Secretion and endocytosis of the cellulose synthase complex (CSC) occurs via microtubules (Paredez et al. 2006; Gutierrez et al. 2009; Crowell et al. 2009). Cortical microtubules may also carry the complexes that secrete mucilage during germination (McFarlane et al. 2008). Endocytosis of the cellulose synthase complex (CSC) does not appear to occur via clathrin-coated vesicles (CCVs) (Crowell et al. 2009), since the smallest plant CCVs observed are 30 nm in diameter, while CSCs are 25 nm. It seems likely, however, that other adaptor or accessory proteins may be required for CSC endocytosis. Microtubules are also required for clarthrin-mediated endocytosis that utilizes dynamins (Konopka and Bednarek 2008). Therefore, microtubules are involved in both clathrin- and nonclathrin-mediated endocytosis, as well as marking the sites of cellulose and pectin secretion.

5 Models of Trafficking

Cellular trafficking occurs in all living cells to maintain homeostasis and respond to cellular communication and biotic and abiotic stimuli. Rates of endocytosis and exocytosis in root hairs and pollen tubes were estimated by Ketelaar et al. (2008). Based on the amount of membranes and cell wall material needed to be inserted for growth and the amount of extra membrane that would need to be recycled via endocytosis, they calculated that there was an excess of 86.7% membranes in root hairs and 79% in pollen tubes. They then calculated that if secretion were inhibited, the cells would continue to grow for 33 more seconds. Ketelaar et al. tested this experimentally, and measured that growth continued for 30–40 s, in the range that their model predicted. Therefore, trafficking to and from the PM is a dynamic process, and there may also be some lag time in responding to the stimuli as vesicles are already in motion. Trafficking to and from the PM may be induced, as observed in receptor-mediated endocytosis, or constitutive as appears to be the case for some of the PM-localized PIN auxin transporters. Other types of trafficking are specialized and specific to certain cell types or developmental processes, such as the polar tip growth

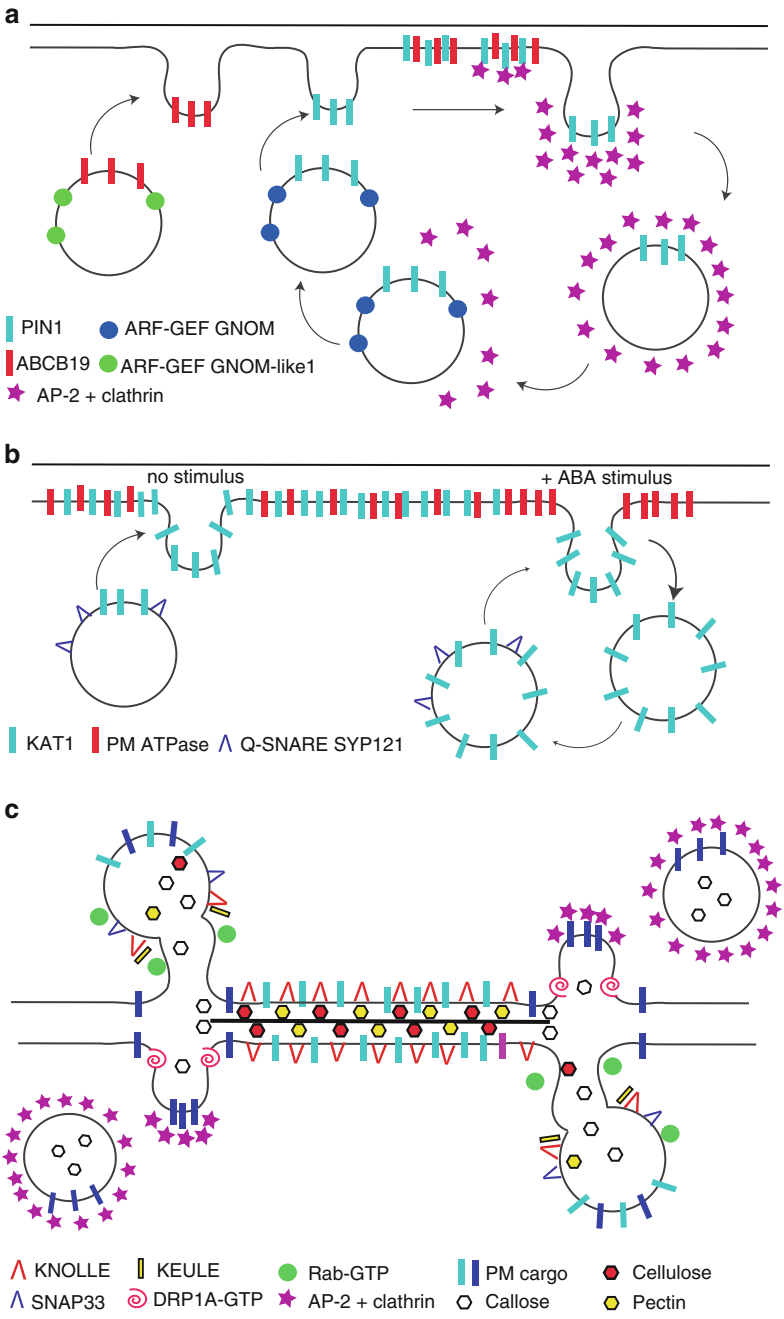

Fig. 3 Models of trafficking. (**a**) Constitutive trafficking. PIN1 is an example of a constitutively trafficked protein. PIN1 trafficking is dependent on the ARF-GEF GNOM. The PIN1 and ABCB19 auxin exporters colocalize on the PM and ABCB19 stabilizes PIN1 at the PM. However, ABCB19

of pollen tubes and root hairs and the formation of the cell plate during cell division, and responses to pathogens. Models of each type of trafficking are presented in Fig. 3.

5.1 Constitutive

An example of a constitutively cycled protein is the auxin efflux carrier PIN1, which also displays polar localization in the stele of root tissues. Proper PIN1 polar localization on the PM is dependent on a brefeldin A-sensitive ARF-GEF GNOM (Geldner et al. 2003). The hormone auxin itself affects the polar localization of PIN1 (Peer et al. 2004), which was attributed to auxin inhibition of endocytosis (Paciorek et al. 2005). Vesicular cycling of PIN1 is also sensitive to the auxin transport and trafficking inhibitor *N*-1-naphthylphthalamic acid and trafficking inhibitors such as brefeldin A (BFA) (Geldner et al. 2003; Peer et al. 2004). Recently, Dhonukshe et al. (2007) manipulated the Adaptor Proteins by over-expression of the heavy clathrin chain and used a kinase inhibitor (tyrphostin A23) that has been shown to inhibit μ-adaptin interaction with cargo (Dhonukshe et al. 2007; Ortiz-Zapater et al. 2006). The result was altered rates of CCV-mediated PIN1 endocytosis, although PIN1 appears to lack a μ-adaptin-binding site. Therefore, polar and asymmetric localization of auxin transport proteins is mediated by CCVs, and specific adaptins/APs are essential to the establishment and maintenance of cellular polarity in plants. As AP-4 in mammals is proposed to have a role in basolateral redirection, AP-4 may function in analogous, but mechanistically distinct, polar trafficking of membrane proteins in plants.

5.2 Induced

KAT1 (K^+ channel) is an example of a PM protein that undergoes induced endocytosis and secretion in guard cells as elegantly demonstrated with the use of functional fluorescently fused proteins, FRAP, and electrophysiological analyses

Fig. 3 (continued) trafficking is mediated by the ARF-GEF GNOM-like1, and ABCB19 appears to be stable on the membrane and does not undergo dynamic cycling. (**b**) Induced trafficking. KAT1 trafficking in guard cells is induced by the hormone abscisic acid (ABA). In the basal or uninduced state, the majority of KAT1 is on the PM, and a subset of KAT1 is in an endosomal population (*left*). Following ABA stimulus, KAT1 is rapidly internalized from the PM into endosomal populations (*right*, *heavy arrow*). This rapid endocytosis is specific to KAT1, since the PM ATPase remains at the PM. After several hours, KAT1 is recycled back to the PM via SYP121-depend trafficking (*light arrows*). (**c**) Specialized trafficking. Cytokinesis is an example of specified trafficking. Cytokinesis requires coordination of KEULE/Sec1, KNOLLE/SYP111, SNAP23, Rab-A2, Rab-A3, Rab-F2, and DRP1A and DRP2B for vesicle fusion and endocytosis for nascent PM formation and secretion of callose, cellulose, and pectin for cell plate formation

(Sutter et al. 2006, 2007). The majority of the KAT1 channels reside on the PM and the remainder are in an endosomal population. Following stimulus by the hormone abscisic acid, KAT1, but not the PM H^+ ATPase, undergoes selective and rapid endocytosis to an endosomal compartment. KAT1 then recycles back to the PM over several hours in a SYP121-dependent pathway. This work also showed that KAT1 is localized on the PM in microdomains of 0.5–0.6 μm in diameter. BRI1, discussed above, is another example of a protein that undergoes induced trafficking.

5.3 Specialized

Specialized trafficking includes polar growth observed in root hairs and pollen tubes, as well as those that occur during cell plate formation during cytokinesis. While the asymmetric localization of PM proteins such as COBRA, PIN1, and PIN2 in the root tip may be considered specialized, little is known about the mechanisms that drive and maintain that localization. Polar tip growth in pollen tubes and root hairs involves coordination of Rabs, Secs, SYPs, and dynamins and microtubules, and has been discussed throughout this chapter.

Cytokinesis also requires coordination of Rabs (Rab-A2, Rab-A3, Rab-F2, Chow et al. 2008), Secs (KEULE/Sec1, Assaad et al. 2001), SYPs (KNOLLE/SYP111, Boutté et al. 2010; Reichardt et al. 2007), and dynamins/DRPs (DRP1A, Konopka and Bednarek 2008; DRP2B, Fujimoto et al. 2008) and microtubules, but additionally, it requires that a cell plate is formed during nascent PM formation to produce two daughter cells. Cell plate formation is dependent on secretion of nascent proteins to the preprophase band and formation of the phragmoplast (a scaffold of the forming cell plate). The targeting of vesicles to the cell plate is viewed as the default pathway in diving cells (Jürgens 2005). Vesicle fusion results in PM formation, localized regions of callose, followed by callose removal and depositions of pectin and hemicellulose/cellulose and cell wall modifying enzymes like KORRIGAN (Zuo et al. 2000; Robert et al. 2005), resulting in membrane partitioning as the cell plate is formed.

KEULE/Sec1 binds KNOLLE/SYP111 to effect vesicle fusion during cytokinesis (Assaad et al. 2001). KNOLLE (KN) has been used to follow the secretory pathway during cytokinesis, and to show that nascent proteins are secreted to form the cell plate, and KN subsequently was found in MVBs bound for the lytic vacuole, and that endocytosis is not necessary in that process (Reichardt et al. 2007). More recently, it was shown that endocytosis plays a role in restricting KN to the cell division plane (Boutté et al. 2010). Polar localization of other proteins at the cell plate is also observed. For example, ABCB19 has polar localization at the newly formed cell plate, but ABCB19 does not colocalize with KEULE, indicating that ABCB19 is not involved in early cell plate formation (Blakeslee et al. 2007). Protein turnover or processing may also take place at the cell plate as aminopeptidase M1 (APM1) is observed at the forming cell plate during cytokinesis, and loss-

of-function mutants show aberrant planes of cell division (Peer et al. 2009). Therefore, APM1 is required early in cytokinesis for proper planes of cell division, whereas KNOLLE, KEULE, and KORRIGAN are required for completion of cell division.

6 Concluding Remarks

Although the past decade has yielded a wealth of new information regarding plasma membrane trafficking mechanisms, there are still many outstanding questions to be resolved. The mechanisms underlying the polar targeting of proteins to PM are yet to be fully elucidated. More substantive elaboration of transcytotic redirection events and evaluation of the contribution of this phenomenon to distinct polar trafficking pathways are required. More extensive characterization of the motif(s) that are required for cargo selection and targeting is a priority, as earlier models assuming that cargo selection is primarily mediated by μ-adaptins must be reevaluated in light of evidence that cargo binding can be demonstrated for all mammalian adaptor protein isoforms. Regulatory combinations of the many potential CLE and LLRs, which act synergistically or antagonistically to maintain the meristem or cell identity must be explored. It must also be determined how many different types of endosomal compartments are really present and whether they are really distinct compartments or a gradient of membrane populations characterized by protein concentrations. Finally, the contribution of membrane subdomains to trafficking mechanisms must be evaluated. Emerging new techniques and technologies to dynamically resolve the identity and spatial distribution of vesicle populations and membrane subdomains as they interact with the cytoskeleton are expected to resolve many of the outstanding questions currently confronting plant cell biologists and provide a framework for the identification and engineering of plants that can meet the needs of a growing human population without sacrificing biodiversity.

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The Plasma Membrane and the Cell Wall

Arun Sampathkumar, Lutz Neumetzler, and Staffan Persson

Abstract Plant cell walls are among the features that distinguish plants from animals. The cell wall contributes to internal cellular turgor pressure while offering protection to the cell from external threats. The cell wall is also flexible, and its content and structures are modulated according to growth and tropisms. A typical plant cell wall is primarily composed of carbohydrates synthesized either at the plasma membrane (PM) or in the *trans*-Golgi network: cellulose and callose are produced at the PM, whereas the matrix polysaccharides, i.e., pectins and hemicelluloses, are synthesized in the Golgi apparatus and are subsequently transported across the PM via exocytosis. The wall also contains enzymes and structural glycoproteins. The cell wall is formed by independent and coordinated mechanisms based on physical properties and self-assembly, as well as the action of cell wall-modifying enzymes. Thus, the plant cell wall physiology is dynamic and includes coordinated sugar synthesis, polymer assembly, trafficking, and modification.

1 Introduction

The plant cell wall is one of the fundamental features that distinguish plants from animals. The wall is essential for plant growth and morphogenesis and therefore constitutes one of the most prominent compartments in plants (Carpita and McCann 2000). The cell wall also counterbalances internal cellular turgor pressure (Somerville et al. 2004), offers protection to the cell from external threats, including pathogens (Vorwerk et al. 2004), stabilizes specialized cells (Turner et al. 2007), and contributes to the determination of extracellular ionic composition (Carpita and McCann 2000).

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Such diversity requires the wall to be quite flexible and therefore to modulate its content and structures according to growth and tropisms. As a natural renewable product, the cell wall and its derivatives are of great importance for various industries, such as paper, textile, food, and biomass/biofuel production (Lapasin et al. 1995; Somerville 2007).

A typical plant cell wall is primarily composed of carbohydrates that are synthesized either at the plasma membrane (PM) or in the *trans*-Golgi network (Carpita and McCann 2000). Accordingly, two cell wall polymers, cellulose and callose, are produced at the PM, whereas the matrix polysaccharides, i.e., pectins and hemicelluloses, are synthesized in the Golgi apparatus (Somerville et al. 2004; Lerouxel et al. 2006). Oligosaccharides from the Golgi apparatus are subsequently transported across the PM via exocytosis and are incorporated into the growing cell wall (Geisler et al. 2008). In addition to the polysaccharides, the wall also contains enzymes and structural glycoproteins that can be subdivided according to their amino acid constitution and their divergent carbohydrate moieties (Seifert and Roberts 2007).

The formation and maintenance of the cell wall is driven by independent, but coordinated, mechanisms. For example, cellulose crystallization is induced by physical strain and is a self-assembling process, while processes such as the reinforcement and remodeling of xyloglucan chains during cell expansion are enzyme mediated (Somerville et al. 2004). Such remodeling is performed by active enzymes that prune cell wall sugars, once they have been deposited. Thus, the plant cell wall physiology is dynamic and includes coordinated sugar synthesis, polymer assembly, trafficking, and modification. Instead of attempting to give a complete overview of cell wall constituents and synthesis, here we focus on cell wall characteristics and processes that are directly or indirectly linked to the plasma membrane (PM).

2 Primary and Secondary Cell Walls

The plant cell wall is a general term used to describe at least three different substructures: the middle lamella, the primary cell wall, and the secondary cell wall (Fig. 1; Carpita and McCann 2000). The middle lamella is deposited during cell plate formation (Matar and Catesson 1988) and is therefore shared by two neighboring cells. This structure is mainly composed of pectic polymers, such as homogalacturonan (HG). The primary cell wall is deposited after cell division and is flexible in nature to accommodate cell elongation. In contrast, the secondary cell wall supplies structural support for specialized cell types, such as xylem vessels, and is deposited after growth cessation (Carpita and McCann 2000). More detailed descriptions of the different polymers incorporated in cell walls can be found in reviews by Mohnen (2008), Carpita and McCann (2000), Obel et al. (2006), Lerouxel et al. (2006), Fry (2004), and Cosgrove (1997).

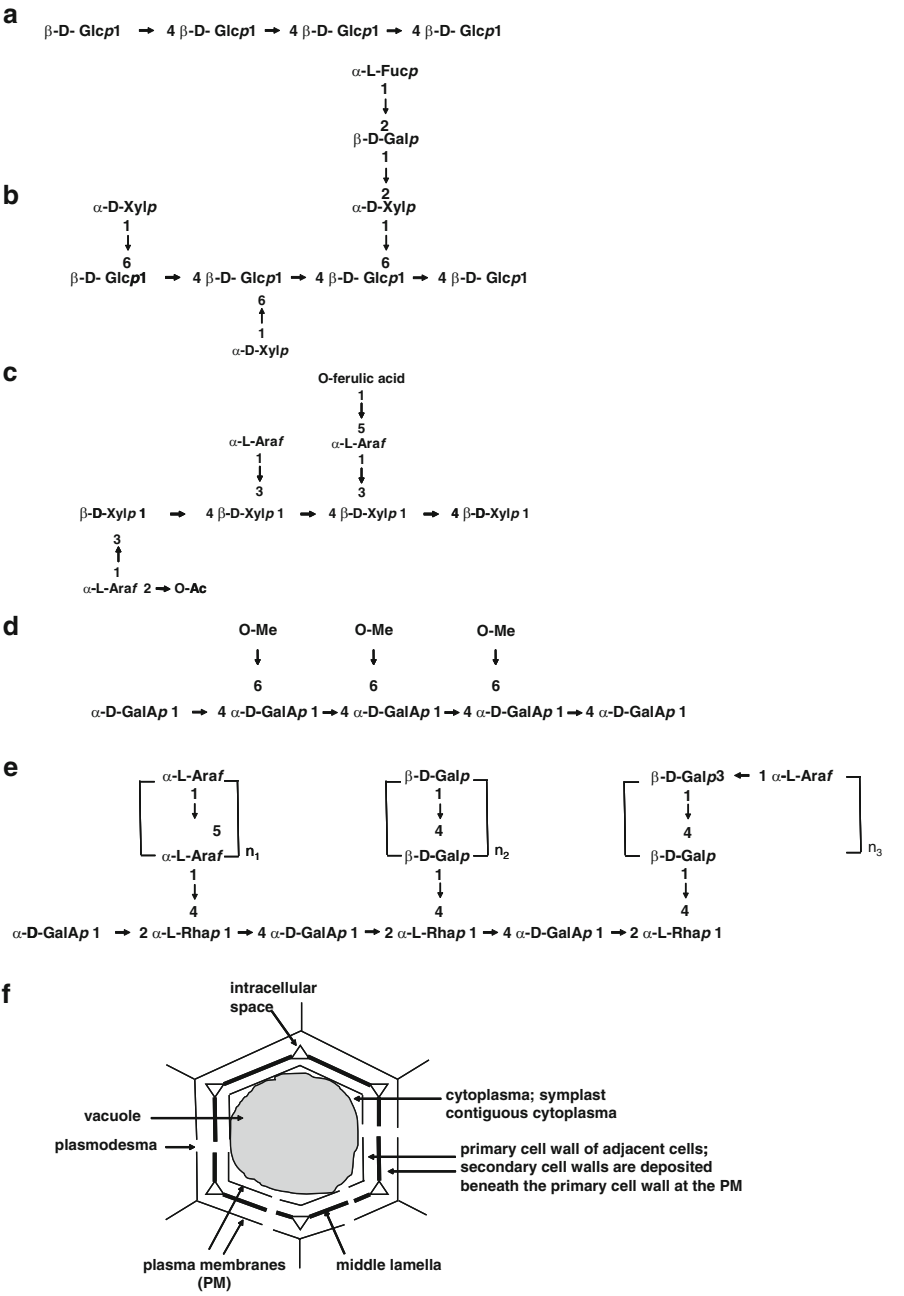

Fig. 1 *Cell wall polymers and their organization in different walls.* (**a**–**e**) Schematic views of different cell wall polymers, including β-1,4-linked glucan (**a**), xyloglucans (**b**), arabinoxylans (**c**), homogalacturonans (**d**), and rhamnogalacturonan I (**e**). (**f**) Schematic view of the different types of cell walls and their relative position to each other

2.1 Primary Cell Walls

The primary cell wall consists of four major components that can interact with each other to form a flexible structure (Fig. 1; Carpita 1996). These components include the polysaccharides cellulose, hemicellulose, and pectin, as well as heavily glycosylated proteins. Growing dicot walls are typically composed of approximately equal parts of each polysaccharide class, as well as 1–5% proteins (Zablackis et al. 1995). However, in graminaceous monocots (grasses), cell wall compositions are more variable (Carpita 1996) and generally include cellulose, the hemicellulosic polymer xylan, and mixed-linked glucan polymers (Yokoyama and Nishitani 2004). Generally, cellulose microfibrils are cross-linked by hemicellulosic polymers and possibly also by pectic polymers, such as arabinans and galactans (Fig. 2; Zykwinska et al. 2005). Hemicelluloses comprise a heterogeneous group of glycans and include xylan, mannan, and xyloglucan polymers, which together with mixed-linked glucans and callose may be woven around and into the cellulose microfibrils (Fig. 1; Lerouxel et al. 2006), explaining the harsh extraction procedures necessary for separation of these polymers.

Hemicelluloses contain cellulose-like structures in their polymer backbones (Lerouxel et al. 2006). One of the more prominent hemicelluloses is xyloglucan in which glucose forms the backbone and xylose, galactose, and fucose are added as side residues (Fig. 1; Obel et al. 2006). The individual sugar moieties may further be modified by acetylation (Pauly et al. 2001a, b). The xylose-based polymer xylan is the most abundant hemicellulose in grasses and hardwoods (Carpita 1996). Xylans are comprised of β-1,4-linked D-xylosyl residues that may be decorated with arabinose and glucuronic acids (Fig. 1). Carpita and Gibeaut (1993)

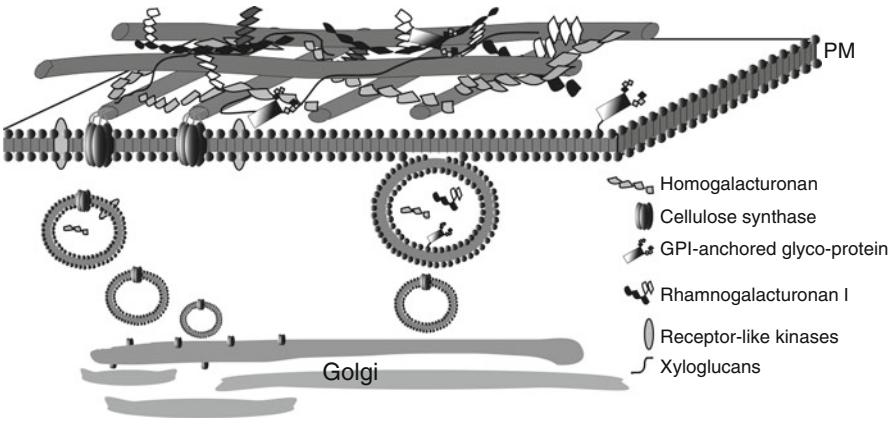

Fig. 2 *Schematic model of a primary cell wall in dicots*. The cellulose microfibrils form the load-bearing backbones of the wall and are cross-linked by hemicelluloses, and possibly pectins. The latter polymers are synthesized in the Golgi and transported to the cell surface via exocytosis. Several other enzymes and structural proteins also play a vital part in the integrity and modification of the cell wall

hypothesized that arabinoxylans may be functionally similar to xyloglucans in grasses, where only small amounts of xyloglucans are present. Three to five percent of the dry weight of most hardwoods consists of glucomannans, which consist of linear β-1,4-linked mannosyl chains with glucosyl residues (Meier 1985).

Mixed-linked glucans are β-(1,3;1,4)-linked polysaccharides which are unbranched and unsubstituted and are thought to be synthesized in the Golgi apparatus. Mixed-linked glucans are one of the major cross-linking polymers in the type II cell walls of grasses, which appear to contain less pectins and only small amounts of xyloglucans (Carpita and McCann 2000). Large amounts of mixed-linked glucans are produced during endosperm maturation and are of concern to the food processing industry as they are thought to hamper brewing processes involving barley (Keegstra and Walton 2006). Mixed-linked glycans are also deposited in primary cell walls in maize coleoptiles (Gibeaut and Carpita 1993).

Pectic polysaccharides are characterized by D-galacturonic acid content (D-GalA; Mohnen 2008). They constitute the most readily soluble part of the cell wall and are generally thought to hold a substantial amount of water. The pectins may be subdivided into three different types of polymers: HG, rhamnogalacturonan I (RGI), and rhamnogalacturonan II (RGII; Mohnen 2008; Fig. 1). HG is an unbranched chain of approximately 70–100 α-1,4-linked D-GalA units (Thibault et al. 1993). These chains may be methyl-esterified and/or O-acetylated (Ishii 1997). Methyl-esterification neutralizes the otherwise negatively charged, acidic carboxyl group of D-GalA and has a major impact on the gelling abilities of pectins, as well as affecting calcium chelation by HG (Mohnen 2008). RGI consists of alternating 1,2-α-L-rhamnosyl-1,4-α-D-galacturonic acid disaccharide subunits (McNeil et al. 1980). In contrast to HG, RGI usually harbors arabinan (α-1,5-linked), galactan (β-1,4-linked), or arabinogalactan side chains which are attached at the C4 position of the rhamnosyl unit (Fig. 1; McNeil et al. 1980). The side chains can be further decorated with ferulic and coumaric acid (Jones et al. 2003), as well as with smaller amounts of fucose, glucose, and glucuronic acid (O'Neill et al. 1990).

RGII has a highly complex structure and is composed of a backbone of at least seven α-1,4-linked-D-GalA units to which four different side chains may be attached (Vidal et al. 2000; Ridley et al. 2001). These side chains hold a variety of sugars, including the more unusual 2-keto-3-deoxy-D-manno-octulosonic acid, aceric acid, 2-keto-3-deoxy-D-lyxo-heptulosaric acid, and D-glucuronic acid (O'Neill et al. 2001). Thus, RGII has the richest diversity of sugars and linkages of any known polysaccharide (Darvill et al. 1978). In the plant cell wall, the pectins are associated with numerous processes, including fruit ripening (Dumville and Fry 2000), cell wall porosity (Baron-Epel et al. 1988), and cell–cell adhesion (Iwai et al. 2002).

2.2 Secondary Cell Walls

The secondary wall is a rigid structure that is deposited after the plant cell has reached its final morphological stage (Turner et al. 2001). This structure mainly consists of cellulose, xylan, the complex polyphenolic structure lignin, and

glycoproteins (Turner et al. 2001). The secondary cell wall confers strength to the encased plant cell and is the major constituent of tracheary elements, which facilitate nutrient and water distribution in higher plants (Turner et al. 2007). Lignified secondary cell walls represent the most abundant biomass component of many plants.

As is the case with the primary cell wall, cellulose microfibrils in the secondary cell wall are believed to be cross-linked by hemicellulosic polymers (Turner et al. 2001). Xylans are the primary hemicellulosic component of the secondary cell wall in dicot plants and the second most abundant biopolymer in plants overall (Turner et al. 2007). The structure of this polymer is shown in Fig. 1. Lignin is a complex polyphenolic polymer that is synthesized by oxidative cross-linking of monolignols. Indirect evidences suggest that hydrogen peroxide (H_2O_2) produced at the PM functions in lignin polymerization (Barceló et al. 2007). These data are supported by the fact that many peroxidases are present during secondary wall formation and may utilize the PM-associated H_2O_2 (Sato et al. 2006).

3 Golgi-Associated Cell Wall Metabolism

A wide array of biological molecules including proteins, hormones, secondary metabolites, and lipids can be modified with sugar moieties that alter the structural conformation of the molecules. These sugars are attached by an armada of glycosyltransferases that monitor structural and physiological requirements for sugar attachments (Lairson et al. 2008). The *Arabidopsis* genome contains more than 400 glycosyltransferases (http://www.cazy.org), and a large proportion of these are required to catalyze the 30 plus linkages observed in the cell wall matrix polysaccharides (Lairson et al. 2008). Many sugar transfer reactions are carried out in the Golgi apparatus where type II membrane glycosyltransferases utilize nucleotide-activated sugars as precursors to processively synthesize different carbohydrate linkages to form hemicellulosic and pectic polymers (Seifert 2004). One example of such type II GTs is the cellulose synthase (*CESA*) gene family (Richmond 2000; Fig. 3), part of the cellulose synthase superfamily which contains eight *cellulose synthase-like* gene families (*CSLA* to *CSLH*). The high degree of sequence similarity observed within the family suggests that all their gene products may synthesize β-1,4-linked, i.e., cellulose-like, backbones of matrix polysaccharides (reviewed by Lerouxel et al. 2006). These predictions have been confirmed for several members from different families, e.g., *CSLA* synthesizes the mannan backbones (Dhugga et al. 2004; Liepman et al. 2005), *CSLF* appears to synthesize mixed-linked glucans (Burton et al. 2006), and members from the *CSLC* family may synthesize the backbone in xyloglucans (Cocuron et al. 2007). These structures are subsequently decorated with other sugar residues and are shuttled to the cell surface by cellular trafficking mechanisms and exocytosis (Figs. 1 and 2; Geisler et al. 2008) where they are deposited in the cell wall matrix.

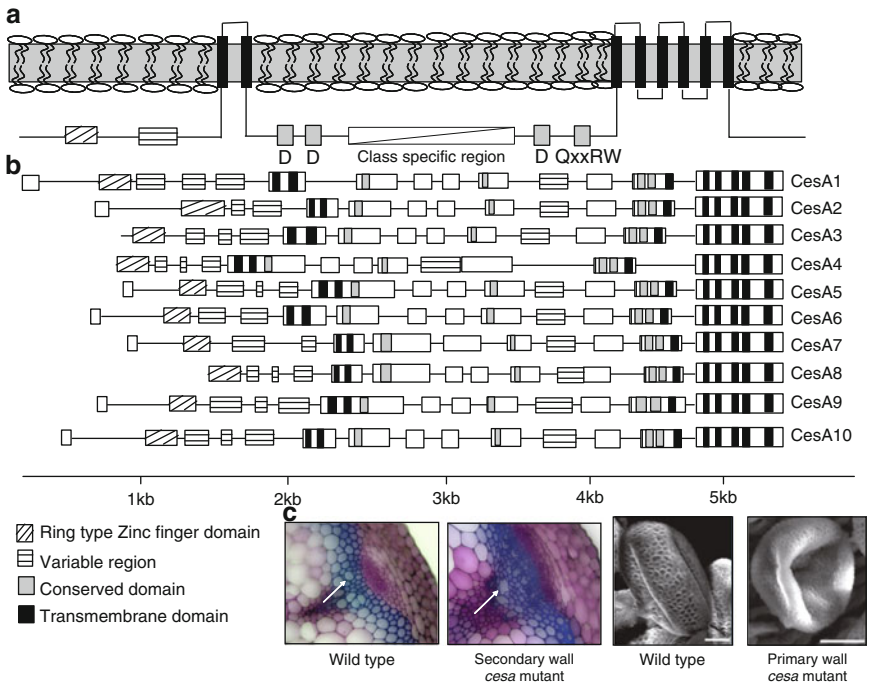

Fig. 3 *The cellulose synthase family*. (**a**) Schematic representation of a typical CESA protein. The approximate locations for different motifs and transmembrane domains are indicated. (**b**) Comparative sketch of the ten CESA proteins in *Arabidopsis*. The scale bar indicates relative size in kilo base pairs. (**c**) Mutant phenotypes for different CESA genes. *Left two panels:* mutations in *CESA4*, *7*, or *8* cause irregular xylem (*irx*) vessels. *Right two panels*: mutations in *CESA1* or *3* cause deformed pollen and result in male gametophytic lethality

Another example of glycosyltransferase activity is the attachment of the galactose residue of xyloglucans, which is the major hemicellulose of primary cell walls in dicots (Lerouxel et al. 2006). The glycosyltransferase that facilitates this attachment is another typical Golgi-localized type II GT (Zhong and Ye 2003; Li et al. 2004) that is encoded by the gene *MURUS3* (*MUR3*; Madson et al. 2003). Plants carrying point mutations in the catalytic site of *MUR3* have defects in xyloglucan synthesis, display epidermal cell swelling close to the apical hook (Pena et al. 2004), and have collapsed trichome papillae (Madson et al. 2003). Interestingly, a missense mutation (*kam1-1*) in *MUR3* and knockout plants of *MUR3*, referred to as *katamari1* (*kam1*), exhibit retarded growth and form large endosomal aggregates (Tamura et al. 2005). This phenotype appeared to be linked to the actin cytoskeleton since treatment of wild-type plants with latrunculin-B, an actin depolymerizing drug, mimicked the *kam1* phenotype (Tamura et al. 2005). These data suggest that the Golgi may have a way to communicate the status of polysaccharide synthesis to actin filaments but could also be due to secondary effects, such as accumulation of insoluble intermediates in the Golgi. In any case, the *MUR3* studies show that

polysaccharide synthesis in the Golgi may be linked to cytoskeletal components and vesicle shuttling to the PM. More comprehensive reviews of glycosyltransferase activity and the synthesis of specific polymers in the Golgi can be found in Keegstra and Raikhel (2001) Scheible and Pauly (2004), Seifert (2004), Lerouxel et al. (2006), and Obel et al. (2006).

4 Apoplastic Components and Processes

Proteins secreted to the apoplastic space are synthesized in the endoplasmic reticulum (ER) and generally contain N-terminal secretion signals (see also chapters "Plasmodesmata and Non-cell Autonomous Signaling in Plants" by Lee et al. and "Plasma Membrane ATPases" by Palmgren et al.). These proteins include enzymes, such as glycosyl hydrolases (http://www.cazy.org; Coutinho and Henrissat 1999), and expansins (http://www.bio.psu.edu/expansins; Kende et al. 2004; Cosgrove 2005), as well as nonenzymatic, structural polypeptides (Carpita and McCann 2000). The latter may be divided into four classes: extensins, proline-rich proteins (PRPs), arabinogalactan proteins (AGPs; Seifert and Roberts 2007; Showalter 2001), and glycine-rich proteins (GRPs), of which the latter generally do not contain carbohydrate moieties. Both enzymatic and structural proteins can be found associated with the plasma membrane linked by a glycosylphosphatidylinositol (GPI) anchor (Fig. 2).

4.1 Expansins and Glycosyl Hydrolases

After secretion, enzymatic proteins such as glycosyl hydrolases (Minic and Jouanin 2006) or expansins may modify sugar chains or interactions between different carbohydrate polymers in the cell wall (Cosgrove 1997). Glycosyl hydrolases and expansins are thought to maintain the stability and plasticity of the cell wall by trimming matrix polysaccharides and loosening hydrogen bonds between cellulose microfibrils and hemicelluloses, which in turn allows the integration of newly formed polysaccharides in the expanding cell wall network (Sampedro and Cosgrove 2005).

The expansin superfamily is divided into four clades based on sequence homology: α-expansins (EXPA), β-expansins (EXPB), and α- and β-expansin-like (EXLA, EXLB) proteins. All expansins share two conserved domains and an N-terminal secretion signal. Domain I is similar to the catalytic domain of glycosylhydrolase family 45, while domain II is similar to a group 2 grass pollen allergen domain of unknown function (Cosgrove 2000). Initially α-expansins were identified as cell wall loosening agents that propagated "acid-induced growth" (McQueen-Mason et al. 1992). Members of the α-expansin family usually show a pH optimum ~4, whereas the β-expansins tend to have a broader pH range with a

peak pH ~5.5 (Li et al. 2003b). These data and expression patterns of genes encoding β-expansins suggest that β-expansins are involved in pollen tube growth and α-expansins are associated with acid-mediated growth.

The pH of the cell wall is mainly determined by PM-localized H^+-ATPases and is usually around 5.5 (see the chapter "The Role of Plasma Membrane Nitrogen Transporters in Nitrogen Acquisition and Utilization" by Tsay and Hsu) but can decrease about 1 unit in response to stimuli, most prominently auxin treatment (Rayle and Cleland 1992 and references within). Such pH change promotes expansin-driven polymer creep and thus expansion. Expansins have been isolated from growing tissues such as the elongation zone of roots in soybean (Lee et al. 2003), elongating cotton fibers (Orford and Timmis 1998) and tomato fruits (Brummell et al. 1999), and germinating maize pollen grains (Cosgrove et al. 1997), suggesting that cell wall expansion is active in a variety of developmental processes and tissue types.

Arabidopsis harbors approximately 380 glycosyl hydrolases, which degrade different polymers in the apoplast during plant growth and development and also in response to pathogens (Minic and Jouanin 2006). The glycosyl hydrolases are divided into 113 classes according to sequence homology and structure (http://www.cazy.org; Henrissat et al. 2001). One class of enzymes that is involved in cell wall hydrolysis and reorganization is xyloglucan endo-transglycosylase/hydrolases (XTHs; Rose et al. 2002). Some XTHs not only have the exceptional ability to function as endohydrolases of xyloglucan chains but also can transfer and reconnect the newly generated reducing end to another xyloglucan chain in a process often referred to as molecular grafting (Fry 2004). Xyloglucan is thought to tether cellulose microfibrils and maintain their spatial arrangement (see Sects. 2.1 and 2.2). Thus, XTHs are able to strengthen, maintain, or loosen the hemicellulosic cross-links between cellulose microfibrils, and thereby, modulate the plasticity of the cell wall. *Arabidopsis* contains 33 *XTH* genes encoding XTH enzymes, which cluster in the glycoside hydrolase family GH16 (http://www.cazy.org). *XTH*s are differentially expressed throughout development (Yokoyama and Nishitani 2001), and their expression responds to stimuli such as mechanical disturbance and multiple hormones (Yokoyama and Nishitani 2001). However, it appears that there is a certain amount of redundancy in XTH regulation and substrate specificity, which, in turn, renders the assignment of an exact physiological role for each individual XTH difficult. The *XTH* gene family exemplifies the redundancy of cell wall-related genes which generally complicates the attempts to study and dissect the function of single family members in plant physiology.

4.2 Nonenzymatic Apoplastic Proteins

Hydroxyproline-rich glycoproteins (HRGPs), PRPs, extensins, and GRPs are non-enzymatic apoplastic proteins that are enriched in the amino acids hydroxyproline/proline (Hyp/Pro), serine/threonine (Ser/Thr), and glycine (Gly). Approximately 10% of the dry weight of a typical cell wall is provided by wall proteins, depending

on the tissue and species. Except for the GRPs, these proteins are highly glycosylated and contain glycan moieties with different composition and complexity (Farrokhi et al. 2006). Their glycan chains are thought to link the proteins to cell wall polymers (pectins and hemicelluloses) and to promote structural integrity. During protein maturation in the ER and the Golgi, sugar moieties are attached to the proteins by distinct Golgi-localized membrane-bound type II GTs (Keegstra and Raikhel 2001; see the chapter "Plasma Membrane ATPases" by Palmgren et al.).

HRGPs are enriched in the amino acids Hyp/Pro, Ser/Thr, and alanine (Ala). Arguably the most prominent types of HRGP are the AGPs, which constitute a subclass among the HRGPs (Seifert and Roberts 2007). In classical AGPs, the glycan chains account for the vast proportion of the molecular weight (>90%; Du et al. 1996) and are attached to the protein via O-glycosylation to hydroxyproline residues in the central domain of the protein (Oxley and Bacic 1999). The glycan chains typically hold highly branched arabinogalactan type II composed of D-galactosyl pyranoses (β-1,3- and β-1,6-linked) with terminal L-arabinosyl furanoses, which are α-1,3-linked to the β-1,6-Gal*p* (Oxley and Bacic 1999). The side chains can further be decorated not only with β-1,6-linked D-glucuronic acid or α-1,4-linked L-rhamnose (Tan et al. 2004) but also with L-fucose and D-xylose (Oxley and Bacic 1999). Variation in glycan chain length is as extensive as variation in sugar content. While Qi et al. (1991) found carbohydrates with an average chain length of 30 sugar residues in gum arabic, Kawasaki (1987) isolated AGPs from cultured tobacco cells with a chain length of 46–140 sugar residues and a 70% saturation of hydroxyproline residues. This may indicate that the carbohydrate moieties can be metabolized and modified during development. AGPs are thought to be involved in cell wall elongation (Park et al. 2003) and other morphogenic processes (reviewed by Showalter 2001). Despite some similarities in carbohydrate and protein sequences, the AGPs are a diverse group of proteins in both sequence and modes of action. The most unifying feature may be the recognition by the β-glycosyl Yariv reagent (Nothnagel 1997) and by antibodies against their glycan epitopes (Seifert and Roberts 2007). Subgroups of AGPs, including the fasiclin-like AGPs (FLAs), have been shown to contain GPI anchors (Oxley and Bacic 1999; Borner et al. 2005; Murphy et al. 2002), which suggests that these AGPs function at the interface of the PM and cell wall and may serve to anchor membrane complexes (Murphy et al. 2002).

4.3 Glycosylphosphatidylinositol Anchors

GPI-anchored proteins (Schultz et al. 1998) are found in plants, animals, and fungi (see also chapters "Plasma Membrane Protein Trafficking" by Peer, "Plasmodesmata and Non-cell Autonomous Signaling in Plants" by Lee et al., and "Plasma Membrane ATPases" by Palmgren et al.). The synthesis and transfer of the anchor to the C terminus of a protein are associated with different stages of protein maturation in the rough ER. The initial reactions involved in GPI synthesis in animals appear to

take place on the cytosolic side of the ER (Udenfriend and Kodukula 1995) where phosphatidylinositol (PI) is attached to a lipid, e.g., diacylglycerols, alkylacylglycerols, or ceramides (Schultz et al. 1998). This glucosamine-PI anchor is then extended with three mannosyl residues inside the ER (Kinoshita et al. 1997). Finally, a terminal phosphoethanolamine is added, which can form an amide bond to the C terminus of the protein after trimming of the protein at the cleavage site ω behind a GPI-anchor signal sequence has occurred (Schultz et al. 1998). A similar process is thought to occur in plants but has not been definitively elucidated to date.

Oxley and Bacic (1999) determined the structure of GPI anchors derived from AGPs of pear suspension cells and found that substitutions of the second mannosyl group sometimes occur. Similar substitutions have also been seen in rat (β-*N*-acetylgalactosaminyl, Homans et al. 1988) and *Trypanosoma* (α-(1,3)-galactosyl, Ferguson et al. 1988). Disruption of GPI anchor synthesis affects a variety of proteins and leads to severe phenotypes, such as *peanut1*, an *Arabidopsis* homolog of the human PIG-M, which transfers the first mannosyl residue to the premature GPI anchor (Gillmor et al. 2005). Further studies of AGPs from pear suspension cells (Oxley and Bacic 1999) revealed that the lipid anchor itself contained tetracosanoic acid, an indicator for ceramides, which may function as signaling molecules in various stress and developmental processes (Sawai and Hannun 1999). It is therefore apparent that some AGPs may be released by phospholipases, i.e., PLC and PLD, in cell signaling events involving either the released lipid moiety or protein (Oxley and Bacic 1999). In addition, the GPI-anchored proteins may facilitate interactions with other PM-associated proteins (Schultz et al. 1998) as was shown for contactin, which is part of a signal transduction complex that mediates signals between glia and neuron cells in rats (Peles et al. 1997). In a proteomic survey of *Arabidopsis* callus cells, 30 GPI-anchored proteins were identified (Borner et al. 2003). Among those were fasiclin-like and other AGPs, endoglucanases, COBRA-like proteins, and receptor-like kinases (RLKs).

5 Callose

The polymer callose consists of β-1,3-linked glucan molecules that appear to form helical duplexes and triplexes, which form a gel when heated. Plants can utilize this property to protect and seal the plasma membrane from physical damage, for example, in response to fungal infection (Vorwerk et al. 2004). Callose is produced during cytokinesis in plants and multicellular green algae (Scherp et al. 2001) and is also produced during cell plate formation in dividing cells (Hong et al. 2001). After cell division has ceased, callose is degraded and rapidly replaced by cellulose as the cell plate fuses with the parental cell (Verma 2001). Callose is also deposited in pollen tube walls during germination and growth of pollen tubes (Dumas and Knox 1983). Although callose constitutes up to 86% of the dry weight in specialized cell walls such as pollen tubes and is deposited in response to wounding or stress (Stone and Clark 1992), the relationship of these responses to specific *callose synthase*

(*CalS*) genes remains largely obscure. *Agrobacteria*, for instance, synthesize curdlan, a β-1,3-D-glucan cell wall polymer. Owing to sequence homology between the *curdlan synthase* (*CrdS*) and *CESA* genes (Stasinopoulos et al. 1999), it has been generally assumed that the callose synthase may be found in one of the related *CSL* families described above. However, although the CESA and CalS proteins share some common features such as the putative catalytic domain (Verma and Hong 2001; Fig. 3), the CalS proteins are approximately 2,000 amino acids in length and are, thus, considerably longer than the CESAs. Additionally, fungal genes encoding 1,3-β-D-glucan synthases (Douglas et al. 1994) were suspected to encode CalS. Homologs of these *glycan synthase-like* (*GSL*) genes are also found in plants (Doblin et al. 2001). However, more recent studies from barley (Li et al. 2003a) and tobacco (Brownfield et al. 2007) provide genetic and biochemical evidence indicating that *GSL*s encode CalS proteins. The *Arabidopsis* genome contains 12 *GSL* genes, which appear to function in CalS complexes in various tissues (Hong et al. 2001). The CalS complex (200–220 kDa; Brownfield et al. 2007) is located at the plasma membrane and contains 14–16 transmembrane domains, a large central cytoplasmic loop that separates two major membrane regions, and an N terminus facing the cytoplasm (reviewed by Verma and Hong 2001).

6 Cellulose

The β-1,4-linked glucan polymer cellulose is the most abundant biopolymer on the planet and constitutes the main load-bearing structure in the plant cell wall (Somerville 2006). The cellulose microfibril is thought to consist of 36 hydrogen-bonded glucan chains, which presumably are synthesized by a PM-localized CESA complex. These chains are insoluble and consist of 500–14,000 linked glucose molecules that are laid down in a twisting, ribbon-like chain (Fig. 3).

6.1 The Cellulose Synthase A Complex

Cellulose synthase A (CESA) complexes contain several structurally similar subunits, which are the only proteins that have been directly associated with cellulose synthesis (Somerville et al. 2004; Somerville 2006). The CESA genes were originally discovered through weak sequence similarities to bacterial cellulose-synthesizing genes (Pear et al. 1996) and belong to glycosyl transferase family 2, which uses nucleotide-diphospho-α-D-sugars to produce β-linked polysaccharides (Henrissat et al. 2001). CESA proteins are believed to form a complex in the Golgi (Taylor et al. 2000) which is subsequently delivered to the cell surface via vesicle trafficking (Paredez et al. 2006). PM-associated CESA complexes have been visualized by immunogold labeling and are arranged as hexagonal "rosettes" measuring approximately 25 nm in diameter (Kimura et al. 1999). It has been speculated that each subunit in the rosette synthesizes six β-1,4-glucan chains (Herth 1983).

The *Arabidopsis* genome contains ten *CESA* genes (Richmond 2000; Somerville et al. 2004; Fig. 3). The gene products of at least three of these are necessary to form functional rosette complexes (Taylor et al. 2000; Desprez et al. 2007). *CESA4*, *7*, and *8* encode CESA proteins that are involved in secondary wall cellulose production in *Arabidopsis* (Turner and Somerville 1997; Taylor et al. 2000), and *CESA1*, *3*, and *6* are required for primary wall cellulose biosynthesis (Arioli et al. 1998; Beeckman et al. 2002; Gillmor et al. 2002; Desprez et al. 2007; Persson et al. 2007). However, one of the subunits, CESA6, appears to be exchangeable in the primary wall rosette (Desprez et al. 2007; Persson et al. 2007). This position is suggested to be occupied by CESA6-like CESAs, i.e., CESA2, 5, and 9, depending on tissue and cell type.

Genes encoding putative CESAs have also been identified in poplar (seven *CESA* genes; Joshi et al. 2004), maize (12 *CESA* genes; Appenzeller et al. 2004), barley (at least eight *CESA* genes; Burton et al. 2004), and rice (at least ten *CESA* genes; Richmond 2000). Based on sequence and mutant analysis of *CESA* genes the existence of two trimeric complexes for primary and secondary walls was identified in other plant species as well (Tanaka et al. 2003; Burton et al. 2004).

6.2 Structural Organization of the CESA Proteins

The CESA proteins contain 985 to 1,088 amino acids in *Arabidopsis* (Richmond 2000) and are predicted to harbor eight transmembrane (TM) domains. These predictions have been confirmed by proteomic analyses (Nühse et al. 2004). Two of the TM domains are predicted to be located approximately 300 amino acids from the N terminus, and the remaining six are positioned closer to the carboxy terminal. The extreme N-terminal portion of the proteins is cytoplasmic and contains a RING-type zinc finger domain or cysteine-rich LIM transcription factor motif. This domain is predicted to function in protein–protein interactions between the individual CESAs (Saurin et al. 1996), which have been experimentally corroborated by expression of the N terminus of cotton CESA proteins in *Escherichia coli* (Kurek et al. 2002).

The two N-terminal TMs are followed by a large globular region of ~500 amino acids that is presumably cytoplasmic (Delmer 1999). This region comprises highly conserved amino acid motifs that are common to all CESA proteins. One such conserved amino acid stretch is the D, D, D, QXXRW motif (Saxena et al. 1995) that constitutes a classical processive glycosyl transferase domain that is thought to transfer sugar residues to the growing end of the polysaccharide backbone. Deletion of the first conserved D residue in the GhCESA1 processive motif was shown to abolish binding of UDP-Glc to the protein, and site-directed mutation of the chitin synthase gene in yeast and SpsA in *Bacillus subtilis* has shown that the conserved Asp motif is required for substrate binding and catalysis (Nagahashi et al. 1995; Charnock et al. 2001). This portion of the protein also contains a hypervariable region of approximately 65–90 amino acids.

6.3 Cellulose Synthesis

Cellulose was first reported to be synthesized from UDP-glucose in vivo and in vitro in *Acetobacter xylinum* (Hestrin et al. 1947). The rate of synthesis and turnover of UDP-glucose in higher plants like cotton corresponds well with rates of cellulose accumulation in the secondary cell wall (Carpita and Delmer 1981). UDP-glucose is generated by the sucrose synthase (SuSy) machinery. Approximately half of the SuSys in cotton fibers were found to be associated with the PM and may be involved in channeling carbon from sucrose to β-glucan synthases such as cellulose and callose (Amor et al. 1995). However, various combinations of mutations in genes encoding SuSys did not produce the dramatic phenotypes (Bieniawska et al. 2007) seen in cellulose-deficient mutants (see below).

It has also been suggested that sterol glucosides might act as primers for cellulose synthesis (Peng et al. 2002). In this model, a glucose residue from cytoplasmic UDP-glucose may be transferred to membrane-bound sitosterol resulting in sitosterol-β-glucoside (see the chapter "Plasma Membrane Protein Trafficking" by Peer). This molecule could subsequently be utilized by CESA proteins to produce cellulose. In addition, lipid-linked cellodextrins are increased in the *korrigan* mutant (Sato et al. 2001), which is defective in a putative endoglucanase (see below). It is therefore plausible that KORRIGAN may hydrolyze the glucan residues from a sterol-linked primer.

It has also been hypothesized that microtubules may guide CESA complexes during cellulose production (Heath 1974). Recent reports support this hypothesis. Paredez et al. (2006) convincingly showed that YFP-labeled CESA6 particles at the PM moved along the microtubules. Disruption of the microtubules by oryzalin, a microtubule-destabilizing drug, also disrupted the orientation of cellulose deposition initially (Paredez et al. 2006). Analogous studies during secondary cell wall formation in developing xylem vessels indicate that microtubules affect cellulose deposition and also that actin may be involved in trafficking of CESAs to the cell surface (Wightman and Turner 2008). The role of cytoskeleton in directing microfibril formation is discussed in Chapter 3.

6.4 Mutational Analyses of Cell Wall Formation

6.4.1 Mutations Affecting Cellulose Formation in the Primary Cell Wall

A vast number of mutants that are deficient in cellulose biosynthesis have been identified (Somerville 2006). These mutants typically exhibit severe growth retardations (e.g., Gillmor et al. 2002; Roudier et al. 2005) or impaired secondary wall integrity (e.g., Turner and Somerville 1997; Brown et al. 2005). The primary wall CESAs are essential for plant growth, and mutations in *CESA1* or *CESA3* result in either gametophytic or embryo lethality (Beeckman et al. 2002; Gillmor et al. 2002;

Persson et al. 2007; Desprez et al. 2007; Mutwil et al. 2008). For example, the temperature-sensitive allele *rsw1-1* (*radial swelling 1-1*) affects *CESA1* and results in swollen cells in the cotyledon and a reduction in cellulose production compared to wild type when grown at 31°C (Arioli et al. 1998). The temperature shift also leads to loss of CESA complexes at the plasma membrane, suggesting that the CESA1 is needed for rosette assembly (Arioli et al. 1998; Williamson et al. 2001). In addition, the point mutations *rsw1-2* and *1-20* affect the catalytic domain of CESA1 and result in embryo lethality (Gillmor et al. 2002; Beeckman et al. 2002). Several other point mutations in *CESA1* and *CESA3* have established links between ethylene and jasmonic acid synthesis and cellulose production (Ellis et al. 2002), and other apparent null mutations result in pollen deficiencies (Persson et al. 2007). However, mutations such as *prc* (*procuste*) in the third primary wall *CESA* protein, CESA6, exhibit only slight dwarfism and lateral cell swelling in seedling roots and etiolated seedlings (Fagard et al. 2000). As noted above, recent studies indicate that these inconsistencies in mutant phenotypes among primary wall CESAs may be due to functional compensations of CESA6-like CESAs, i.e., CESA2, 5, and 9 (Desprez et al. 2007; Persson et al. 2007). Of the latter CESAs, CESA2 and CESA5 were shown to be able to partially rescue *prc* mutants when driven by the CESA6 promoter.

Apart from the *CESA* genes, several other mutants have been identified which affect primary wall cellulose production. Mutations in *KORRIGAN* (*KOR*) are dwarfed and exhibit anisotropic cell swelling (Nicol et al. 1998; Lane et al. 2001; Sato et al. 2001). *KOR* was found to encode a putative endo-(1,4)-β-D-glucanase and is expressed heavily in rapidly growing regions and localized to the PM (Brummell et al. 1997; Zuo et al. 2000). Although the in vivo function of KOR is presumed, the soluble domain of KOR from *Brassica napus* expressed in yeast hydrolyzed cellulose, while other polymers such as xyloglucans were unaffected (Mølhøj et al. 2001). In addition, a KOR ortholog from poplar was found to have the same activity (Master et al. 2004). Other mutant alleles corresponding to KOR have also been identified, including the temperature-sensitive *rsw2* (Lane et al. 2001), *lions tail* (*lit*) (Hauser et al. 1995), and *altered cell wall1* (*acw1*) (Mølhøj et al. 2002). These *kor* mutants generally exhibit reduced levels of cellulose and may be defective in cytokinesis (Zuo et al. 2000). A direct interaction between the CESAs and KOR has been proposed (Somerville 2006). However, KOR does not copurify with secondary or primary CESA proteins, suggesting that its interactions with these proteins are not strong (Szyjanowicz et al. 2004; Desprez et al. 2007).

COBRA is a GPI-anchored protein that affects cellulose deposition and production (Schindelman et al. 2001; Roudier et al. 2005). Reporter gene fusions with the *COBRA* promoter showed that *COBRA* is highly expressed in rapidly expanding tissues that correspond to high levels of cellulose deposition. COBRA was detected in discrete domains adjacent to the PM where it was oriented perpendicular to the longitudinal growth axis similar to cortical microtubules (Roudier et al. 2005). Putative null mutants of *COBRA* lead to embryo lethality and disorganized cellulose microfibrils. Based on the timing of disorganization and reduction in cellulose

synthesis, it has been suggested that COBRA may provide a feedback system for the disordered cellulose microfibril (Roudier et al. 2005).

The PM-localized protein KOBITO (KOB) also affects cellulose production and mutations in the gene result in embryo lethality (Pagant et al. 2002). The *kob* mutant exhibits randomized cellulose microfibrils that appeared to be blocked by pectic material (Pagant et al. 2002). Additional *KOB* alleles have been identified, such as *elongation deficient* (*eld*; Lertpiriyapong and Sung 2003) and *abscissic acid-insensitive8* (*abi8*; Brocard-Gifford et al. 2004). However, the function of KOB remains elusive and is further complicated by dissimilar localizations observed for the protein in two different studies. In one study, the protein was reported to be localized in the apoplast (Lertpiriyapong and Sung 2003), but in another study it was reported to display a punctate cytoplasmic pattern in the root elongation zone (Brocard-Gifford et al. 2004).

Other mutants that have cellulose deficiencies include *pom1*/*ctl1* (Hauser et al. 1995; Zhong et al. 2002), *knopf* (Gillmor et al. 2002), and *pnt* (Gillmor et al. 2005).

6.4.2 Mutations Affecting Secondary Cell Wall Cellulose

The first mutant that was shown to affect secondary cell wall formation in *Arabidopsis* was the *tbr* (*trichome birefringence*) mutant (Potikha and Delmer 1995). Chemical analyses of the mutant revealed that it was unable to deposit cellulose in specific cell types such as trichomes, while the cellulose content in, for example, the xylem vessels remained unaffected (Potikha and Delmer 1995). Subsequent forward genetic screens have generated a variety of *Arabidopsis* mutants that exhibit collapsed xylem vessels (Turner and Somerville 1997). These mutations were coined *irregular xylem* (*irx1*, *2*, *3*, and *5*) as the xylem vessels in these mutants are not able to withstand the negative pressure generated during water transport in the stem. The cellulose content in some of these mutants was reduced by nearly 70% when compared to wild type (Turner and Somerville 1997), while the primary wall cellulose levels remained unaffected (Turner and Somerville 1997). Electron microscopic analyses of the vascular cell walls showed them to be thin and of uneven thickness (Turner and Somerville 1997). The genes affected in *irx1*, *3*, and *5* encode the three secondary CESAs, *CESA4*, *7*, and *8* (Taylor et al. 1999, 2000, 2003). Since all three mutants exhibit similar phenotypes and expression patterns, it has been suggested that the function for each of these genes is unique. Mutations in *CESA7* and *8* were also found among the *fragile fiber* (*fra*) group that exhibit reduced mechanical stem strength (Zhong et al. 2003). Corresponding secondary cell wall mutants have also been found in rice (Tanaka et al. 2003), and, based on expression patterns, similar secondary wall CESA triplexes are anticipated in other species including poplar (Geisler-Lee et al. 2006) and barley (Burton et al. 2004). More recently, several other *irx* mutants were obtained through transcriptional coordination analyses, and several of these were shown to be associated with the synthesis of xylans (Brown et al. 2005; Persson et al. 2005).

The similarity between primary and secondary wall cellulose synthesis in the form of the two orthologous rosette triplexes is further corroborated by other mutations. For example, mutations in the *COBRA-like 4* (*COBL4*) gene cause secondary wall cellulose deficiencies (Brown et al. 2005), reminiscent of the *cobra* mutations in the primary wall. In addition, a *CTL1-like* gene (*CTL2*) is coexpressed with the secondary *CESA*s, suggesting that *CTL*s represent another orthologous gene pair that is associated with the primary and secondary wall cellulose synthesis. Interestingly, mutations in *KOR* may cause cellulose deficiencies in both primary and secondary walls, as well as cell (wall) integrity (Szyjanowicz et al. 2004). Mutants from the *fra* group have also linked secondary cell wall production with phosphoinositides, microtubules, and actin filaments (Zhong et al. 2004).

6.5 Inhibitory Drugs

Several drugs that affect cellulose production have been described. One of the well-characterized drugs is the herbicide isoxaben, which appears to either directly or indirectly interact with the CESAs (Scheible et al. 2001). This was inferred through forward genetic screens for isoxaben-insensitive mutants in which mutations in *CESA3* and *CESA6* were found to confer resistance to the drug (Scheible et al. 2001; Desprez et al. 2002). These mutants were referred to as *isoxaben-resistant* (*ixr*) *1* and *2*, respectively. Isoxaben has subsequently been shown to completely abolish CESA complexes from the PM (Paredez et al. 2006; DeBolt et al. 2007a) and to destabilize the microtubules (Wasteneys 2004; Paredez et al. 2008).

Another drug widely used to disrupt cellulose production is 2,6-dichlorobenzonitrile (DCB; Hogetsu et al. 1974). Although the target of this drug is not known, DCB appears to induce CESA complex accumulation at distinct foci at the cell surface (DeBolt et al. 2007a), suggesting a role in vesicle shuttling or actin stabilization. Among the other drugs morlin, a coumarin derivative with unknown mode of action (DeBolt et al. 2007b), and thaxtomin A, a phytotoxin that causes severe root swelling and inhibits cellulose production at nanomolar concentration (Scheible et al. 2003). Forward genetic screens have identified at least one locus, *txr1*, which is resistant against the drug. The corresponding gene *TXR1* was found to encode a novel protein of unknown function (Scheible et al. 2003). In addition, a recently discovered drug cobtorin causes disruption of cortical microtubules and also inhibits cellulose production (Yoneda et al. 2007).

7 Cell Wall Signaling

It is apparent that the synthesis of cell wall components occurs in two different compartments and that the cell wall structure needs to be flexible and responsive. It is therefore evident that there has to be quite an elaborate feedback system between

the two synthesizing compartments. In addition, the building blocks for the cell wall components are supplied by a multitude of metabolic pathways that also need to sense such demands. Recent reports show that several RLKs may sense the integrity of the cell wall and transmit signals that notify the cell's interior about the status of the wall.

7.1 Receptor-Like Kinases

RLKs comprise a group of signal mediators with more than 600 members in *Arabidopsis* and over 1,100 genes in rice (Shiu et al. 2004). Hence, the RLKs comprise the largest gene family of receptors in plants and are further divided into several subclasses according to their kinase domains (reviewed by Morillo and Tax 2006). RLKs are involved in the regulation of various different processes such as perception of brassinosteroids (Kinoshita et al. 2005), determination of meristem organization (Suzaki et al. 2004), and perception and response to pathogen attack (Gómez-Gómez et al. 2001).

Recently, a RLK, THESEUS (THE1), which appears to sense cell wall integrity, was identified (Hématy et al. 2007). Mutations in *THE1* suppress growth defects in several cellulose-deficient mutants, such as *prc1-1*. Interestingly, the suppression was not due to increased cellulose production (Hématy et al. 2007; Fig. 4b), suggesting that THE1 may tell the cell to grow despite the lack of cellulose production. The mutations in *THE1* appeared to activate various pathogen-related genes as assessed by microarrays (Hématy et al. 2007). THE1 is a member of the CrRLK family that also holds FERONIA, which affects pollen tube growth cessation after it has reached the female gametophyte (Escobar-Restrepo et al. 2007). It is anticipated that a multitude of other RLKs also may contribute to the signaling between the cell wall and the interior of the cell (Hématy and Höfte 2008).

7.2 Wall-Associated Kinases

Wall-associated kinases (WAKs) were initially discovered through immunolabeling of the cell wall in plasmolyzed cells using antibodies against a serine/threonine kinase domain (Kohorn et al. 1992). Kohorn et al. then isolated the first cDNA that corresponded to a wall-associated kinase (*WAK1*), which later was shown to be PM associated (He et al. 1999). WAKs generally contain a TM domain that connects the cytoplasmic C-terminal serine/threonine kinase domain to an apoplastic domain that consists of an epidermal growth factor (EGF)-related motif (He et al. 1999; Fig. 4a). This motif is followed by a divergent N-terminal segment. EGF-like repeats generally contain six cysteine residues involved in the formation of three disulfide bonds that change the conformation of the domain. Work on EGF repeats in metazoans has shown that these repeats facilitate protein–protein interactions

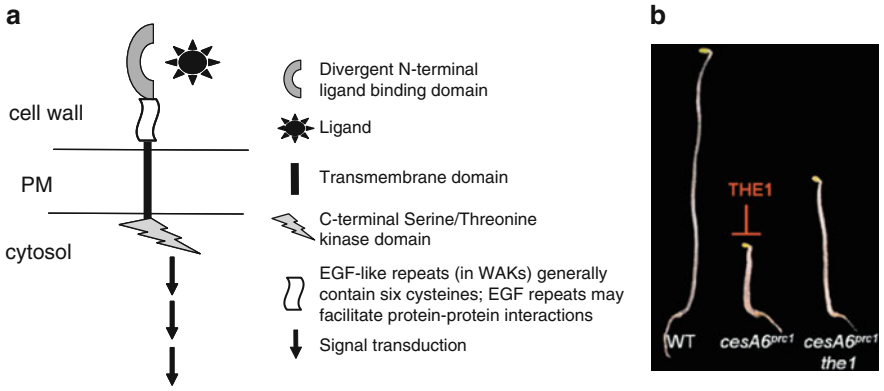

Fig. 4 *Receptor-like kinases sensing cell wall alterations*. (**a**) Schematic model of an RLK. The RLK may bind a ligand that will cause a downstream signal cascade. The EGF is found in the WAKs. (**b**) The RLK THE1 suppresses the cellulose-deficient mutant *prc*. Mutations in the *THE1* result in restoration of growth in the *prc1-1* background (taken from Hématy et al. 2007)

(Davis et al. 1987). Consistent with these results the N terminus of WAK1 could bind to cell wall-localized GRPs in *Arabidopsis* (Park et al. 2001). The notion that WAKs may interact with cell wall components was further corroborated by experiments in which an unpurified cell wall degrading enzyme solution was able to release WAKs from the wall (He et al. 1996). It was later shown that the cell wall component responsible for this interaction is likely to be a pectin, as pectinase could release the WAK from the cell wall fraction (Wagner and Kohorn 2001). In addition, signals from western blots using antibodies against HG (JIM5, JIM7; Knox 1997) coincided with WAKs (Wagner and Kohorn 2001).

WAK1 clusters together with four other RLK genes on chromosome 1 (*WAK2*–*WAK5*; He et al. 1999). All of them contain 11 highly conserved serine/threonine domains and share 86% identity over the entire C-terminal part. The extracellular N-terminal portion of the protein is more variable (40–64% sequence identity). The *WAK*s are generally expressed at organ junctions, in root and shoot apical meristems, and in expanding tissues such as leaves (Wagner and Kohorn 2001). Further expression analyses showed that *WAK1*, 2, *3*, and *5* are mainly expressed in leaves and stems, while *WAK4* is specifically expressed in siliques. Plants transformed with an antisense construct targeting all *WAKs* were dwarfed, without alterations in cell number suggesting a reduced cell expansion (Wagner and Kohorn 2001). Transgenic RNA interference (RNAi) plants targeting either *WAK1* or *WAK2* confirmed that the WAKs may regulate cell expansion, and promoter GUS fusions suggested that these WAKs have distinct, but perhaps partially overlapping, functions (He et al. 1999; Wagner and Kohorn 2001).

WAK1 appears to be induced by pathogen stress (He et al. 1998), and pathogen-induced expression of *WAK1* to *3,* and *WAK5* could be mimicked by treatment with salicylic acid (SA) and 2,2-dichloroisonicotinic acid (INA), which is an analog of SA (He et al. 1998, 1999). In a yeast two-hybrid survey, Park et al. (2001) provided

data showing that the extracellular domain of WAK1 binds specifically to GRP3 without interacting with the over 50 other GRPs present in *Arabidopsis*. Furthermore, Park et al. (2001) showed that WAK1 forms complexes with GRP3 and another kinase-associated protein phosphatase (KAPP), which interacts with several other kinases (Braun et al. 1997). Interestingly, *wak2* null mutants displayed reduced expression of a vacuolar invertase that mediates solute status and may connect WAK function to cell expansion (Kohorn et al. 2006). Taken together, these results suggest that WAK may function in transcriptional regulation and may link cell wall-associated biotic and abiotic stimuli with gene expression.

8 Outlook

The last 5 years have seen a considerable increase in the understanding of the composition and synthesis of the cell wall. However, over 1,000 genes in the *Arabidopsis* genome are believed to be associated with the synthesis and modification of the cell wall (Somerville et al. 2004), and it is therefore clear that we are still just scratching the surface. For example, very little is currently known about the components and the mechanisms, for how the need of synthesis, or modification, of cell wall constituents is transmitted, and how the components are transported from the Golgi to the cell surface. In addition, very little is known about the transcriptional regulation of cell wall synthesizing and modifying genes. Together with a continued effort to dissect the synthetic cell wall apparatus, these tasks may shed new light into how the cell wall is constructed and re-modeled.

It is likely that such efforts will be considerably expanded in the near future as a result of tremendous increases in funding of plant cell wall research driven by increased demand for biofuels and enhanced biomass production. Major new research institutes dedicated to elucidating cell wall biology have been formed in the United States utilizing funding streams from government, private foundations, and industry. The future of the cell wall field therefore looks quite bright, and we believe that there will be considerable advances in the field in the near future.

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Plasmodesmata and Non-Cell-Autonomous Signaling in Plants

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Abstract Plasmodesmata are fundamental intercellular communication channels in plants that are essential for coordination of physiological and developmental signaling processes across cellular boundaries. The fact that creation of these membranous structures is considered one of the most crucial factors in the evolution of higher plants clearly states their unparalleled significance in plant systems. Plasmodesmata are also unique in their structure, in that they establish an endomembrane as well as a cytoplasmic continuum throughout the whole plant body by forming cytoplasmic strands that are lined with plasma membrane externally and endoplasmic reticulum internally. These structures, when assembled during cytokinesis by entrapment of endoplasmic reticular strands in the expanding cell plate, are called primary plasmodesmata. It is remarkable that plant cells have acquired an additional mechanism to produce secondary plasmodesmata by de novo biosynthesis postcytokinetically across existing cell walls, which in essence is necessary to maintain or increase symplasmic connectivity between expanding cells. This process is thought to occur through cell wall loosening and membrane fusion followed by deposition of new cell wall materials around the nascent protoplasmic strands. Perhaps, it is one of the most fascinating discoveries in plant biology that plasmodesmata are highly dynamic channels with the capacity to dilate and facilitate macromolecular trafficking despite the physical constraint imposed by the surrounding cell wall. It is also this activity through which plasmodesmata can act as supracellular checkpoint over intercellular transfer of signaling or information molecules. Exciting future discoveries in plasmodesmal biology are expected to be made by uncovering the molecular composition, anatomy, and transport mechanism of this fascinating yet incredibly recalcitrant biological structure.

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1 Introduction

Effective intercellular communication is essential for the survival of multicellular organisms. Without such mechanism, coordination of their growth, development, and appropriate responses to various environmental challenges and stimuli is simply impossible. For this reason, both plant and animal systems have developed unique cell-to-cell communication strategies that conform to their distinct cellular architecture and organization. In animal cells, symplasmic connections that permit the diffusion of small molecules such as ions and metabolites are afforded through gap junctions. These structures are built by the assembly of connexon hemichannels between adjacent cells, plasma membranes (PMs) of which are in direct contact (Meiners et al. 1991). In plants, constructing this type of intercellular channel is physically impossible due to the separation of PMs by the cell walls from the onset of cell division. To establish and maintain intercellular connections under such morphological constraint, plants have adopted a mechanism of producing plasma membrane (PM)-lined symplasmic connections, i.e., plasmodesmata (PD) across cell walls. PD have evolved from structurally and mechanically simple forms found in primitive organisms to highly complex and diverse entities more prevalent in higher plants. One of the most crucial modifications introduced to the higher plant PD may have been the entrapment of the endoplasmic reticulum (ER) within the PM-lined pores (Robards and Lucas 1990; Lucas et al. 1993; Lucas and Wolf 1993). This new feature is thought to enhance, to a certain extent, structural stability of PD and provide an additional regulatory mechanism for the selectivity and size exclusion limit (SEL) of macromolecules that move cell to cell.

PD are dynamic channels, in that their structure, function, and biogenesis are under constant readjustments throughout the plant development (Robards and Lucas 1990; Overall and Blackman 1996; Zambryski and Crawford 2000; Ehlers and Kollmann 2001; Roberts and Oparka 2003). Based on the morphology and the nature of biosynthesis, PD are classified as primary or secondary forms. Primary PD denote morphologically simple types that are assembled during cytokinesis by entrapment of ER strands, which become appressed by the deposition of cell wall materials (Hepler 1982). Secondary PD mainly represent those produced de novo across existing cell walls during cell expansion and development. They often show more complex, branched morphologies, which cannot easily be differentiated from structurally modified primary PD. What controls innate PD frequency and activation of secondary PD formation is not yet understood, but the development of complex PD is thought to be an important mechanism for controlling their SEL.

PD of higher plants can dilate and facilitate selective trafficking of macromolecules in addition to providing a basal cell-to-cell permeability of molecules that are smaller than ~800 Da (Ding 1997; Haywood et al. 2002; Heinlein 2002; Heinlein and Epel 2004; Kurata et al. 2005a). It is now well established that PD play a fundamental role in trafficking of endogenous signaling molecules including not

only proteins and RNAs (Jackson 2001; Haywood et al. 2002; Oparka 2004) but also infectious viral materials (Wolf et al. 1989, 1991; Atkins et al. 1991; Deom et al. 1992; Citovsky 1999).

In this chapter, we aim to provide an overview of the structural characteristics of the PD in an evolutionary context, describe two major modes of PD biosynthesis, and present models illustrating potential mechanisms involved in de novo formation of PD. We will then focus our discussion on a few selected examples that highlight the functional significance of PD-mediated cell-to-cell trafficking of non-cell-autonomous proteins (NCAPs) and the identification of PD-associated proteins. Finally, we will close this chapter by drawing a potential parallel between PD and the PM-lined intercellular channels of animal cells, tunneling nanotubes.

2 Plasmodesmata Are Membrane-Lined Cytoplasmic Channels

PD are specialized intercellular channels that establish both cytoplasmic and endomembrane continuity in plants (Robards and Lucas 1990; Lucas and Wolf 1993; Roberts and Oparka 2003). Unlike animal cells, plant protoplasts are encased by rigid cell walls, preventing the PMs of neighboring cells from making direct contact. This physical limitation imposed upon plant cells may have driven the creation of membrane-lined cytoplasmic pores as means of a symplasmic communication, which is essential to function as a complex multicellular organism. Consequently, this event is considered a major factor in the evolution of higher plants (Lucas et al. 1993).

At the ultrastructural level, individual primary plasmodesma in higher plants is composed of tubular cytoplasmic channels that are lined externally with the PM and internally with the ER membrane (Ding et al. 1992a; Overall and Blackman 1996; Lee et al. 2000) (Fig. 1). The cytoplasmic space between the PM and ER membrane is divided into microchannels (~3–4 nm in diameter) by crosslinking of presumed globular proteins that are embedded within the PM inner leaflet and ER outer leaflet (Robards and Lucas 1990; Overall and Blackman 1996). The basal SEL of these microchannels is estimated to be ~800 Da, which is sufficient for the diffusion of small molecules such as nutrients and ions. The central or core region of a plasmodesma is occupied by an appressed form of the ER, which is connected to the cytoplasmic ER cisternae. This unique structural organization enables the establishment of an endomembrane continuum between adjoining cells throughout plant tissues or symplasmic domains (Overall et al. 1982; Robards and Lucas 1990; Roberts and Oparka 2003). The internal structure of PD corresponding to the appressed ER was originally termed desmotubule (Robards 1968) because the appearance of the structure was interpreted as something similar to cytoplasmic microtubules. Later, Lucas et al. (1993) proposed renaming it to “appressed ER” to reflect the fact that the core region constitutes a modified form of ER strand continuous with

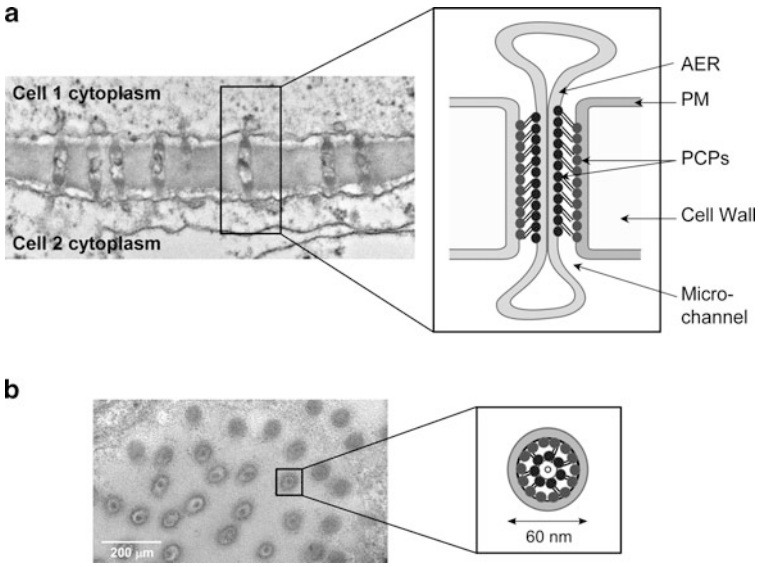

Fig. 1 Ultrastructure of PD. Transmission (**a**) and scanning (**b**) electron micrographs showing *side* and *top views*, respectively, of numerous PD, clustered in a pit field. Corresponding schematic diagrams (*right panels*) illustrate basic architecture of a plasmodesma embedded in the cell wall and delimited by PM inner and ER outer leaflets (modified from Lee et al. 2000). These membranes appear to contain electron-dense globular proteins (Ding et al. 1992a) that may constitute putative plasmodesmal channel proteins (PCPs)

the cortical ER. Appressed ER is thought to provide PD channels with structural stability within the rigid cell wall environment. In addition, the presence of proteinaceous molecules, apparently embedded in the appressed ER membrane, raises the possibility that the appressed ER may enable PD to control SEL and facilitate macromolecular trafficking (Lucas et al. 1993).

Another important role provided by the appressed ER is a functional membrane coupling between neighboring cells. Fluorescently tagged lipid molecules that label the ER membrane in a target cell were shown to spread into and label the ER membrane in neighboring cells, supporting the idea that the ER membrane is functionally coupled through PD (Grabski et al. 1993; Martens et al. 2006). By contrast, no occurrence of a membrane coupling through the PM has been reported. Interestingly, the extent of ER membrane coupling is not constant across the boundaries of different cell types. A higher degree of the coupling was revealed at the cell junctions between the companion cell (CC) and the sieve element (SE) than other junctions (Martens et al. 2006). Consistent with previous structural observations that the appressed ER structurally lacks the lumen space in general (Overall et al. 1982; Ding et al. 1992a), the ER coupling through the lumen was not detected even at the CC-SE junction when a ER targeted green fluorescent protein (GFP) reporter was employed (Martens et al. 2006).

3 Formation of Plasmodesmata

Plant cells grow and develop by division, expansion, and differentiation. In order to create and maintain specific cell types, adjacent cells are required to successfully process physiological and developmental information through coordinated cell-to-cell communication. Accordingly, higher plants have evolved mechanisms by which symplasmic connectivity is tightly controlled. For example, the frequency of PD is determined by dynamic process of generation and degeneration of PD under specific physiological conditions or developmental stages in plants. In addition, existing PD can undergo structural modifications regardless of their origin to confer a differential permeability or to temporally arrest symplasmic connectivity.

3.1 Primary Plasmodesmata

Primary PD in higher plants are produced by insertion of the ER within the newly forming cell plate in a dividing cell (Hepler 1982). During cytokinesis, a cell plate develops through the fusion of both endosomal and Golgi-derived vesicles containing cell wall materials, and eventually coalesces with the PM to separate the two daughter cells. This process is guided by the phragmoplast, a scaffold composed of cytoskeletal elements and the ER, which is oriented perpendicular to the developing cell plate. Cortical ER strands trapped and appressed within the expanding cell plate of the newly forming daughter cells then lead to the formation of primary PD (Fig. 1). This event which would determine the initial density of primary PD in newly divided cells, is unlikely a random event. However, whether and how this process is controlled remain to be elucidated. As cells mature, primary PD can be structurally modified to acquire complex and branched morphologies.

3.2 Secondary and Modified Plasmodesmata

The mechanism by which secondary PD are formed is crucial for plant cells to maintain a certain level of cell-to-cell connectivity and to establish new symplasmic connections during normal physiological and developmental progression. It is also vital when heterotypic cell unions are produced, for example, at a graft junction or host–parasite interface (Ehlers and Kollmann 2001). Structurally, secondary PD are more complex than cylindrical primary PD, in that they usually contain multiple cytoplasmic strands interconnected in the middle lamellar region of cell walls, developing median cavities (Ding et al. 1992b). Detailed ultrastructural analysis performed on heterograft unions indicated that de novo synthesis of PD involves cell wall loosening and degradation as well as membrane fusion events (Kollmann and Glockmann 1991). This process initiates with a thinning and removal of the

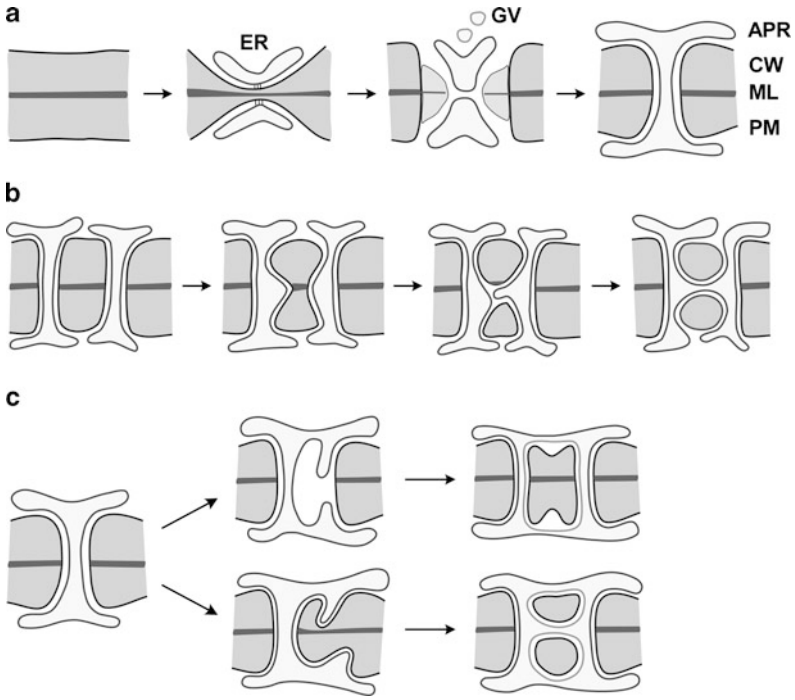

Fig. 2 Models depicting potential mechanisms by which secondary or modified PD are thought to form. (**a**) Secondary PD formation during establishment of graft junctions appears to involve cell wall (CW) loosening and degradation followed by ER attachments to both sides of the cross wall. Subsequently, fusion of membranes and deposition of new cell wall materials will occur by Golgi-derived vesicles (GV) delivered to the site, completing the insertion of the appressed ER (APR) within the newly formed PD. Adopted from Ehlers and Kollmann (2001). (**b**) Lateral fusion of primary PD. AER strands in neighboring primary PD may expand through middle lamella (ML) region, accompanying cell wall loosening and digestion until they fuse to form H-shaped PD. Adopted from Lucas et al. (1993). (**c**) Formation of secondary PD by twinning or fission. Secondary PD may be formed by inserting new PD next to primary or preexisting PD acting as templates (twinning) or by splitting them in two (fission) (*top*). Alternatively, they may be formed de novo by an insertion of cortical ER into either side of the cell wall next to a preexisting PD and a subsequent fusion of the ER strands at the middle lamella (*bottom*)

cross wall followed by attachment of the ER membranes to the PM, while Golgi vesicles deliver new membrane and cell wall materials to the site (Fig. 2a). This process continues until the PM and ER membranes from each side of the cross wall finally fuse. Rebuilding of cell walls around the PM leads then to a completion of de novo plasmodesmal connections between neighboring cells.

Branched PD can also be formed by the modification of preexisting PD (Lucas et al. 1993; Ehlers and Kollmann 2001; Faulkner et al. 2008) While primary PD elongate during cell wall expansion and thickening, cortical ER continuous to the appressed ER may become entrapped within the cell wall, creating additional

cytoplasmic connections (Ehlers and Kollmann 2001). Alternatively, the PD containing complex central cavities in the middle lamellar region may be formed by plasmodesmal dilation or lateral fusion of simple primary PD, followed by the apposition of new cell wall materials (Lucas et al. 1993; Ehlers and Kollmann 2001). Here, multiple branches of PD may be formed through the entrapment of ER strands extended from the PD at either side of the middle lamella, or through the insertion of additional PD branches upon localized breakdown of the cell wall. Lateral fusion of primary PD, especially, is thought to give rise to H-shaped form through a specific wall digestion at the middle lamellar region around the PD (Fig. 2b). Initially, wall digestion at the middle lamella may force the PMs of neighboring PD to extend out into this region. Subsequently, turgor pressure-mediated PM fusion may take place when the middle lamella is completely removed, and additional branching of the secondary PD may occur by the insertion of protoplasmic bridges to the existing PD (Lucas et al. 1993).

An ultrastructural study employing root cells from various plant species has shown that a significant number of PD is newly inserted, altering the pattern of PD distribution in expanding walls (Seagull 1983). Individual and/or paired PD dispersed throughout the cell wall area prior to cell elongation became clustered within pit fields in the fully expanded cell walls. These observations suggested that insertions of new secondary PD may occur at the vicinity of preexisting PD. A similar mechanism was also proposed as "PD twinning model", based on recent microscopic observations of freeze-fractured tobacco trichome basal cell walls and related computational simulation (Faulkner et al. 2008). Here, a relatively even distribution of mainly single PD was observed in the newly formed trichome cell walls. As the cell enlarged, the total number of PD in the cross wall increased, indicating the formation of secondary PD. Moreover, closely paired or twinned PD occurred at an increasing frequency within pit fields. These observations led to the speculation that secondary PD may be inserted proximal to certain primary or preexisting PD by using them as templates. Intriguingly, some of the twinned PD share the same wall collars, leading the authors to propose a "fission" model. Here, two individual PD are synthesized by the insertion of a new ER strand into a preformed plasmodesmal pore, which splits subsequently due to the deposition of new cell wall materials (Fig. 2c).

4 Plasmodesmata in Lower Plants

Land plants (embryophytes) including vascular and nonvascular species are thought to be evolved monophyletically from charophycean green algal ancestors (Graham et al. 2000; Karol et al. 2001). Consistent with this notion, the Charales are identified as the closest extant relatives of land plants on the basis of comparative phylogenetic analysis utilizing DNA sequence information of selected genes (Karol et al. 2001). It is notable that the development of primary PD, a common feature shared between Charales and land plants, is considered one of the fundamental changes in the basic body plan and regulatory system during the evolution of land plants.

4.1 *Brown Algae:* Laminaria

Primitive forms of PD are found in some species of brown and green algae but not in red algae (Lucas et al. 1993). Multicellular cyanobacteria and red algae both form some type of intercellular pores between neighboring cells; however, the multicellular brown algae are considered the earliest ancestors of plants that produce PM-lined symplastic connections (Cook et al. 1997). The existence of PD in brown algae, which divide into two daughter cells by centripetal annular furrowing, suggests that the origin of their PD is secondary, i.e., produced by de novo synthesis of PD postcytokinetically. A comprehensive ultrastructural study of PD in the brown algae *Laminaria hyperborean* and *L. saccharina* revealed that they are structurally very simple pores (Schmitz and Kuhn 1982). Although small vesicles and ER were often found to associate with the PD in pit fields, appressed ER as seen in PD of higher plants was clearly absent within these PD (Schmitz and Kuhn 1982). The PD found in *Laminaria* were distributed throughout meristoderm, cortex, and medulla tissues. They ranged from 30 to 60 nm in diameter and evenly dispersed within pit fields at a high frequency of 153 μm^{-2}. A wide distribution of these PD throughout the algal body is thought to play an important role in establishing a symplast in *Laminaria* for both short and long distance transport of photoassimilates.

4.2 *Green Algae:* Chara

The green algae range from unicellular flagellates to multicellular forms with a varying degree of structural complexity and tissue differentiation. In the green algae that form PD, cell division occurs only through cell plate formation mediated by either phragmoplast as in higher plants or by another cytoskeletal structure called phycoplast (Franceschi et al. 1994; Cook et al. 1997). The occurrence of primary PD in green algae is thought to reflect an evolutionary transition from annular furrowing to the development of cell plate-mediated cytokinesis (Lucas et al. 1993). However, the fact that the presence or structure of PD in different classes of green algae is heterogeneous suggests that the origin of PD within this group of plants is not monophyletic (Cook et al. 1997).

An extensive ultrastructural study performed on characean green algae showed that a complete separation of daughter cells in *Chara corallina* occurs by sealing off the cytoplasm between the daughter cells through the formation of cell plate (Franceschi et al. 1994). In this species, however, PD were not inserted during cell plate development but rather formed by invagination of the PMs from either side of the completed wall followed by membrane protrusion into the wall. The PMs were found to form funnel-like shapes that continue to extend through the new cell wall until the membranes fuse to form tube-shaped simple secondary PD with inner diameters of 20–25 nm. The newly formed PD lacked appressed ER, but

highly branched complex PD derived from large central cavities were found in mature cell walls. The structure and secondary origin of PD observed in *C. corallina* was found essentially the same in two other *characean* species, *C. braunii* and *Nitella translucens* (Franceschi et al. 1994). By contrast, primary PD were found to form during cytokinesis in *C. zelanica* (Cook et al. 1997). In this species, not only the entrapment of the ER in a developing cell plate but also the formation of simple primary PD in a new cross wall of the daughter cells were observed. These PD were shown to have neck constrictions and spoke-like structures. However, a higher resolution will be necessary to help determine whether the appressed ER within the PD of *C. zelanica* is structurally intact.

4.3 Bryophytes

The PD of the three bryophyte genera, Monoclea, Notothylas, and Sphagnum, were found to contain appressed ER that are connected to the cytoplasmic ER, similar to the basic structure of PD in seed plants (Cook et al. 1997). Neck constrictions were not found in any of these bryophytes, but spoke-like structures radiating from appressed ER to the PM were found in Notothylas and Sphagnum genera. In addition, ring-like wall specializations and branched PD were found in Sphagnum and Monoclea, respectively. These observations suggest that the complex modification of PD structure found in land plants may have been introduced before the evolutionary branching of bryophytes and seed plants.

5 Proteins Localized at or Near Plasmodesmata

To date, the molecular composition of PD channels remains unknown. However, the list of proteins that are directly or indirectly associated with PD structure and/or function is growing as a result of various approaches combining molecular, genetic, cell biological, and biochemical techniques (Lucas and Lee 2004; Oparka 2004; Maule 2008). Immunological studies have shown that a few proteins are found at or around PD. Some of these include cytoskeletal elements (White et al. 1994; Ding et al. 1996; Blackman and Overall 1998), a pathogenesis-related protein from maize (Murillo et al. 1997), and centrin-like proteins (Blackman et al. 1999). Among the proteins that were shown to localize around PD, the following groups are of a particular interest given that they may constitute structural and/or regulatory elements: cell wall modifying enzymes including β-1,3-glucanase (Levy et al. 2007) and pectin methyl esterase (Chen et al. 2000); cytoskeletal elements including unconventional myosin VIII (Volkmann et al. 2003), actin (White et al. 1994), actin-related proteins and myosin-like protein (Radford and White 1998); calcium-binding proteins including centrin (Blackman et al. 1999) and calreticulin (Baluska et al. 1999); and PD-localized protein 1 (Pdlp1, Thomas et al. 2008). Isolation of

purified, intact PD is technically not feasible because PD do not have a membrane-enclosed boundary and are embedded in cell walls. However, a combination of proteomics and cellular studies seems promising for isolating PD-associated proteins.

The β-1,3-glucanase has long been assumed to play an important role in PD regulation by hydrolyzing β-1,3-glucan polymer, callose, which restricts PD permeability when accumulates around the neck region of PD. A large number of genes (~48) are predicted to encode members of β-1,3-glucanase (AGI 2000). Among these, the Arabidopsis β-1,3-glucanase BG_ppap was isolated from a proteomics study and was shown to localize to both PD and the PM in tobacco when transiently expressed as a GFP-fusion protein (Levy et al. 2007). Analysis of a T-DNA insertional knockout mutation in BG_ppap revealed that the mutant plants had a reduced PD permeability to GFP and an increased accumulation of callose around PD, supporting a role for this gene in regulating the SEL of PD.

Filamentous actin, unconventional myosin VIII, centrin, and calreticulin have also been shown to localize to PD and are proposed to act as structural/regulatory components (Oparka 2004; Maule 2008). The exact roles of these cytoskeletal elements in relation to PD structure or function have yet to be established, but it is plausible that they may play a role in anchoring or directing the cytoplasmic F-actin cables to PD. It is also possible that they, together with a contractile protein, centrin, may be involved in regulating PD permeability or providing a track through the PD for the trafficking of macromolecules.

Pdlp1 belongs to a group of ~30–35 kD, type 1 membrane proteins. They contain a plant-specific, cys-rich extracellular domain at the N-terminus and a C-terminal transmembrane domain followed by a very short (<20 amino acid residues) cytoplasmic extension (Thomas et al. 2008). Pdlp1 fused to GFP was shown to be targeted to PD through a secretory pathway that is sensitive to brefeldin A, an inhibitor of endomembrane vesicle trafficking from ER to Golgi. Overexpression or knockout of Pdlp1 was found to correlate with reduced or increased PD permeability, respectively, suggesting that Pdlp1 plays a negative role in controlling PD. Surprisingly, a patch of 21 hydrophobic amino acid residues comprising the transmembrane domain of Pdlp1 was found to be not only essential but also sufficient for PD localization. Based on this result, it is tempting to speculate whether lipid composition of the PM lining and/or nearby PD is highly specialized to provide a unique local membrane environment for recruiting and stabilizing PD proteins.

6 PD Mediate Macromolecular Trafficking

A significant body of evidence supports the concept that PD are not just simple, diffusional channels but rather highly dynamic channels that establish a unique supracellular control system (Ding 1998; Zambryski and Crawford 2000; Haywood et al. 2002; Roberts and Oparka 2003; Lucas and Lee 2004). PD in higher plants can

facilitate trafficking of proteins and RNA/protein complexes from the cells where they are produced to the neighboring cells where they can function in a non-cell-autonomous manner. Pioneering studies employing viral proteins including the movement protein (MP) encoded by *Tobacco mosaic virus* (TMV) have demonstrated that plant viruses exploit endogenous PD trafficking machinery to spread infectious materials cell to cell (Wolf et al. 1989, 1991; Fujiwara et al. 1993; Noueiry et al. 1994; Waigmann et al. 1994; Wolf and Lucas 1994; Waigmann and Zambryski 1995). Macromolecules that have the capacity to interact with and pass through PD are termed non-cell-autonomous proteins (NCAPs) (Haywood et al. 2002; Lucas and Lee 2004). It is now well known that certain NCAPs that play important roles in cell differentiation and patterning include various types of transcription factors, e.g., KNOTTED1 (KN1), a maize homeobox protein controlling the maintenance of shoot meristem identity (Jackson et al. 1994; Lucas et al. 1995); LEAFY, the *Arabidopsis* homolog of snapdragon FLORICAULA, which controls floral meristem identity (Perbal et al. 1996; Sessions et al. 2000); SHORT-ROOT (SHR), a GRAS family transcription factor required for the specification of endodermis (Helariutta et al. 2000) (Nakajima et al. 2001); and CAPRICE (CPC), a Myb transcription factor important for the specification of hair-forming cells (Wada et al. 2002).

6.1 KN1 Moves Through Plasmodesmata to Act Non-Cell-Autonomously

It had been known that PD have the capacity to potentiate intercellular movement of viral proteins, but the maize transcription factor KN1 was the first endogenous protein that was shown to move through PD in microinjection studies (Lucas et al. 1995). Initial clue to KN1 acting as an NCAP was provided by the finding that KN1 protein is present in both epidermal L1 and inner L2 layers of the maize shoot meristem, although *KN1* mRNA is detected only in the L2 layer. Subsequently, microinjection studies employing fluorescently tagged recombinant KN1 revealed that KN1 is capable of not only trafficking cell to cell through PD but also increasing SEL by inducing dilation of PD in tobacco mesophyll cells. Later on, it was also shown that recombinant KN1 (and its Arabidopsis homologs) fused to GFP is able to move between Arabidopsis cells both in leaf and shoot meristem, and that the direction of KN1 movement was regulated in a tissue-specific manner (Kim et al. 2002, 2003, 2005).

The homeo domain of KN1 was shown to be the essential minimal sequence that is sufficient for cell-to-cell movement in an elegant genetic complementation study. Here, KN1 mutant constructs were expressed as translational fusion to GLABROUS1 (GL1) in the Arabidopsis trichome mutant *glabrous1 (gl1)* background (Kim et al. 2005). GL1, a member of the Myb transcription factor family is required, in a cell-autonomous manner, for the trichome initiation. Following the

expression of protein in subepidermal layers of *gl1* as a fusion to KN1 derivatives, restoration of trichome production from the *gl1* epidermis was assayed. GL1 fused to full-length KN1 or homeobox domain of KN1 alone was shown to traffic from L2 layer to L1 layer and rescue tricomeless *gl1* mutant phenotype, demonstrating that the homeobox domain is sufficient for the cell-to-cell movement activity of KN1. The homeobox domain is comprised of 65 amino acid residues including a nuclear localization signal, which, when mutated, negates cell-to-cell movement of KN1 (Lucas et al. 1995). This result suggests a potential coupling between nuclear localization/accumulation of KN1 and its non-cell-autonomous function. The coupling phenomenon was also observed with other transcription factors, CPC (Kurata et al. 2005b) and SHR (Gallagher et al. 2004) (see Sect. 6.2). Modifications or mutations introduced to partition these proteins exclusively to the nucleus or the cytoplasm resulted in a loss of their intercellular movement activity as well as their cellular function, denomstrating again the importance of the coupling between their nuclear and presumed PD trafficking activities.

What is the significance of KN1 movement for its cellular function? An important insight into this question was provided by a genetic study in which an Arabidopsis mutant of KN1 homolog, *shootmeristemless* (*stm*) was utilized for complementation assays (Kim et al. 2003). When a fluorescently tagged, movement-competent KN1:GFP was expressed under the control of an L1-layer-specific promoter in the *stm-11* mutant background, the *stm* mutant phenotype was partially rescued. By contrast, a movement-impaired fusion, KN1:GUS, failed to complement the mutant phenotype. Moreover, when KN1:GUS was ectopically expressed under the control of the 35 S promoter throughout the whole meristem tissue of the *stm-11* mutant, it was not able to fully rescue the mutant phenotype. These results are consistent with the view that the activation of KN1 as a transcription factor in the nucleus may require preceeding cell-to-cell trafficking.

6.2 CPC and SHR

In Arabidopsis roots, the fate of epidermal cells to develop either as root hair (H) or nonhair (N) cells is specified by their position in relation to the underlying cortex cells (Dolan 2006; Guimil and Dunand 2006; Ishida et al. 2008) (Fig. 3a). This positional information is believed to be transduced via an upstream leucine-rich repeat receptor kinase, SCRAMBLED (SCM), to activate an array of downstream transcription factors including GLABRA2 (GL2) (Ishida et al. 2008; Kwak and Schiefelbein 2006) (Fig. 3b). The signaling process involves an elaborate regulatory circuit that integrates apoplasmic and symplasmic communication pathways in a reciprocal manner across cellular boundaries. Among the transcriptional regulators crucial for this regulatory circuit, CPC is preferentially expressed in presumptive N cells and act as a non-cell-autonomous factor that determines H cell fate (Schellmann et al. 2002; Wada et al. 2002; Ishida et al. 2008).

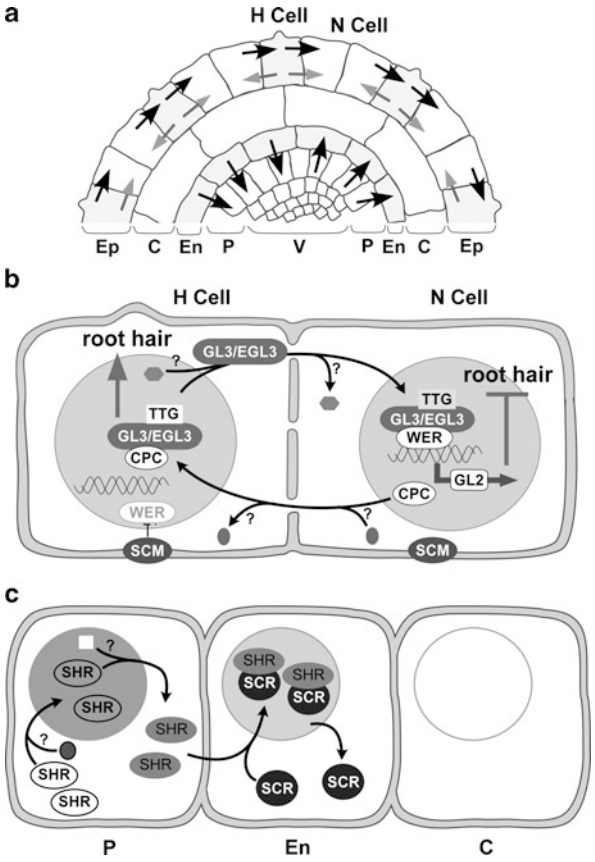

Fig. 3 Role for cell-to-cell trafficking of non-cell-autonomous transcription factors in specification of root cell types. (**a**) A diagram illustrating Arabidopsis root architecture from a cross sectional view. *Arrows* indicate directions of non-cell-autonomous trafficking pathways across specific cellular boundaries required for specifying epidermal hair-cell or endodermal cell type. *H* hair cell, *N* nonhair cell, *Ep* epidermal cell, *C* cortex, *En* endodermis, *P* pericycle, *V* vasculature. (**b**) Root hair cell formation is controlled by a complex pathway involving both noncell-autonomous trafficking and receptor-mediated signaling. Among the transcription factors identified in this process, CPC and GL3/EGL3 act as NCAPs and cross cellular boundaries in a reciprocal manner. Unlabeled objects illustrate chaperone molecules involved potentially in this process. Positional information necessary to specify hair-cell fate is thought to be generated by the cortex cells underlying epidermal cells and the signal mediated by SCM, a receptor kinase, in epidermal cells. The transcriptional complex formed by CPC, GL3/EGL3, and TTG in the cells at the hair-cell position suppresses induction of GL2, expression of which is required to block hair-cell fate. (**c**) Endodermal cell formation involves both non-cell-autonomous and cell-autonomous factors, SHR and SCR, respectively. SHR produced in stele cells move one cell layer outward and enters into the nuclei to stimulate expression of SCR, which in return traps incoming SHR into the nuclei of endodermal cells, preventing further movement of SHR beyond this cell layer. Unlabeled objects illustrate chaperone molecules that may be involved in coupling of SHR nuclear trafficking to its non-cell-autonomous activity within stele cells

The experimental evidence that CPC is a mobile factor is provided by the finding that fluorescent signals produced by CPC:GFP are detected in all epidermal cells when the fusion protein is expressed under the control of its own promoter, activity of which is specific to N cells. Surprisingly, CPC was not the only mobile factor in the signaling network specifying H cell fate. In situ hybridization and promoter fusion studies revealed that *GL3*/*EGL3* mRNAs are specifically expressed in H cells; however, the protein products were found in N cells as well, suggesting their cell-to-cell trafficking activity (Bernhardt et al. 2005). It is notable that the movement of CPC (and *GL3*/*EGR3*) is limited to the cells within the epidermal layer without crossing the boundary between epidermis and cortex, which suggests that the non-cell-autonomous activity of CPC is specific to the boundary between H and N cells. Moreover, CPC remains cell-autonomous when ectopically expressed in other cell types within the root (Kurata et al. 2005b). These observations raise the possibility that the difference between periclinal and anticlinal cell wall boundaries as well as specificity of PD type/structure may determine the extent to which directional PD trafficking of a certain NCAP may be allowed. Alternatively, CPC may require a chaperone molecule or a cofactor, expression of which is epidermal cell-specific.

SHR represents another example of an NCAP showing a directional or controlled movement. It acts non-cell-autonomously as a mobile signal that delivers positional information from the stele cells, where it is expressed, to the single cell layer external to the stele (Nakajima et al. 2001). This specifies the adjacent cell layer to develop as endodermal cells (Fig. 3c). Within the stele cells, SHR shuttles freely between the nucleus and the cytoplasm; however, it becomes confined to the nucleus of endodermal cells after intercellular movement. What mechanism controls or limits SHR to move just one cell layer? It turns out that the nuclear shuttling of SHR is essential for its non-cell-autonomous activity (Gallagher et al. 2004). In addition, confinement of SHR into the nuclei of endodermal cells by another GRAS family transcription factor, SCARECROW, blocks penetration of SHR further into the neighboring cell layer (Cui et al. 2007). It is conceivable that an unknown nuclear factor(s) in the stele cells may recognize and chaperone nuclear imported SHR out into the cytoplasm to couple with the PD trafficking pathway. Or, posttranslational modification of SHR within or during nuclear export may exist to allow the modified form to specifically interact with the PD trafficking pathway. Future studies may unfold a novel regulatory mechanism by which cell-to-cell trafficking of CPC or SHR is controlled through multiple factors and/or posttranslational modulation.

6.3 Mechanism of PD Trafficking

To map minimal sequence elements and identify specific factors involved in PD trafficking, molecular analyses of various NCAPs and PD-associated proteins have been performed, but the data collected so far suggest that the emerging picture is not

so simple. Mutational analyses performed with various NCAPs including KN1, CPC, and SHR have mapped amino acid residues that are important for their movement function (Lucas et al. 1995; Gallagher et al. 2004; Kim et al. 2005; Kurata et al. 2005a, b). Interestingly, 36 unique amino acid residues are identified from a phloem NCAP, CmPP16 that are not only necessary for its own cell-to-cell movement but also sufficient for providing a cell-autonomous protein with intercellular movement activity (Taoka et al. 2007). However, no consensus sequence or structural feature among these NCAPs has been identified. Together with molecular analyses performed on other NCAPs, these results denote that there may not be a common motif that functions as a hypothetical "PD trafficking signal."

Posttranslational modification is gaining an attention as a potential mechanism by which PD-mediated cell-to-cell movement of specific NCAPs is controlled. It was demonstrated that the viral protein TMV MP undergoes phosphorylation at Ser/Thr residues within its C-terminal tail, which negatively affects its movement activity (Waigmann et al. 2000). A member of casein kinase 1 was identified from tobacco as an endogenous protein kinase that phosphorylates the C-terminal tail of TMV MP (Lee et al. 2005). Glycosylation appears to be also an important mechanism for modulating PD trafficking of the phloem-derived NCAP, CmPP16 (Taoka et al. 2007). CmPP16 interacts with *Nicotiana tabacum* NON-CELL-AUTONOMOUS PATHWAY PROTEIN1 (NCAPP1) to move cell to cell (Lee et al. 2003). For this interaction and movement activity, both phosphorylation and glycosylation were shown to be essential (Taoka et al. 2007). Understanding whether posttranslational modification is a prevalent mechanism by which a dynamic recognition occurs between NCAPs and non-cell-autonomous pathway proteins such as NCAPP1 would fill important gaps in our knowledge of PD trafficking.

Experimental evidence described above supports the idea that intercellular trafficking of specialized NCAPs is necessary to determine cell fates and to establish cellular boundaries. In terms of the mechanism underlying cell-to-cell trafficking, it seems most feasible that the non-cell-autonomous transcription factors move directly through PD. However, whether there exists an additional cell-to-cell trafficking pathway involving an unidentified secretion mechanism remains in contention. Employing microinjection would be one approach to test whether their movement is mediated by PD. Future developments of innovative new tools and approaches as well as molecular understanding of PD structure and biogenesis would be also crucial to gain better insight into this quandary.

7 Tunneling Nanotubes: Animal Analog of Plasmodesmata?

Cell-to-cell trafficking of transcription factors (especially homeobox proteins) and their non-cell-autonomous function may not be a phenomenon unique to plant systems. Animal transcription factors including a homeoprotein, Engrailed, were shown to move between cultured cells (Derossi et al. 1996; Maizel et al. 2002;

Prochiantz and Joliot 2003). It was also reported that the plant homeodomain protein KN1 can move cell to cell in cultured animal cells (Tassetto et al. 2005). How does KN1, a homeoprotein from plant kingdom, move between animal cells? The exact mechanism of intercellular transport of transcription factors in animal cells is not yet understood. However, a recent discovery that cultured animal cells form thin PM-lined tubes that connect neighboring cells (Rustom et al. 2004) implies that these so-called tunneling nanotubes (TNT) may provide a mechanism for intercellular macromolecular trafficking.

TNT are a novel type of cell protrusion comprised of an outer phospholipid layer continuous with each cell's PM and an actin-based inner core (Rustom et al. 2004). TNTs were shown to not only provide membrane continuity between connecting cells but also facilitate selective transfer of membrane-bound proteins, vesicles, and organelles without allowing small molecules to freely diffuse. In terms of the basic design and potential roles in cell-to-cell communication, there lies some striking similarities between TNT and PD. For example, plant cells can form tubular PD structures lined by PM but lacking appressed ER, through which viral tubules formed by *Cowpea mosaic virus* (CPMV) can pass (Vanlent et al. 1991). These TNT-like tubular structures protruding from the cell surface were found to form in protoplasts of host or nonhost cells and even in insect cells, suggesting that their formation could be independent of the presence of PD. However, the tubular protrusion of PM was induced by multimeric assembly of the CPMV MPs at the cell periphery, which does not require involvement of cytoskeletal components or secretion pathways (Wellink et al. 1993; Pouwels et al. 2002, 2003, 2004). These studies suggest that de novo production of PM-lined intercellular connections may be an intrinsic property of both animal and plant cells. Understanding this mechanism may shed new light on a potential parallel between TNT and PD, two fascinating intercellular communication systems that have evolved independently.

8 Concluding Remarks

PD play a fundamental role in potentiating cell-to-cell trafficking of signaling molecules, including a special class of NCAPs that play important roles in cell fate determination and developmental patterning. It is clear that a great deal of new insight into this unique intercellular signaling system will be only afforded by the molecular elucidation of biogenesis, composition, architecture, and transport mechanism of PD. These tasks are extremely challenging due to the nature of PD being recalcitrant to both biochemical and genetic approaches. However, fast-growing genomic resources and development of innovative tools may help to overcome the experimental hurdles associated with the PD research and allow exciting new discoveries to be made in this field. Information gained by taking various approaches will become instrumental in delineating the evolutionary origin of PD and perhaps deciphering an "enigma" of plant biology.

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Posttranslational Modifications of Plasma Membrane Proteins and Their Implications for Plant Growth and Development

Christian Luschnig and Georg J. Seifert

Abstract Posttranslational modifications of plasma membrane (PM) proteins are essential for a variety of processes that take place at the interface between cells and their environment. A plethora of PM protein modifications have been identified, several of which have been characterized for their role in the regulation of PM-protein fate. In this chapter, we will focus on a number of selected protein modifications that have been shown to affect protein targeting. Both reversible and irreversible covalent protein modifications appear to affect distinct steps in protein trafficking, and regulate protein association with hydrophobic environments, thereby defining the impact of those proteins on a large variety of plant growth responses.

1 Control of Protein Targeting via Covalent Protein Modifications

Proper targeting of both intrinsic and peripheral membrane proteins to the PM is essential for a normal plant development and mediates communication of a given plant cell with its environment. Among various targeting mechanisms, covalent lipid modifications are now recognized as important determinants of numerous plant signaling events. N-myristoylation, palmitoylation/S-acylation and prenylation of intracellular proteins, and attachment of a glycosyl-phosphoinisitidyl (GPI) lipid anchors (Thompson and Okuyama 2000) are modifications that exert a profound impact on protein targeting and function. It has recently been suggested that PM protein phosphorylation functions as a regulatory switch that is essential for establishment of cell polarity (Michniewicz et al. 2007), and variations in phosphorylation status appear to influence intracellular targeting of a plant-specific family of cell

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polarity determinants (Jürgens and Geldner 2007). Recent characterization of molecular players that control protein modification places them in fundamental cellular processes such as establishment of cell polarity and the assembly of signaling complexes.

1.1 Myristoylation

The term N-myristoylation describes cotranslational addition of the saturated C-14 fatty acid myristate to an absolutely required N-terminal glycine, which is part of a somewhat conserved domain that is essential for recognition by myristoyl-CoA: protein *N*-myristoyltransferase (NMT, Maurer-Stroh et al. 2002a, b). After cotranslational removal of the initiator methionine, endoplasmic reticulum (ER) – resident NMT catalyzes the formation of an irreversible amide bond between the N-terminal glycine and myristate (Wilcox et al. 1987). As a result, N-myristoylated proteins have a significantly higher tendency to associate with hydrophobic structures such as lipid bilayers or protein interaction domains (Bhatnagar and Gordon 1997).

In *Arabidopsis*, two NMT homologs have been identified, which have potentially nonoverlapping roles in the control of plant development (Qi et al. 2000; Pierre et al. 2007). Severe phenotypes of *nmt1* loss-of-function mutants indicate that *NMT1* is required for vital aspects of early plant development. In contrast, a loss of *NMT2* function caused growth deficiencies that are restricted to the control of flowering time (Pierre et al. 2007). Phenotypes of *nmt1 nmt2* double mutants were similar to those of *nmt1*, and ectopic expression of *NMT2* under control of the *NMT1* promoter did not rescue *nmt1* mutants, further suggesting nonredundant functions of these loci (Pierre et al. 2007).

Prediction-based and experimental assessments of the *Arabidopsis* proteome led to the identification of a surprisingly large set of proteins that might undergo N-myristoylation (Maurer-Stroh et al. 2002b; Boisson et al. 2003). A number of protein families that function in signaling events were overrepresented in the *Arabidopsis* myristoylome, including calcium-dependent kinases, several small G-proteins, and proteins predicted to function in pathogen defense responses (Boisson et al. 2003). This may indicate a more prominent role for myristoylation in plants than in other organisms, but experimental confirmation of lipid modification of a larger sample of *Arabidopsis* proteins predicted to be myristolylated is still missing.

1.2 Prenylation

Protein prenylation provides another means for protein targeting to hydrophobic surfaces (Schafer et al. 1990; Randall et al. 1993; Zhu et al. 1993). These lipid modifications involve posttranslational attachment of either a C-15 farnesyl or a C-20 geranylgeranyl group to a cysteine (Thompson and Okuyama 2000; Roskoski 2003; Galichet and Gruissem 2003). In plants, three enzyme activities, highly

conserved among eukaryotes, have been shown to catalyze protein prenylation via formation of a thioester bond at cysteine acceptor sites (Maurer-Stroh et al. 2003). A heterodimeric protein farnesyltransferase (PFT), consisting of α and β subunits that transfer the farnesyl group from farnesyl pyrophosphate to target proteins bearing a C-terminal CaaX motif (where "a" generally represents an aliphatic amino acid and "X" corresponds to M, S, C, A or E) has been characterized (Yang et al. 1993; Qian et al. 1996; Cutler et al. 1996). In addition, a heterodimeric type I protein geranylgeranyltransferase (PGGT I), which shares the α subunit of PFT, but has a distinct β subunit, appears to function in plants (Caldelari et al. 2001; Johnson et al. 2005). This complex uses geranylgeranyl pyrophophate as a substrate for protein prenylation at a C-terminal Caa motif (Caldelari et al. 2001). Arabidopsis orthologs of subunits of the aa heterotrimeric type II protein geranylgeranyltransferase (PGGT II) complex initially described in nonplant eukaryotes have also been identified (Maurer-Stroh et al. 2003). PGGT II prenylation of three tomato Rab proteins could be detected in suspension culture cells, thus providing biochemical evidence for functional PGGT II in plants (Loraine et al. 1996; Yalovsky et al. 1996).

Subsequent protein maturation steps that are essential for proper targeting and function of prenylated proteins appear to be highly conserved in eukaryotes. After cysteine prenylation, three C-terminal amino acids are proteolytically removed by CaaX proteases (Tam et al. 2001; Cadinanos et al. 2003a). In *Arabidopsis*, STE24 and FACE-2, which are orthologs of yeast Ste24 and Rce1, appear to catalyze this processing step (Bracha et al. 2002; Cadinanos et al. 2003b). Subsequently, STE14 prenyl cysteine α-carboxyl methyltransferase, orthologs of which have been identified in plants, catalyzes carboxyl methylation of now C-terminal cysteine (Crowell et al. 1998; Rodriguez-Concepcion et al. 2000).

Mutants deficient in the β subunit of *Arabidopsis* PFT were identified in diverse genetic screens, consistent with an involvement of protein farnesylation in multiple developmental and environmental response mechanisms. Initially identified in screens of Arabidopsis mutants that are deficient in abscisic acid (ABA) signaling (Cutler et al. 1996; Pei et al. 1998), the *ERA1* (*ENHANCED RESPONSE TO ABSCISIC ACID 1*) gene, which encodes PFT, was subsequently implicated in the control of meristem development (*WIGGUM*; Ziegelhoffer et al. 2000; Bonetta et al. 2000; Yalovsky et al. 2000) and in the control of plant pathogen interactions (*MODIFIER OF SNC1 8*; Goritschnig et al. 2008). Loss of the *Arabidopsis* locus encoding the β subunit of PGGT I results in subtle deficiencies in the control of ABA responses and in auxin-mediated control of lateral root formation (Johnson et al. 2005), suggesting nonredundant roles for protein farnesylation and geranylgeranylation in higher plants. Consistently, double mutants deficient in β subunits of both PFT and PGGT1 appear to exhibit additive defects in ABA responsiveness as well as in shoot and floral meristem development (Johnson et al. 2005). These phenotypes resemble the phenotypes of *pluripetala* (*plp*), a mutant deficient in the α subunit shared by PFT and PGGT I (Running et al. 2004).

Motif-based searches for C-terminal prenylation signatures revealed that a large fraction of proteins might undergo this modification in plants (Galichet and Gruissem

2003; Maurer-Stroh et al. 2007). Although farnesylation is suggested to facilitate association with membranes for most predicted *Arabidopsis* proteins, farneslyation of the APETALA1 transcription factor appears to be required for determination of transcription factor activity or binding specificity (Yalovsky et al. 2000), suggesting a more diverse role for this type of lipid modification.

1.3 S-Acylation

Whilst myristoylation and prenylation promote hydrophobic protein interactions, these modifications appear to be insufficient to maintain a stable membrane association (Bhatnagar and Gordon 1997; Shahinian and Silvius 1995). S-acylation, the attachment of fatty acids, like C-16 palmitate (palmitoylation) or C-18 stearate via a thioester bond to cysteines, renders a protein less likely to partition into the aqueous phase, and allows for a more stable membrane anchoring, specifically in combination with another lipid modification (such as myristoylation or prenylation; Shahinian and Silvius 1995). Moreover, unlike other lipid modifications discussed here, S-acylation of proteins is reversible, which makes it a potent regulatory switch, when it comes to the coordination of protein targeting, essential for regulation of intracellular signaling events (Rocks et al. 2005).

Experimental analysis of protein S-acylation has so far mainly been performed in nonplant organisms, but several lines of evidence indicate that a similar order of catalytic events takes place in plants (Hemsley and Grierson 2008). S-acylation involves the activity of protein S-acyltransferases (PATs) that make use of acyl-CoA as an acyl group donor (Roth et al. 2002). No clear consensus sequence for protein S-acylation has been determined. Instead, this protein modification was found at a large variety of distinct recognition sites (Magee and Seabra 2005). For example, di-cysteine motifs and cysteine "strings" consisting of more than 10 cysteines have been identified as targets for PAT activity (Babu et al. 2004; Sun et al. 2004). Moreover, S-acylation of intrinsic membrane proteins that has been described occurs at cysteines both within transmembrane regions and in hydrophilic domains (Resh 1999; Tanimura et al. 2006). Reversion of S-acylation appears to be mediated by acyl protein thioesterase activity (Camp and Hofmann 1993; Duncan and Gilman 1998), which, together with protein S-acylation activities, constitutes a protein acylation cycle crucial for protein localization and activity (Rocks et al. 2005; see below).

Plant genome sequencing has revealed the presence of several loci presumably involved in protein thioacylation and deacylation. In *Arabidopsis*, 23 putative PAT genes have been found, and all are predicted to encode integral membrane proteins (Hemsley and Grierson 2008).To date, only a single locus, *TIP GROWTH DEFECTIVE 1* (*TIP1*), has been analyzed in detail, and it was shown to function in the control of the fundamental processes of cell expansion and polar growth control (Schiefelbein et al. 1993; Hemsley et al. 2005). Similarly, only limited information concerning protein deacylation in plants is currently available. A loss-of-function

allele of *SUPPRESSOR OF AVRBST-ELICITED RESISTANCE 1* (*SOBER1*), a gene encoding a carboxyesterase predicted to hydrolyze acyl groups from proteins, interferes with the pathogenicity of a type III effector protein encoded by the plant pathogen *Xanthomonas campestris*, thus suggesting a role for protein deacylation in the control of pathogen responses in higher plants (Cunnac et al. 2007).

1.4 Lipid Modification Targets

Consequences of deficiencies in protein lipid modifications have been studied by site-directed mutagenesis that eliminates covalent lipid attachment of a number of plant proteins. These experiments demonstrated that such modifications can control protein activity and localization. For example, SALT OVERLY SENSITIVE 3 (SOS3) was among the first myristoylated proteins identified in plants (Ishitani et al. 2000). *SOS3* belongs to the calcineurin B-like (CBL) family of plant calcium sensors, which shows an increased tendency to associate with CBL-interacting protein kinases (CPIKs) with elevated calcium levels (Albrecht et al. 2001; Kolukisaoglu et al. 2004). Such CBL/CPIK complexes have been shown to function as regulators of salt tolerance in *Arabidopsis* (Albrecht et al. 2003; Kim et al. 2007). Mutagenesis of the penultimate N-terminal glycine completely abolished SOS3 myristoylation and interfered with complemention of a *sos3* loss-of-function allele after transformation with the construct (Ishitani et al. 2000). In another study, replacement of the penultimate N-terminal glycine in the β1 and β2 subunits of the heterotrimeric SnRK1 kinase was found to interfere with the PM association of GFP-tagged reporter proteins, demonstrating that myristoylation of these proteins is crucial for proper localization of the SnRK1 complex (Pierre et al. 2007; Polge and Thomas 2007).

A requirement for dual lipid modifications in the control of proper intracellular targeting and activity has recently been demonstrated in plants (Hemsley and Grierson 2008). CBL1, which, like SOS3, belongs to a family of plant calcium sensor proteins, is not only myristoylated at the penultimate N-terminal glycine but also undergoes S-acylation, presumably at a cysteine adjacent to its myristoylation site (Batistic et al. 2008). Remarkably, S-acylation of CBL1 appears to depend on prior myristoylation, since a mutation of the penultimate N-terminal glycine interferes with both myristoylation and S-acylation, whereas mutagenesis of the S-acylation site blocks S-acylation without impacting on protein myristoylation (Batistic et al. 2008). In line with a sequential order of protein modification events, myristoylation of CBL1 appears to be essential for its delivery to the ER, whereas subsequent targeting to its ultimate localization at the PM strictly depends on S-acylation (Fig. 1a; Batistic et al. 2008). Importantly, mislocalization of CBL1 was also shown to interfere with PM localization of already preassembled CBL/CPIK complexes (Fig. 1b), demonstrating a role for lipid modifications in proper targeting of the entire signaling complex (Batistic et al. 2008).

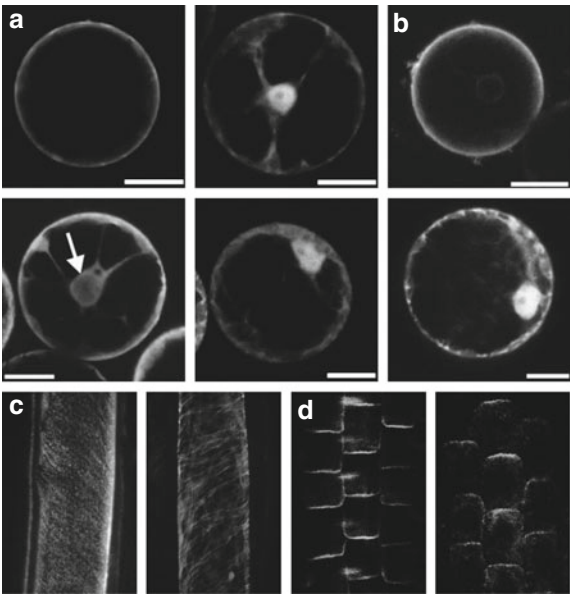

Fig. 1 Consequences of protein modifications on their subcellular localization. (**a**) Localization of GFP-tagged versions of CBL1 in tobacco protoplasts: CBL1:GFP (*top*, *left*) localizes to the PM; interference with myristoylation in CBLG2A:GFP results in intracellular accumulation of the reporter protein (*top*, *right*). Blocking of CBL1 S-acylation in CBL1C3S:GFP causes accumulation of the protein at the nuclear envelope (*bottom*, *left*; *arrow*). Interference with myristoylation and S-acylation in CBL1G2AC3S:GFP results in intracellular accumulation of the reporter (*bottom*, *right*), similar to the distribution of CBL1G2A:GFP. Images from Batistic et al. (2008); with kind permission from the authors (**b**) Localization analysis of a CBL1/CIPK1 complex in tobacco protoplasts by bimolecular fluorescence complementation. Coexpression of CBL1:SPYCE and CIPL1:SPYNE demonstrates interaction of these proteins at the PM (*top*). Upon coexpression CBL12GA:SPYCE and CIPL1:SPYNE, intracellular accumulation of the protein complex can be observed (*bottom*), suggestive of a role for CBL1 myristoylation in proper targeting of the CBL1/CIPK1 complex. Images taken from Batistic et al. (2008); with kind permission from the authors. (**c**) Localization of CaMV promoter driven 35S FLA13:YFP (*left*) and tau-YFP (*right*) reporter constructs in elongating hypocotyl epidermis cells. FLA13:YFP displays a punctate, striated distribution oriented roughly transverse to oblique to the elongation axis. In a comparable cell, the overall orientation of microtubules highlighted by tau:YFP shows a similar orientation although at lower density. Clare Simpson and Kim Baumann (both at the John Innes Centre, Norwich, UK) kindly provided FLA13:YFP and tau:YFP, respectively. (**d**) Localization of PIN1 to the basal (*lower*) pole of root stele in wild type (*left*) is shifted to a predominantly apical localization in *PID* gain-of-function background (*35S::PID*; *right*). Images kindly provided by Jiri Friml (VIB Ghent, BEL)

Dual lipid modifications have also been shown to regulate the trafficking of components of plant G-protein signaling. ROP6, a small G-protein involved in abscisic acid signaling and root hair growth (Lemichez et al. 2001; Molendijk et al. 2001), appears to be prenylated and, depending on its signaling status, can also undergo S-acylation (Sorek et al. 2007). Activated ROP6 appears to be S-acylated, whilst GDP-bound, inactive ROP6 is not. As a likely consequence of S-acylation,

active ROP6 copurifies with detergent-resistant membrane fractions, indicating that this lipid modification promotes association of ROP6 with specific membrane domains where signaling complex formation might take place (Sorek et al. 2007). Thus, as is seen with Ras-type GTP-binding proteins in mammals in which reversible thioacylation regulates subcellular localization, regulation of acylation /deacylation of ROP6 may determine its signaling function (Rocks et al. 2005; Sorek et al. 2007).

1.5 GPI Anchors

GPI-anchoring has been shown to be associated with the assembly of lipid subdomains ("lipid rafts") and polar sorting in animal cells (Schuck and Simons 2004; Jacobson et al. 2007). As the machinery for GPI biosynthesis is well conserved between animals, yeast, and plants, it has been speculated that the biological roles of GPI-anchoring might be conserved across kingdoms (Schultz et al. 1998; Schindelman et al. 2001; Gillmor et al. 2005). GPI-anchors in eukaryotes consist of a lipid tethered to the C terminus of peptides by a linker of the stereotypical structure: protein → ethanolamine → PO_4 →6 Man 1→2 Man 1→6 Man 1→4 GlcN 1→6 *myo*-inositol 1→ PO_4 → lipid. A variety of additional side chains, such as phosphoethanolamines or sugars linked to mannosyl residues and acyl groups linked to inositol, has been observed. Except for the initial enzymatic reactions, the biosynthetic steps of GPI assembly and subsequent attachment to the C terminus of target proteins take place in the lumen of the ER. The genes encoding the participating enzymes have been characterized in mammals and in yeast (Orlean and Menon 2007), and a high degree of sequence conservation allows for the identification of the corresponding plant genes (Table 1).

Although GPI-anchors possess a conserved core domain, GPI-anchored proteins (GAPs) in various kingdoms represent heterogeneous mixtures with respect to glycan core modifications and acyl moieties (Paulick and Bertozzi 2008). Detailed studies of the precise GPI structures in plants are scarce. However, there is circumstantial evidence of structural heterogeneity. In an investigation of plant GPI-structures, it was shown that about 50% of GPI-anchors released from membrane arabinogalactan-proteins (AGPs) contain β(1→4) galactose side chains at the 6-linked mannose residue of the core glycan, a plant-specific modification (Oxley and Bacic 1999). GPI-anchored AGPs from rose suspension cells treated with phosphotidylinositol-specific phospholipase C (PI-PLC) released only a subset of AGP-species, suggesting the existence of structural variants insensitive to the enzyme (Svetek et al. 1999). In proteomics studies, when microsome-bound GAPs were released alternatively using PI-PLC or phospholipase D (PLD), slightly different sets of peptides were identified (Borner et al. 2003; Elortza et al. 2003, 2006). As suggested by comparable findings using reversed phase chromatography of AGPs (Svetek et al. 1999), the differential sensitivity to cleavage of GAPs by PI-PLC and the less specific PLD could be caused by heterogeneity of GPI modifications such as glycosylation or acylation.

Table 1 Mammalian and yeast components of the GPI biosynthetic pathway, based on Orlean and Menon (2007), and their closest *Arabidopsis thaliana* homologs

Step	Reaction	Human locus	Yeast locus	Arabidopsis locus
1	GlcNAc-PI synthesis	PIG -Ab	Gpi3pb	At3g45100 SETH2
		PIG-C	Gpi2p	At2g34980 SETH1
		PIG-H	Gpi15p	nf
		PIG-P	Gpi19p	At1g61280
				At2g39445
		PIG-Q	Gpi1p	At3g57170
		PIG-Y	Eri1p	nf
		DPM2	–	At1g74340
2	GlcNAc-PI de-N-acetylation	PIG-Lb	Gpi12pb	At3g58130
				At2g27340
3	GPI flipping	?	?	
4	Inositol acylation	PIG-W	Gwt1p	At4g17910 PNT5
5	α1,4 mannosyltransfer	PIG-M	Gpi14p	At5g22130 PNT1
		PIG-X	Pbn1p?	At5g46850
6	Etn-P transfer to Man-1	PIG-N	Mcd4p	At3g01380
7	α1,6 mannosyltransfer	PIG-V	Gpi18p	At1g11880
8	α1,2 mannosyltransfer	PIG-B	Gpi10p	At5g14850
9	Etn-P transfer to Man-3	PIG-O	Gpi13p	At5g17250
		PIG-F	Gpi11p?	At1g16040
y	α1,2 mannosyltransfer	hSmp3	Smp3p	nf
10	Etn-P transfer to Man-2	hGpi7	Gpi7p	At2g22530
11	GPI transamidase	PIG-F	Gpi11p?	At1g16040
		PIG-Kb	Gpi8pb	At1g08750
		GAA1	Gaa1p	At5g19130
		PIG-S	Gpi17p	At3g07180
		PIG-T	Gpi16pb	At3g07140
		PIG-U	Gab1p	At1g63110
p	Inositol deacylation	PGAP1	Bst1p	At3g27325
p	sn-2 deacylation	PERLD1/PGAP3	Per1p	At1g16560
				At5g62130
p	sn-2 C26 acylation		Gup1p	At1g57600
p	sn-2 acylation	PGAP2	Cwh43p?	nf

The initial two steps take place at the cytoplasmic face of the ER. The molecular basis of step 3 the flipping of the lipid head from the cytoplasmic to the luminal face of the ER membrane is unknown. y: In yeast and in some human tissues, a fourth mannosyl residue is attached. This step is essential only in yeast but is apparently absent from Arabidopsis. p: Steps of modification after the GPI structure is attached to the protein. For virtually every known component of GPI anchor biosynthesis and remodeling of yeast or mammals, there exists a single close Arabidopsis homolog. The presence of three pairs of two equally close Arabidopsis homologs indicates recent gene duplication events. For four Arabidopsis genes, SETH1, SETH2, PNT1, and PNT5, there is experimental support for a role in GPI-biosynthesis (see references in text and for PNT5 http://www.arabidopsis.org). ?: Not known

The importance of GPI anchor biosynthesis for plants has been demonstrated by the families of *seth* and *peanut* mutants in *Arabidopsis thaliana*. The *seth1* and *seth2* mutants showed distinctively reduced male transmission due to reduced pollen germination and pollen tube elongation (Lalanne et al. 2004). *SETH1* and

SETH2 encode two components of the initial step in GPI-anchor biosynthesis (Table 1). Numerous GAPs were identified in elongating pollen tube microsomes, and it was hypothesized that GPI anchoring mediated polar targeting of more than one of these GAPs to the pollen tube tip and is required for normal pollen tube elongation in processes related to cell wall biosynthesis or remodeling (Lalanne et al. 2004). Five *peanut* mutants representing distinct genetic loci were identified in a screen for embryo mutants and were found to essentially lack GAPs (Gillmor et al. 2005). *PNT1* encodes an intermediate step in GPI anchor biosynthesis (Table 1). Consistent with a role for GAPs in polarized growth, *pnt* mutants display pollen defects similar to *seth1* and *seth2*. Homozygous *pnt1* mutants undergo pleiotropically aberrant embryo development and display seedling lethality. The biochemical defects of *pnt1* mutants suggest a role for GAPs in cell wall assembly and remodeling. Interestingly, *pnt1* tissue can be propagated in callus culture, indicating the specific requirement of GAPs for organized cell division and expansion processes, rather than for basic cell survival and mitosis (Gillmor et al. 2005).

1.6 GPI-Anchored Proteins

Bioinformatic and proteomic analyses have identified 248 GAPs in *A. thaliana* (Borner et al. 2002, 2003; Eisenhaber et al. 2003; Elortza et al. 2006). Several plant GAPs have been genetically shown to fulfill essential roles, which could be based on biochemical processes that are compartmentalized to distinct PM domains.

The initial characterization of the Arabidopsis *cobra* (*cob*) mutant alleles suggested a role for COBRA in the highly polarized expansion of root cells during root elongation. Root epidermal cells in *cob* mutants appeared to expand in the radial instead of the normal longitudinal direction giving rise to the hypothesis that *COB* might be involved in specifically instructing cells into which direction to expand (Hauser et al. 1995). Detailed investigation suggested that *COB* might be essential for normal cellulose deposition as implied by a strong reduction of cellulose content in *cob* mutants (Schindelman et al. 2001). COBRA appears to be required for the normal orientation of cellulose microfibrils perpendicular to the main axis of cell elongation (Roudier et al. 2005). *COB* encodes a GAP (Schindelman et al. 2001; Roudier et al. 2005) containing a weakly conserved cellulose-binding domain. The failure of an artificial *COB* variant lacking a GPI-modification motif to complement the *cob* mutant suggests that the lipid anchor is essential for subcellular targeting, stability, or biochemical function (Roudier et al. 2005).

Next to the founder gene, 11 additional *COB-like* (*COBL*) genes have been identified in Arabidopsis (Roudier et al. 2002) and similar numbers of related genes exist in maize and rice (Brady et al. 2007). By combining transcript profiling and reverse genetics, essential roles for *COBL4* and *COBL9* in secondary cellulose formation (Brown et al. 2005) and root hair elongation (Jones et al. 2006) were identified, respectively. While COBL4 has no apparent GPI anchoring motif, its functional orthologs from rice, BRITTLE CULM 1 (OsBC), and maize, BRITTLE

STALK 2 (BK2), are GAPs, and it remains to be experimentally tested whether GPI-anchoring is optional in this subclade of secondary cell wall-specific COBL genes. It is possible that Arabidopsis COBL4 might contain a cryptic GPI-modification signal that is "invisible" to available algorithms. Mutations in *COBL9* result in a dramatic root hair phenotype in Arabidopsis (Jones et al. 2006), thus evoking a potentially wider role of GAPs in polarized tip growth. Transcripts of numerous GAP-encoding genes were found to be enriched during root hair formation, and another GAP mutant, *mrh5,* which is defective in a GPI-anchored glycerophosphoryl diester phosphodiesterase-like gene, also displayed defective root hair morphogenesis (Jones et al. 2006). A ubiquitous requirement of GAP function for normal root hair elongation among flowering plants was recently indicated by the finding that the maize *ROOTHAIRLESS* 3 locus (*rth3*) encodes a monocot-specific COBL gene (Hochholdinger et al. 2008). Several COBRA-like proteins, including Arabidopsis COBL9, have also been identified in elongating pollen tubes (Lalanne et al. 2004). Together with the pollen elongation defect in the *seth* and *peanut* mutants (Lalanne et al. 2004; Gillmor et al. 2005), this finding suggests that a shared set of GAPs, encoded by paralogous genes, act in the two paradigms of polarized tip growth in plants. In summary, COBL proteins are generally GPI-anchored and function in the polarized processes of oriented cellulose deposition and root hair formation in a fashion that suggests an indirect role in controlling cell wall deposition.

AGPs are a structurally and functionally diverse group of glycoproteins (Nothnagel 1997; Showalter 2001; Seifert and Roberts 2007) that were among the first plant GAPs to be characterized (Oxley and Bacic 1999). Later work demonstrated a tight biological relationship between two seemingly unrelated posttranslational modifications of cell surface proteins – GPI anchoring and arabinogalactan (AG) glycosylation. It was found that approximately 40% of all *A. thaliana* GAPs are predicted to be AG-modified (Borner et al. 2002, 2003). However, most characterized AGPs are GPI-anchored (Schultz et al. 2002). The biological significance of GPI-anchoring of AGPs remains to be elucidated; however, the lectin-like properties of AGPs and their high degree of glycosylation could play a role in interactions between GPI-enriched PM domains and the periplasmic space/cell wall (Lamport et al. 2006; Seifert and Roberts 2007). Moreover, as is sometimes the case in animals (Potter et al. 2006), glycosylation might be an additional factor required for polarized localization of some GAPs.

The fasciclin-like AGPs (FLAs) are an AGP subgroup containing a fasciclin-like domain potentially involved in protein–protein interactions (Schultz et al. 2002). FLAs have been shown to be essential for normal root elongation in Arabidopsis under salt stress, as the *salt overly sensitive 5* (*sos5*) mutant carries a missense mutation in the fasciclin-like domain of *FLA4* (Shi et al. 2003). Reminiscent of the *cob* phenotype, *sos5* mutant roots exhibit radial expansion under restrictive salt conditions. However, it remains to be determined whether FLA4 and COB both act in a similar fashion in the regulation of polarized cellulose deposition.

Xylogen, a GPI-anchored AGP with similarity to nonspecific lipid-transfer proteins (ns-LTP), was initially isolated as a soluble factor inducing xylem differentiation in *Zinnia* cell culture (Motose et al. 2004). Arabidopsis *xyp1 xyp2* double

mutants lacking the two closest xylogen homologs are characterized by a lack of vascular continuity, and it was concluded that xylogen might function as a mediator of inductive cell–cell interactions in vascular development. In addition to *XYP1* and *XYP2,* there are at least 13 additional members of the xylogen AGP family in Arabidopsis, all of which are predicted to be GAPs.

The protein encoded by the *SKU5* locus is relatively abundant in the PM and is frequently identified in proteomic surveys of GAPs (Borner et al. 2003; Elortza et al. 2003, 2006) and detergent-resistant membranes (Borner et al. 2003; Shahollari et al. 2004). *SKU5* is required for normal root responses to mechanical stimulation (Sedbrook et al. 2002). *SKU5* encodes a GAP similar to copper oxidase and is a member of a family of 19 genes, only three of which are predicted or confirmed GAPs (*SKU5*, *SKS1*, *SKS2*).

The *PMR6* locus encodes the only predicted GAP in a family of 27 pectate lyase-like Arabidopsis proteins (Vogel et al. 2002). *PMR6* is required for normal cell wall structure, as pectin is enriched in mutants. This apparently contributes to the ability of *pmr6* mutants to directly suppress mildew invasion of host cells or indirectly induce resistance in a jasmonate, salicylic acid, and ethylene-dependent manner.

NDR1, an Arabidopsis gene required for resistance mediated by *RPS2*, *RPM1*, and *RPS5* (Coppinger et al. 2004), encodes a PM-localized GAP that retains its N terminus, indicating dual PM-anchoring by the noncleavable N-terminal signal anchor and by the C-terminally attached GPI-anchor. This rare topology enables NDR1 to directly interact with the cytosolic resistance protein RIN4 (Day et al. 2006) and to thereby activate disease resistance signaling. GPI-anchoring at the PM possibly enables NDR1 to function as an intercellular transducer of pathogen signals or to directly interact with the pathogen (Coppinger et al. 2004).

Given the sparse information that is available on the cellular localization of GAPs, it is still too early to conclusively support or reject the hypothesis that the GPI-modification is sufficient for targeting to specific cellular domains. COB and SKU5 are both relatively abundant GAPs that are localized to the PM of elongating root cells (Sedbrook et al. 2002; Roudier et al. 2005) and both appear to be localized in a nonuniform fashion. However, while COBRA was primarily localized in a striated pattern along the lateral PM domain, SKU5 was more uniformly distributed with occasional apical-basal localization. The distinct localization of COB and SKU5 in elongating root epidermis cells might indicate that, although GPI-anchoring is necessary for transport to the PM, additional factors such as glycosylation, oligomerization, or heterophilic interactions are required for recruitment into specific PM subdomains. This is sometimes the case in animal systems (Benting et al. 1999; Paladino et al. 2004; Potter et al. 2006). Alternatively, remodeling of GPI anchors through differential ethanolamine substitution, attachment of sugar side chains, or inosityl acylation might contribute to targeting of GAPs to specialized PM domains.

GPI-anchored AGPs have mostly been visualized using ectopically expressed fluorescent protein fusions. In a majority of these studies, the fusion proteins appear evenly distributed throughout the PM (Nguema-Ona et al. 2007; Sardar et al. 2006). However, the localization of xylogen in vascular cells, visualized using specific antibodies, is strikingly polar (Motose et al. 2004). In immature xylem cells,

xylogen is found concentrated at the apical side of the PM. In a carrot cell culture undergoing somatic embryogenesis, an AGP-specific carbohydrate epitope was found to be distributed in a polar fashion in premitotic cells and appeared to predispose the differential developmental fate of the resulting daughter cells (McCabe et al. 1997). Although the precise molecular nature of the AGP(s) visualized in this study is unknown, GPI-anchoring provides an attractive mechanistic model for polar distribution of a specific group of cell surface glycoproteins.

There is mounting momentum for the idea that some GAPs might mediate the relationship between the cell wall and the cytoskeleton (Baskin 2001; Wasteneys and Fujita 2006). The localization of LeAGP1 was altered by cytoskeleton-disrupting drugs in tobacco cells (Sardar et al. 2006) while chemical disruption of AGPs caused a disorganization of microtubules (MTs) and bundling of actin in the same system and in *A. thaliana* roots (Nguema-Ona et al. 2007). Furthermore, FLA13: yellow fluorescent protein fusion appeared in a striated pattern reminiscent of transverse MTs in elongating hypocotyl cells (Fig. 1c; GJ Seifert, unpublished observations). The similar striated localization of COBRA transverse to the elongation axis of the root epidermal cells is rapidly disrupted by chemical disruption of MT (Roudier et al. 2005). On the one hand, the postulated interaction with the cytoskeleton could provide a rationale for the polar and uneven localization function of numerous GAPs. On the other hand, a presumed strict localization of GAPs at the outer leaflet of the PM obviates the requirement for additional factors that have the capacity to interact with the cytoskeleton as well as with the GAPs. These hypothetical factors could either be transmembrane proteins such as receptor-like kinase (RLKs) or lipid microdomain-associated membrane-embedded proteins or lipid-anchored cytosolic proteins (Sardar et al. 2006), and interestingly, the GAP FLA2 cofractionated with ABCB19, an ATP-binding cassette P-glycoprotein possibly acting in auxin polar transport (Murphy et al. 2002; Titapiwatanakun et al. 2009). A clear demonstration of plant GAPs localization to the external leaflet of the PM and elucidation of RLK function could be crucial in the understanding of the role of GAPs in cellular polarity.

1.7 Phosphorylation as a Determinant for Membrane Protein Targeting

PIN auxin transport proteins represent well-characterized polarity determinants in higher plants (Benjamins et al. 2005; Petrasek et al. 2006; Vieten et al. 2007). Several of the *Arabidopsis* PIN proteins have been shown to occupy an asymmetric, highly polarized location at the PM (Gälweiler et al. 1998; Müller et al. 1998; Friml et al. 2002, 2003). Moreover, manipulation of such polar PIN distribution was demonstrated to interfere with the establishment of auxin gradients and growth responses, indicating that subcellular PIN localization provides vectorial

information for the directional distribution of the growth regulator (Wisniewska et al. 2006).

In recent years, a plethora of regulatory switches has been found to control expression and subcellular distribution of PIN proteins (Benjamins et al. 2005; Vieten et al. 2007). PIN phosphorylation, for example, is suggested to act as a potential determinant for polar PIN localization (Friml et al. 2004; Michniewicz et al. 2007). PINOID (PID), a member of the AGCVIII family comprising plant orthologs of PKA, PKG, and PKC protein kinases (Galvan-Ampudia and Offringa 2007), is apparently a mediator of such phosphorylation-dependent PIN relocalization events. This is supported by the analysis of PID gain- and loss-function alleles, both of which give rise to a switch in the subcellular localization of PINs (Friml et al. 2004). A loss of PID activity results in relocation of PIN1 from the apical to the basal side of shoot epidermis cells, whereas PID overexpression mediates a basal-to-apical shift of PINs in the stele and in ground tissue of roots (Friml et al. 2004; Fig. 1d). Remarkably, a similar basal-to-apical shift in the localization of PIN proteins was detectable in mutants deficient in PP2A phosphatase activity, suggesting that elevated PID kinase activity, either as a result of PID overexpression or, alternatively, caused by reduced phosphatase activities, results preferentially in an apical localization of PIN proteins (Michniewicz et al. 2007). Compelling evidence for regulatory cross-talk between PINOID and PINs came from experiments demonstrating PID-mediated phosphorylation of PIN1 in a protoplast system, indicating that PINs might represent direct targets for PID kinase activities (Michniewicz et al. 2007; Kleine-Vehn and Friml 2008).

Additional reports demonstrated a quite general role for cargo phosphorylation as polarity determinant in eukaryotes. Studies of immunoglobulin receptors in polarized epithelial and endothelial cells revealed phosphorylation-dependent transcytosis from the basolateral to the apical surface (Casanova et al. 1990; Apodaca and Mostov 1993). These observations parallel the effects of PIN phosphorylation, suggesting that phosphorylation acts as a universal signal for cargo delivery to apical destinations in polarized cells.

At present, PINs represent the only class of plant proteins, polar delivery of which appears to be affected by their phosphorylation status. Yet, only little is known about mechanisms that might control phosphorylation and associated intracellular relocation of PIN proteins (Kleine-Vehn and Friml 2008). A connection with phospholipid signaling was established based on the finding that an *Arabidopsis* 3-phosphoinositide-dependent kinase binds to and modulates activity of PID (Zegzouti et al. 2006). In addition, transcription of PID was found to be induced by auxin (Benjamins et al. 2003), suggesting that variations in the subcellular distribution of PIN proteins that have been observed in response to variations in auxin concentrations could arise as an indirect consequence of altered *PID* expression (Sauer et al. 2006).

In addition to phosphorylation, members of the PIN protein family also undergo ubiquitination (Abas et al. 2006), a covalent modification that, besides its well-characterized role in proteasome-mediated protein degradation, acts as a signal for internalization and endosomal sorting of PM proteins (Hicke and Dunn 2003;

Haglund and Dikic 2005). In *Arabidopsis*, ubiquitination of PIN2 has been related to the control of protein localization as well as to its degradation, highlighting partially overlapping roles for phosphorylation and ubiquitation in the control PINs (Abas et al. 2006). Strikingly, ubiquitination of some PM proteins was demonstrated to depend on their phosphorylation status (Hicke et al. 1998; Shenoy et al. 2001). Similar crosstalk between PIN phosphorylation and ubiquitination might determine PIN distribution and steady-state levels. Yet, experimental confirmation of such interactions has not been provided so far.

2 Concluding Remarks

A large variety of signals and stimuli are perceived at the PM, which functions as the interface crucial for a cell's communication with its biotic and abiotic environment. Such multitasking requires numerous signal recognition and transduction modules, also reflected in the fact that the PM is a highly heterogeneous subcellular compartment consisting of functionally diversified macro- and microdomains that not only interact with other PM domains but also with the cell wall and the cytoskeleton. Posttranslational modifications, such as those discussed here, are instrumental to maintain such functional diversity, which is a prerequisite for the remarkable developmental plasticity and adaptability of plants in an ever-changing environment.

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Section II
Plasma Membrane Transporters

Functional Classification of Plant Plasma Membrane Transporters

Burkhard Schulz

Abstract A short overview of the very large and diverse group of plasma membrane transport systems is presented here. Emphasized are the transporters with important physiological functions in higher plants, which are localized to the plasma membrane. Members of gene families or transporters that do not localize to the PM are generally not discussed in this overview. In most cases, structural features of transporters rather than their transport activity were used for functional classification because many groups and families of transporters have a broad transport spectrum and can assume characteristics of biphasic or high- and low-affinity transporters. Expression data was mentioned if it helped to indicate possible functional aspects of transporters.

1 Introduction into Plasma Membrane Transport

The plasma membrane (PM) is the ultimate isolation of cell content from its proximate environment, the cell wall, and extracellular space. Cell metabolism and the flow of information as well as material for developmental processes require this boundary to serve as a selective barrier for molecules across this membrane in both directions, which means it is not completely impenetrable. This is the point where cytoplasmic composition is regulated and changing environmental signals are communicated to the cell. Transporters and channels are important elements of this cell-to-cell and environment-to-cell communication in plants. They can be understood as gatekeepers regulating molecule transport across the PM.

Decades of studies using labeled transport substrates, electrophysiological approaches, pharmacological research with transport inhibitors, in vivo imaging of

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intracellular ions, reconstitution of transporters in isolated membranes and vesicles, heterologous expression and transport measurements, as well as experiments describing the structure of transporters have contributed greatly to the analysis and understanding of molecule transport through the plant PM. Specific structures, such as the plant intrinsic plasmodesmata, which represent cell-to-cell connections through the cell walls of neighboring cells, connect the cytoplasm and membrane systems of both cells with each other. Plasmodesmata are very sophisticated constructions and dynamic structures, which play an important role in controlling the exchange of mRNA and proteins between cells. These are not specifically included in this list as they are the subject of chapter "Plasmodesmata and Non-cell Autonomous Signaling in Plants" by Lee, Cho, and Sager.

Whole plant genome sequencing projects, beginning with the groundbreaking publication of the complete *Arabidopsis* genome sequence (AGI 2000), have enabled us to tap into a treasure chest of genetic information. This has led to novel methods for gene discovery using forward and reverse genetics with never before seen efficiency and enabled us to mine gene data with bioinformatics tools. On the flip side, we now have the new "problem" of having nearly too many identified transporters to employ them all for a thorough analysis of transporter genes and their function.

This chapter provides a short overview of the families of PM transporter systems. The proteins and their genes are characterized by their functional classifications rather than their sequence and structural homology. The functional role that transporters play in developmental processes and signaling is established mostly by mutational analysis. However, the proper attribution of transporters in functional developmental networks is often lacking, leaving an open playing field for further research. Emphasis must be laid on the mechanism of transport rather than the molecules that are transported because large transporter families, which have a common structure but a very broad spectrum of transport substrates, can hardly be categorized based on their transport activity. The most striking example for this challenge is the ATP-binding cassette (ABC) transporter superfamily, which has more than 130 family members in eight subfamilies in *Arabidopsis* and rice with a broad spectrum of transport substrates (see chapter "ABC Transporters and Their Function at the Plasma Membrane" by Knöller and Murphy; Rea 2007; Schulz and Kolukisaoglu 2006; Verrier et al. 2008). A majority of these transporters have not even been localized to their cellular compartments. PM, tonoplast, mitochondrial, and peroxisomal ABC transporters have been found (Rea 2007).

2 Types of Membrane Transport

2.1 Passive Transport

Membrane transport enables the entry and exit of molecules and ions into or out of cells. One cannot begin to address membrane transport without mentioning the four primary mechanisms it uses. Diffusion is the simplest way of transport

through a membrane. Nonpolar, noncharged, and hydrophobic small molecules can move from regions of higher concentration to regions of lower concentration through a permeable membrane. They use the concentration gradient as a driving force to achieve equilibrium. The concentration difference dictates the amount of movement of molecules through membranes by diffusion. The variety of molecules, which are able to enter plant cells by diffusion, is rather small. Gases such as O_2, CO_2, N_2 and small molecules like ethanol can permeate the cell via diffusion. Even small, uncharged but polar molecules such as water and urea rely on transport systems for efficient transport. As soon as molecules surpass a particular size (glucose, fructose, etc.) or carry an electrical net charge (anions and cations), they are excluded from the cell and can no longer enter the cell through diffusion. The separation of opposite charged molecules through a biomembrane leads to the buildup of an electrical membrane potential, which is generated by the electrical transmembrane gradient. This electrical gradient, together with the concentration gradient, is defined as electrochemical potential across a cellular membrane. Transport of charged or polar molecules through a biomembrane requires three distinct phases: (a) The hydration shell that surrounds the charged molecule in the cytoplasm must be removed. (b) The charged molecule must migrate through the apolar lipid bilayer by diffusion. (c) The molecule must be surrounded with a new cover of water molecules to form a new hydration shell. This process is unlikely because the activation energy necessary to strip away the hydration shell and transport the polar molecule through the lipid phase is very high.

Second, membrane proteins can facilitate the migration of charged molecules through membranes by lowering the necessary activation energy. These transport facilitators serve as carriers, pores, or channels and allow the passive transport of molecules through the isolating barrier of a biomembrane. Transport is still driven by concentration differences and occurs along a concentration gradient through the membrane. The transport rate depends on the number of transporters per area in a membrane and is limited to a maximum rate. Transporters often form channels as integral membrane proteins to create a hydrophilic environment inside the pores, which allow permeation through the membrane. This is accomplished by replacing the hydration shell with weak hydrophilic interactions during the passage through the membrane, resulting in a greatly facilitated diffusion of hydrophilic molecules through the membrane. Carriers have higher transport substrate specificity, in many cases even a high stereospecificity, whereas channels show less substrate specificity and facilitate high diffusion rates of molecules through the membrane. Movement of molecules through a membrane against a concentration gradient is the hallmark of active membrane transport performed by energized pumps. This process requires cellular energy in the form of ATP equivalents or the establishment of electrochemical gradients. The transport limit depends on the number of available pump proteins in the membrane.

2.2 Active Transport

Primary active membrane transport is the third mechanism, and it is always directly coupled with an energy-generating reaction – in most cases ATP hydrolysis. The common transport systems of primary active transport are the ABC transporters, which hydrolyze ATP and use the energy to power the transport of molecules against a concentration gradient through the PM. ABC transporters are one of the largest gene families in plants. They have a vastly diverse transport substrate spectrum. The next largest group of representatives for primary active transport is p-type ATPases.

The fourth and the last transport mechanism is secondary active transport. This requires the establishment of an electrochemical gradient of a charged molecule through primary active transport prior to the secondary active transport. The energy of this electrochemical gradient is subsequently used for an "uphill" transport of a second molecule against a concentration gradient. Secondary active transport always requires the cotransport of two or more molecules. This process can take place as symport (unidirectional transport) or antiport (counter exchange in different directions).

Another way to functionally group membrane transporters is to classify them into uniporters and cotransporters. Uniporters transport only a single substrate in one direction, whereas cotransporters can be characterized as symporters and antiporters. The first class transports at least two different transport substrates in the same direction. The latter facilitate the movement of at least two different molecules in the opposite direction (counter exchange) during the transport process. Both classes – uniporters and symporters – can be either facilitated diffusion transporters (passive transport that does not require energy) or energized transporters (active transport that relies on energy input).

3 Passive Transport Through the PM

The simplest case of passive transport through the PM is diffusion of molecules. Diffusion through membranes is only effective for nonpolar molecules following a concentration gradient. The solute moves from a compartment of higher concentration to a region of lower concentration across the membrane. Diffusion comes to an end when both compartments possess the same concentration of solutes. Charged molecules such as ions are excluded from the cell and need specific transporter molecules that allow passage through the membrane without the high input of free energy that would be necessary to directly move the charged molecules through the membrane. Transporters or permeases that facilitate transport through the membrane can be grouped in carriers and channels. Both types of membrane proteins form multiple membrane spanning regions that form a passageway for charged molecules with a greatly reduced free energy ΔG to facilitate transport of charged molecules. As mentioned before the hydrated solute gets dehydrated upon entry into the transporter. Weak noncovalent interactions with the dehydrated solute allow passage through the

transporter. This provides a more hydrophilic environment for transfer through the membrane than can be found in the very hydrophobic lipid bilayer of the membrane. After passage through the transporter, the solute is rehydrated again in the cytoplasm.

3.1 Channels

Channels and pores are proteins, which do not present a great obstacle against the free flow of substances through membranes. They allow high rates of transmembrane passage, which exceeds one of the more specific carriers by orders of magnitude. As they are passive transporters, their transport direction is determined by differences in solute concentration and electrochemical potential. In the case of channels, we can distinguish between cation and anion channels. Yet channels can also transport water (aquaporins or water channels) or hydrophilic small molecules along a concentration gradient or following an electrochemical potential gradient in the form of a facilitated transport. The membrane proteins that form a channel create a hydrophilic pore or gateway through the PM, which is used by water molecules or ions to pass through the membrane. Channels that are permeable most of the time are called ungated channels in contrast to channels that open upon an electrical or chemical signal. These channels are called gated channels. Both types of channels can be very selective for the transport molecule whose passage through the membrane they facilitate.

3.1.1 Anion Channels

Anion channels are present in the PM and have also been found in all other plant cell membranes. They play important roles in ion and pH homeostasis as well as signaling processes that allow plants to adapt to biotic (pathogens) and abiotic (salinity, temperature, drought) environmental stresses. A classification of these transport proteins has been made possible through electrophysiological characterization. Patch clamp analysis (Neher and Sakmann 1976) adopted in plant systems allowed the characterization of single channels and their electrophysiological behavior (Moran et al. 1984; Schroeder et al. 1984; Hedrich and Schroeder 1989). Several anion channels have been characterized physiologically, yet the number of cloned and characterized genes for anion channels is still rather small.

Anion channels of the PM can be ascribed to four different classes. Two different classes of voltage-dependent rectifying channels are characterized by activation through depolarization or hyperpolarization. Anion channels activated by light and those activated by mechanical stretch of the membrane (Cosgrove and Hedrich 1991) complete the groups of channels found in the PM (Roberts 2006; De Angeli et al. 2007). Depolarization-activated channels occur as slow-activating (S-type) and rapid-activating (R-type) anion channels. Both types have been found in guard cells, root epidermis cells, hypocotyls, and cell cultures. Selectivity for the transport substrate

differs between both channel types. Rapid-activating channels are selective for nitrate, chloride, malate, sulfate, and citrate (Kohler and Raschke 2000; Diatloff et al. 2004). S-type channels show the highest selectivity for chloride and, to a lesser extent, for citrate and phosphate (Zhang et al. 2004). Light-activated ion channels have been described in plants as well as animals (Banghart et al. 2004; Spalding 2000). In plants, the most prominent processes that involve light-activated anion channels are blue light-induced growth responses of hypocotyls and stems, and stomata movements. Using patch clamp technology, a PM-localized Cl^- channel has been identified as blue light inducible in etiolated *Arabidopsis* hypocotyls upon blue light irradiation (Cho and Spalding 1996). Mechanosensitive anion channels in plants have been first reported by Falke et al. (1988) in tobacco cell cultures and by Cosgrove and Hedrich (1991) in guard cell PM. Being stretch-activated channels, these anion transporters are likely involved in osmosensing in guard cells and osmoregulation in tobacco cell cultures as their respective opening times differ dramatically. Qi et al. (2004) show in a recent report the existence of mechanosensitive anion channels in protoplasts of *Arabidopsis* mesophyll cells. This channel was activated through positive pressure on outside-out patches in patch clamp experiments, indicating that this channel is a negative regulator for cell volume control during cell growth or osmotic stress. The molecular identity of these channels is not yet established.

3.1.2 Aluminum-Activated Malate Transporters

ALMTs are aluminum-activated malate transporters, which are implicated in stomata closing as well as conferring resistance toward increased levels of soil aluminum. Soluble aluminum Al^{3+}, which is mobilized by acidification of soils, is toxic to the root apex. Many plants show resistance toward high Al^{3+} levels by exporting organic anions from the root apex. TaALMT1 from wheat was the first cloned plant Al^{3+}-resistance gene (Sasaki et al. 2004). Its role as aluminum-activated malate permease was shown by heterologous expression in tobacco and barley, which resulted in increased aluminum resistance in both cases. Additional members of the ALMT protein family have been characterized from *Arabidopsis*, brassica, and rye (Delhaize et al. 2007). In *Arabidopsis*, ALMTs form a small gene family with 14 members, which are localized to different membrane compartments (Kovermann et al. 2007). ALMTs have six to eight predicted transmembrane (TM) domains and show inducible malate transport when expressed in *Xenopus laevis* oocytes, which indicates that the ALMT proteins themselves also act as aluminum stress sensor. Recent data from Piñeros et al. (2008) indicate that ALMTs can also be involved besides aluminum resistance in plant nutrition and ion homeostasis as they are implicated in selective transport of anions.

3.1.3 Slow Anion Channel-Associated 1

Anion channels are the essential means for the control of stomata closing and anion efflux from plant cells. Most anion channels are characterized only on a

physiological level using electrophysiological methods. A gene encoding a PM protein in guard cells has recently been isolated from genetic screens for mutants that show increased sensitivity toward ozone and reduced sensitivity toward CO_2 (Vahisalu et al. 2008; Negi et al. 2008). The slow anion channel-associated 1 (SLAC1) protein is mostly expressed in guard cells and is related to fungal and bacterial dicarboxylate/malic acid transporters. *Arabidopsis* mutants in *SLAC1* are impaired in activity of slow anion channels (S-type channels). Mutations in *SLAC1* do not affect rapid anion channels (R-type) or Ca^{2+} channels. Mutant analysis showed that SLAC1 integrates signal transduction that leads to stomata closure in response to ozone, CO_2, nitric oxide, abscisic acid (ABA), changes in light regime and humidity, and calcium ions. SLAC1 belongs to a small protein family with five members in *Arabidopsis* and nine members in rice (Vahisalu et al. 2008). Three of the proteins, including SLAC1, have a long hydrophilic N-terminal tail, whereas two have only the TMs. SLAC1 has nine to ten predicted membrane helices and long hydrophilic tails at the amino and carboxy terminus. All SLAC proteins are PM localized but are expressed in different tissues (Negi et al. 2008). The association of SLAC1 – a membrane protein with organic acid transporter features – with S-type anion channels, which act as malate permeases, suggests an important role for SLAC1 in S-type anion channel activity.

4 Cation Channels

4.1 K^+ Channels

Potassium is one of the most prominent components in plant dry matter and a very important nutritional component for plants. Membrane polarization and osmoregulation, specifically control of water potential and turgor, are functions that rely on availability and transport of K^+ in and out of the cell. Long-range transport processes such as photosynthate transport from sink to source tissues and root pressure buildup for transpiration-independent transport out of roots depend on K^+. Regulation of stomata opening through guard cell movements is another important process that uses K^+ as a driving force. Uptake systems for K^+ are described as high-affinity and low-affinity transport systems.

4.1.1 Shaker-Type K^+ Channels

Inward-Rectifying Shaker-Type Channels

The guard cell system of leaf stomata has been proven to be a highly yielding experimental system for K^+ channel research. The first K^+ channel has been identified using patch clamp technology on protoplasts of guard cells (Schroeder et al. 1984). Molecular complementation of yeast mutants allowed the identification

of KAT1 and AKT1 – the first two K^+ channels from plants. These channels – together with KAT2 and SPIK (shaker pollen inward K^+ channel, also known as AKT5) – show the characteristics of voltage-dependent and hyperpolarization-activated inward-rectifying channels (Anderson et al. 1992; Sentenac et al. 1992; Müller-Röber et al. 1995).

Shaker-like K^+ channels have six TMs. Voltage sensitivity for all shaker-type channels is mediated by the fourth TM through a number of positively charged amino acids. A loop region between TM5 and TM6 forms a pore domain. The cytoplasmic C terminus contains a putative cyclic nucleotide-binding domain and an ankyrin domain (see chapter "Biology of Plant Potassium Channels" by Hedrich et al.). It has been shown that a functional pore is formed by tetrameric complexes of similarly structured monomers. The cytosolic C terminus is required for assembly of the complex. Interaction studies using yeast 2-hybrid as well as the split ubiquitin system revealed the ability for physical interaction and heteromerization of AtKC1, AKT1, and KAT1 (Mäser et al. 2001; Daram et al. 1997; Obrdlik et al. 2004; Zimmermann et al. 2001), which expands the possibilities for differential complex formation through heteromerization. Regulation of K^+ channel activity through Ca^{2+} has been reported for shaker-like K^+ channel (Hedrich and Kudla 2006; Xu et al. 2006; Li et al. 2006). Calcium sensors of the calcineurin class in concert with their interacting Ca^{2+}-binding protein kinases (CIPKs) activate shaker-like AKT1 channel under low K^+ conditions. Interestingly, a similar interplay between calcineurins and CIPKs activates the Na^+/H^+ exchanger SOS1 under high Na^+ conditions, resulting in export of toxic Na^+ from the cytosol. Dephosphorylation of the related shaker-like channel K^+ AKT2 by CIPK/PP2C complexes negatively regulates its activity (Batistic and Kudla 2004), which suggests a general principle of Ca^{2+}-mediated CIPK/calcineurin regulation of shaker-like K^+ channel.

Outward-Rectifying Channels

Further members of this plant transporter family, which has nine members in *Arabidopsis*, are the outward-rectifying channels (guard cell outward-rectifying K^+ channel) GORK and stellar K^+ outward (rectifier) SKOR (Mäser et al. 2001). These outward-rectifying channels are activated via membrane depolarization and belong to the group of shaker-like potassium channels. SKOR allows mostly unidirectional efflux of K^+ from cells into the xylem of the root vascular tissue – a function of root to leaf long-distance transport. Mutations in skor lead to reduced K^+ conductance and reduced K^+ content in stems and leaves (Gaymard et al. 1998). The guard cell outward-rectifying K^+ channel (GORK) is the most prominent outward-rectifying K^+ channel in guard cells and essential for stomatal movements (Ache et al. 2000). GORK is activated through anion efflux from guard cells, which results in membrane depolarization. Water loss and stomata closure are subsequent results of GORK activation. Loss-of-function mutants in gork are impaired in stomata closure (Hosy et al. 2003). The outward-rectifying channels GORK and SKOR are also able to form heteromeric channels when expressed in heterologous

systems, which indicates also for this subgroup of shaker-like potassium channels a greater functional diversity through combination of different monomers (Dreyer et al. 2004). It has been shown that opening and closing of voltage-gated shaker-type K^+ channel is mediated by the voltage sensor of the fourth TM. The voltage signal has to be transmitted from the voltage sensor to the pore-forming loop between TM5 and TM6. Recent results by Li et al. (2007) indicate that interaction of several TMs and the pore loop contributes to the voltage sensitivity and directionality of shaker-like K^+ channels. The outward rectifier SKOR could be converted into an inward rectifier and an inactive SKOR/LKT1 hybrid channel was converted into an active inward rectifier by single amino acid mutations in TM5 and the loop region.

Tandem Pore K^+ Channel

Tandem pore K^+ channels (TPKs) are K^+ channel with four TMs with a pore loop (P-loop) after the first and the third TM. A C-terminal EF-hand motif suggests channel activation through elevated Ca^{2+} levels, which have been shown to activate vacuolar TPKs localized in guard cell vacuoles. The activation of TPKs of the tonoplast leads to massive efflux of K^+ into the cytosol and from there into the apoplast, which results in stomatal closure (Ward and Schroeder 1994; Gobert et al. 2007). A 14-3-3 protein-binding site in the C terminus of TPKs mediates channel activation through 14-3-3 proteins (Latz et al. 2007). These TPKs are mostly voltage insensitive, slow vacuolar "open rectifiers," which are represented with TPK4 as their only PM-localized member. TPK4 is only present in pollen and seems to be important for maintaining ion homeostasis during pollen tube growth (Becker et al. 2004).

High-Affinity K^+ Transporters

High-affinity K^+ transporters HKT in plants are structurally related to K^+ channels. They consist of four pairs of TMs with a pore loop between the two TMs that form each pair and are found in bacteria, fungi, and plants. Their structure resembles an equivalent of the tetrameric KcsA-type channels, which are represented only by a single gene Kir1/KCO3 in *Arabidopsis* and are absent in poplar and rice genomes (Ward et al. 2009). The first cloned plant HKT gene was TaHKT1 from wheat (Schachtman and Schroeder 1994). Surprisingly, this transporter acts as high-affinity K^+/Na^+ cotransporter, which turns into a low-affinity Na^+ uniporter at high ion strength (Rubio et al. 1995). Subsequent characterization of HKT orthologs from different plant species revealed a mix of Na^+ transporters, Na^+/K^+ cotransporters, and Na^+-coupled K^+ transporters for these genes (Gierth and Maeser 2007). Potassium channel P-loops allow penetration of the K^+ cation into the hydrophobic membrane space. The P-loops end up in the so-called filter residues gly-tyr-gly (GYG), which are able to bind K^+ ions with carbonyl backbone groups.

This interaction is weak and allows subsequent release of the K^+ ion (Doyle et al. 1998; Morais-Cabral et al. 2001). The GYG motif is highly conserved in all K^+ channels. This, however, is not the case for a subgroup of plant HKTs that are predominantly transporting Na^+ instead of K^+. These proteins have a filter glycine in the first P-loop replaced by a serine residue (Mäser et al. 2001). Site-directed mutagenesis and functional testing revealed that a filter glycine in the first P-loop determines K^+ specificity, whereas replacement of this position with a serine renders the channel into a Na^+ transporter.

KUP/HAK/KT Permeases

The family of KUP/HAK/KT plant permeases has been identified through its homology to K^+ uptake permeases from bacteria (KUP) (Schleyer and Bakker 1993) and high-affinity K^+ transporters from fungi (HAK) (Banuelos et al. 1995). KUP/HAK/KT genes have been cloned from different plant species and tested via molecular complementation in *Escherichia coli* and other heterologous systems for potassium transport (Quintero and Blatt 1997; Gierth and Maeser 2007). All KUP/HAK/KT transporters contain 10–14 TMs without P-loops and can be found as small gene families in *Arabidopsis* (13 predicted genes) and rice (25 predicted genes) (Schwacke et al. 2003). The mechanism of K^+ transport through KUP/HAK/KTs remains unclear. Functional characterization *in planta* through overexpression or mutant analysis revealed for KUP1, KUP4, and KUP5 from *Arabidopsis* high-affinity uptake kinetics for K^+. Analysis of *Arabidopsis* mutants *tiny root hair 1 (trh1)* and shy3-1 showed in both cases impaired growth of root hair tips and dark-grown hypocotyls, respectively (Rigas et al. 2001; Elumalai et al. 2002). However, in both cases, K^+ transport was not the causative reason of impaired growth, which indicates possible interactions of KUP/HAK/KTs with other components that are required for cell elongation.

5 Ca^{2+} Channels

A number of calcium channel activities have been characterized in plants including Ca^{2+} influx and efflux transporters (see chapter “Mechanism and Evolution of Calcium Transport Across the Plant Plasma Membrane” by Connorton et al.). The corresponding genes for these activities have not yet been identified. Candidate genes that could encode plant PM Ca^{2+} channels belong mainly to the families of ionotropic glutamate receptor channel homologs (GluR) (Lacombe et al. 2001) with 20 members and the gene family of cyclic nucleotide-gated channels (CNGs), which also have 20 family members in *Arabidopsis*. In both cases, ample circumstantial evidence indicating activity as calcium channels for these proteins has been gathered.

5.1 Cyclic Nucleotide-Gated Channels

Plant CNGs are implicated in Ca transport but show also additional cation transport activities such as K^+, Na^+, and other monovalent cations (Kaplan et al. 2007). CNGs have six TMs, which form a selective pore that can be opened and closed due to binding of cyclic nucleotides to the nucleotide-binding domain in the C-terminal extension of CNGs (Kaplan et al. 2007). Heterologous expression of plant CNGs in yeast, oocytes, and mammalian cells for transport studies reveals a relatively broad selection of ions used as transport substrates. Mutational analysis in *Arabidopsis* shows that CNGs are involved in processes that require Ca flux and signaling such as pathogen responses and pollen tube growth (Ali et al. 2007; Frietsch et al. 2007).

5.2 GluR (Ionotropic Glutamate Receptor Channel Homologs)

The gene family of GluR-like proteins has 20 members in *Arabidopsis*. So far heterologous expression of plant GluRs using oocytes or other expression systems to investigate ligand-binding or ion channel specificity has not been successful. It is possible that heteromer formation is essential for functionality because channel or additional protein factors that are not yet known to interact with GluRs in plants are required. It has been shown that glutamate and glycine stimulate Ca^{2+} influx in *Arabidopsis*. Dennison and Spalding (2000) report that application of glutamate to root tips triggers membrane depolarization and rapid increase in cytoplasmic Ca^{2+}. Overexpression studies and mutant analysis in *Arabidopsis* indicate that plant GluRs are involved in C influx upon glutamate treatments (Kang et al. 2006; Qi et al. 2006). However, none of these studies clearly identifies a single plant GluR as Ca^{2+} permease.

5.3 Annexins and Ca^{2+} Transport

Annexins are soluble proteins that are able to bind or insert into the PM in a calcium-dependent way. Structural motifs in annexins suggest that they are able to transport calcium. In a recent study, highly purified ANN33/35 from maize was used on root epidermal protoplasts or *Arabidopsis* expressing the calcium reporter apoaequorin. This treatment resulted in elevated cytosolic Ca^{2+}. Incorporation of annexins in lipid bilayers generated Ca^{2+} conductance, which indicated a functional nonselective Ca^{2+} channel (Laohavisit et al. 2009). The genuine role of annexins in calcium transport must be further established using functional analysis of mutants and isolated proteins in homologous systems.

5.4 Voltage-Dependent and Mechanosensitive Ca^{2+} Channels

Voltage-dependent Ca^{2+} channel can be classified as hyperpolarization-activated Ca^{2+}-permeable channel (HACC) and depolarization-activated Ca^{2+}-permeable channel (DACC) (White et al. 2002). Mechanosensitive Ca^{2+} channel (MCC) seems to play a role in mechanoresponses in plants. Increase in Ca^{2+} upon mechanostimulus has been reported (Monshausen et al. 2007). Genes of the MSL gene family and MCA1 and MCA2 from *Arabidopsis* are suggested as good candidates for MCC, but the identity of these channels is not yet known.

6 Pumps

6.1 P-Type ATPases

Plant P-type ATPases pump protons across the PM to concentrate them in the cell wall space. This results in the generation of an electrochemical gradient across the membrane, which provides the driving force for secondary active transport. The classification as P-type pumps comes from the fact that autocatalytic phosphorylation of the pump results in a required structural change for active transport. Dephosphorylation resets the pump to its nonactive stage after transport has taken place.

6.1.1 P_{3A}-Type H^+-ATPase

Most of the PM-localized ATPases generate electrochemical gradient potential, which are utilized for the export of calcium, zinc, and heavy metals from the cell. The P-type ATPases of the P_{1B}-(Zn^{2+}, Cd^{2+}), the P_{2B}-(Ca^{2+}), and the P_{3A}-type (H^+) are the groups of P-type ATPases, which are localized to the plant PM. The major pump of the plant PM is the P_{3A}-type H^+-ATPase, which was cloned nearly two decades ago as one of the first plant membrane transporters. Its pumping activity establishes an electrochemical gradient across the PM, which powers secondary transport systems involved in uptake of macro- or micronutrients including heave metals, Na^+/K^+, H^+/K^+, Ca^{2+}, or solute efflux. They are present in most phyla, except animals. In plants, the H^+-ATPase (AHA) is found in small gene families of 9–12 members (Baxter et al. 2003). A functional overlap and compensation due to the redundancy in expression can be assumed because all of these genes are expressed in either specific or broad expression patterns, often several AHA genes are expressed in the same tissue and cells. A fitting explanation for the great diversity in this gene family has not yet been proposed (see chapter "Plasma Membrane ATPases" by Palmgren et al.). The entire gene family of plasma membrane H^+ pumps is expressed in guard cells where the stomatal opening is

dependent on uptake of potassium and water, which is mediated by outward proton pumping through H^+-ATPases. Constitutively activated H^+-ATPases upon binding of the fungal toxin fusicoccin lead to a permanently opened stomatal pore (Schaller and Oecking 1999).

6.1.2 P_{2B}-(Ca^{2+}) ATPase

Of the three major subfamilies of Ca^{2+} ATPases, only the P_{2B}-(Ca^{2+}) ATPases (ACA) are localized to the PM. They are related to mammalian PM calmodulin-stimulated ATPases; however, plant P_{2B}-(Ca^{2+}) ATPases have a calmodulin-binding autoinhibitor domain at the N terminus. The gene families of P_{2B}-(Ca^{2+}) ATPase contain 11 members in rice and 10 members in *Arabidopsis*. Sequence alignments and intron structure allows to group the P_{2B}-(Ca^{2+}) ATPase into four subgroups (Baxter et al. 2003). They have ten TMs and two characteristic cytoplasmic loops, as well as long N- and C-terminal extensions. The functional role for these ATPases is the control of calcium homeostasis in the cell. The export of calcium from the cell generates gradients, which are used for rapid cellular signaling processes. Aside from stress signaling (Qudeimat et al. 2008), growth processes of pollen and inflorescences are regulated by the activity of (Ca^{2+}) ATPases (Schiott et al. 2004). Ca^{2+}/calmodulin binds the autoinhibitory domain and prevents interaction of the autoinhibitory domain with the pore domain, thereby activating the pump (Boursiac and Harper 2007; see chapter "Plasma Membrane ATPases" by Palmgren et al.).

6.1.3 P_{1B}-Zn^{2+}-ATPase

P_{1B}-Zn^{2+}-ATPases are ubiquitous heavy metal transporter, which transport mainly Zn, Co, Cd, Pb, Cu, and Ag (Baxter et al. 2003). In *Arabidopsis*, two of eight P_{1B}-Zn^{2+}-ATPases are located at the PM of root xylem parenchyma and pericycle cells. These transporters are able to transport Zn and Cd across the PM. Mutant analysis corroborated transport studies in heterologous expressing yeast because the null mutants of HMA2 and HMA4 displayed a zinc-deficient phenotype (Hussain et al. 2004).

6.2 H^+-Pyrophosphorylase

H^+-pyrophosphorylases (H^+-PPases) have 14–17 TMs, which act as homodimerized proton pumps. They are energized by pyrophosphate (PPi). The family of plant H^+-PPases can be distinguished into type I PPases that are potassium activated and less-sensitive toward calcium ions and type II PPases that are potassium insensitive and hypersensitive toward calcium. It has been speculated that plant H^+-PPases are

redox regulated via inactivation through H_2O_2 and reactivation by glutathione (Gaxiola et al. 2007). Studies in *Arabidopsis* indicate an essential role for type I H^+-PPase AVP1 because overexpression leads to increased auxin transport, increased cell division, and hyperplasia. Null mutants in *avp1* show reduced polar auxin transport, stunted root, and shoot development. It seems likely that AVP1 facilitates the trafficking of P-type H^+-ATPase to the PM, which results in apoplastic pH changes and increased auxin transport. PM localization of H^+-PPase was also correlated with high-capacity polar auxin transport (Mitsuda et al. 2001; Langhans et al. 2001).

6.3 ABC Transporters

ABC transporters make up the largest membrane transporter family in monocots and dicots (Martinoia et al. 2002; Jasinski et al. 2003; Garcia et al. 2004; Rea 2007; Verrier et al. 2008). They are active transport pumps, which are energized by ATP hydrolysis. All ABC transporters consist of at least two transmembrane-spanning hydrophobic domains (TMDs), as well as two cytoplasmic nucleotide-binding folds (NBFs). The latter are responsible for binding and hydrolyzing ATP to generate energy for the transport. Additional domains at the N- or C terminus can be found. Most ABC transporters have six predicted TMs in each TMD, which would bring the total number of transmembrane-spanning regions TMs to 12. Single ABC transporter-domain transporters have one TMD and one NBF. Two of these single-domain transporters form a functional entity, which is able to perform the transport of specific substrates. The process of reiterated ATP binding and substrate transport includes the induced ATP hydrolysis by high-affinity substrate binding with subsequent changes in the conformation of the ABC transporter complex. This leads to the translocation of the substrate into the extramembrane space or the upper leaflet of the bilayer of the membrane. Dimerization of the NBFs favored by ATP binding and subsequent dissociation of NBF dimers upon ATP hydrolysis are the steps that have been postulated from structural studies using crystallized NBFs and ABC transporters from prokaryotes and mouse (Hung et al. 1998; Locher et al. 2002; Dawson and Locher 2007; Aller et al. 2009). Following the translocation of the substrate, ATP hydrolysis at the second NBF reconstitutes the former open structure of the ABC transporter and allows further binding of transport substrate (Higgins and Linton 2004). Of all eight defined subfamilies of ABC transporters that can be found in plants, only the families of ABCB [also known as p-glycoprotein transporters (PGPs)] and ABCG, which contain the pleiotropic drug resistance (PDR) transporters, are represented in the PM. Of the 29 ABCB transporters in plants, 21 encode full-length ABC transporters. Four of them (ABCB1, ABCB4, ABCB14, ABCB19) have been characterized by mutant analysis and functional expression in heterologous systems.

6.3.1 Phytohormone Transport and ABC Transporters

Auxin is by its molecular nature a weak acid. It is partly protonated in the acidic apoplastic space and therefore able to permeate the PM. In the cytoplasm, with its more neutral pH, it will dissociate and become a membrane-impermeable anion, which is no longer able to leave the cell via permeation. Active transport systems must be in place to facilitate the export of auxin from the cells (Kramer 2006). Transport through entire files of cells to create auxin gradients in tissues requires a coordinated interplay of export and import transport systems. In the case of auxin, a plethora of plant developmental processes and environmental responses of plants are regulated through differential distribution and changes in its cellular concentration. To generate these auxin gradients, several families of PM transporters function as auxin transporters (Zazímalová et al. 2010). Export of auxin anions is facilitated through several ABC transporters of ABCB and ABCG families. The plant-specific PIN-FORMED (PIN) transporter family has eight members in *Arabidopsis* and has been implicated in auxin transport for nearly two decades (Okada et al. 1991). The active uptake of auxin into cells involves transporters of the AUX/LAX family of proton-symport amino acid permeases (Parry et al. 2001) alongside ABCB transporters. The most prominent function of PM-ABC transporters is auxin transport. ABCB1 and ABCB19 function as auxin exporters, whereas ABCB4 plays a role in auxin import (Titapiwatanakun and Murphy 2008). It seems that auxin transport directionality for ABCB4 is ambiguous because import and export functions have been described for it depending on a threshold concentration of the substrate (Terasaka et al. 2005; Santelia et al. 2005; Lewis et al. 2007; Yang and Murphy 2009). ABCB14 acts as a malate importer, which attributes it a role in stomata regulation during CO_2 uptake (Lee et al. 2008). The unusual functional dichotomy as importers (auxin import via ABCB4, alkaloids via cpABCB1, malate import via ABCB14) and apparent exporters of substrates (auxin export through ABCB1 and ABCB19) makes plant ABC transporters of the ABCB subfamily unique in this respect. ABC transporters in eukaryotic systems have been characterized as substrate exporters until now. Prokaryotic ABC transporters that have been described as importers for nutrients and ions rely on interaction with specific substrate-binding proteins, which facilitate import through these ABC transporters (Oldham et al. 2007). In the plant ABCB transporter family, we have the situation that structurally related transporters such as the guard cell-specific malate importer ABCB14, on the one hand, and the auxin exporters ABCB1 and ABCB19, on the other hand, show different transport directionality and relatively strict substrate specificity. ABCB14 seems to transport only malate and fumarate, whereas related succinate and oxalate show less transport affinity. ABCB1 and ABCB19 as auxin transporters show substrate specificity for IAA, 1-NAA (ABCB1), and auxin conjugates, but not the artificial auxin 2,4-D (Titapiwatanakun and Murphy 2008). This makes this ABC transporter family a group of relative specific transporters. From the subfamily of ABCG transporters, ABCG36 has been identified in *Arabidopsis* to be involved in the regulation of efflux of the auxin precursor indole-3-butyric acid (IBA) (Strader and Bartel 2009). A related member of this subfamily ABCG has

been shown to act specifically on the efflux of the synthetic auxin 2,4-D. Plants that are defective in ABCG are hypersensitive to the toxic herbicide 2,4-D, whereas a dominant gain of function mutation confers increased resistance to 2,4-D. The transport and homeostasis of internal IAA and related auxins are not affected by these mutations (Ito and Gray 2006). The ABCG subfamily also contains the transporters ABCG11 and ABCG12, which are required for the transport of wax precursors and extracellular cutin accumulation (Pighin et al. 2004; Panikashvili et al. 2007; Ukitsu et al. 2007; Bird et al. 2007; Luo et al. 2007). Abscisic acid (ABA), which has been shown to act in long-distance signaling as phytohormone response to universal stresses, is loaded into the root xylem under mild drought stress and transported throughout the plant (Jiang and Hartung 2008). Two recent reports for ABCG25 and ABCG40 identified these transporters as PM-localized ABA exporter and ABA uptake transporter, respectively. This implicates these ABC transporters in cell-to-cell signaling of ABA (Kuromori et al. 2010; Kang et al. 2010). One can get the impression that ABCGs have a broader transport spectrum than ABCB transporters. However, further research and discovery in the entire ABC transporter family will likely reveal a more complex picture in this respect.

7 The PIN-FORMED (PIN) Transporter Family

The family of PIN transporters represents plant-specific membrane proteins – most of them localized to the PM – that use auxin as their transport substrate. This attributes an important function to this family of transport proteins as the activity of PIN proteins generates auxin gradients and flux maxima. They are essential for regulating processes such as morphogenesis, flowering, embryo development, organ patterning, and primordia development as well as a diverse suite of environmental responses from gravitropic and phototropic movements to pathogen response (Vieten et al. 2007; Křeček et al. 2009; Petrasek and Friml 2009). Interestingly, PIN transporters were only found in land plants and seem to represent a group of transporters with reduced sensitivity to plant-specific flavonoids, which inhibit the activity of the ancient group of ABC transporter-based auxin transporters (Zazímalová et al. 2010). The founding member of the PIN family is PIN1, which was identified as null mutant in *Arabidopsis*. The dramatic phenotype of this loss-of-function mutation shows frequent embryo and seedling defects, failure to develop intact flowers, and a bare inflorescence, which resembles a pin-like stem caused by extreme apical dominance (Okada et al. 1991; Gälweiler et al. 1998). Function of the PIN transporters as controlling elements of auxin gradients and auxin flux in plants is tightly linked with their subcellular localization. The so-called *long PINs* are a subgroup of PIN transporters comprised of two hydrophobic domains with five transmembrane-spanning regions each and a hydrophilic cytoplasmic loop that connects both domains. Long PINs are localized in the PM and have been shown to act as auxin exporters. In most cases, these PINs show

polarized localization at specific regions of the cell. This localization pattern determines the direction of auxin flux and the function of auxin for the development of a specific organ (Křeček et al. 2009).

8 Amino Acid/Auxin Permease Transporters

Plant amino acid transporters have been found in at least five gene families (Rentsch et al. 2007). These transport proteins show different selectivities and affinities for their transport substrates, as well as distinct localization in the cell. Most AA transporters belong to two major protein superfamilies: the amino acid transporter family (ATF1) and the amino acid–polyamine–choline (APC) transporters (Wipf et al. 2002).

8.1 Amino Acid–Polyamine–Choline Transporters

Plant APC transporters are categorized into two subfamilies as cationic amino acid transporters (CATs) and L-type amino acid transporters (LATs). Both plant transporter families are identified according to their homology to mammalian transporters (Verrey et al. 2004). The members of the CAT subfamily have 14 putative TMs and mediate Na^+-independent uptake of cationic amino acids. Nine family members have been found in the *Arabidopsis* genome (AtCAT1-9). CAT1 and CAT5 show activity as high-affinity transporters for basic amino acids (Su et al. 2004), whereas CAT6 is a low-affinity transporter for lysine and neutral amino acids (Hammes et al. 2006). Most CATs are localized to the PM, but tonoplast localization has also been found (CAT2) (Su et al. 2004). LATs have 12 TMs and are represented with six predicted genes in *Arabidopsis*. They are related to APCs from yeast (Wipf et al. 2002). Amino acid transporters of both families mediate H^+- or Na^+-coupled sym- or antiport of amino acids. These transporters still await functional characterization *in planta*.

8.2 Amino Acid Transporter Family

The majority of all characterized plant amino acid transporters belongs to the ATF group, which comprises 46 predicted members in the *Arabidopsis* genome (Schwacke et al. 2003). This superfamily of transporters is subdivided into the plant-specific lysine/histidine transporters (LHTs), amino acid permease (AAP), ProT, AUX/LAX, and the aromatic and neutral amino acid transporters (ANTs) and GABA transporters (GATs) that were also found in animals and yeast (Wipf et al. 2002).

ATF1 transporters contain 9–11 TMs with a cytosolic N terminus and an apoplastic C terminus (Chang and Bush 1997).

8.2.1 Amino Acid Permeases

Amino acid permeases (AAPs) are represented by eight genes in *Arabidopsis*. They mediate Na^+-independent, H^+-coupled low- or medium-affinity transport of basic amino acids (AtAAP3 and AtAAP5). AAPs transport neutral, acidic, and cationic amino acids, including the major transport forms of glutamine, asparagine, and glutamate. Increasing proton concentrations strongly activate transport of amino acids. The only exception is AAP6, which shows high-affinity transport kinetics for neutral and acidic amino acids and also transports aspartate (Fischer et al. 2002). Analysis of *ataap1* mutant in *Arabidopsis* confirmed the transport data from expression studies in heterologous systems, which indicated that neutral, basic, and uncharged amino acids are cotransported with protons in a one for one fashion (Lee et al. 2007; Boorer et al. 1996; Boorer and Fischer 1997).

8.2.2 AAAP Transporters Involved in Auxin Transport

As pointed out above, auxin has only a limited ability to enter the cell via diffusion because only a fraction of the extracellular auxin is protonated and therefore able to permeate without transporter proteins. Influx carriers facilitate increased import of auxin in cells where a rapid and active uptake of auxin is required. During gravitropic responses in lateral root cap cells, AUX1 plays a major role in changing the direction of polar auxin fluxes. In 1980, Maher and Martindale described an *Arabidopsis* mutant that was isolated in a screen for mutants that showed 2,4-D-resistant root elongation (Maher and Martindale 1980). This auxin-resistant 1 (AUX1) mutant also exhibited delayed responses to inhibitory concentrations of IAA (Evans et al. 1994). A striking phenotype of this mutant was a total lack of root gravitropism after reorientation of roots. This defect could be complemented by exogenous application of the membrane-permeable auxin 1-NAA (Marchant et al. 1999; Yamamoto and Yamamoto 1998). Using T-DNA insertion mutants, Bennett et al. (1996) isolated the *AUX1* gene and identified it as a member of the amino acid/auxin permease (AAAP) family of PM-localized proton symporters (Wipf et al. 2002). This family of transporters seems likely to contain auxin transporters as auxin is derived from the amino acid tryptophan, which is also transported by this transporter family (Wipf et al. 2002). Knockout of the *AUX1* gene leads to the disruption of the basipetal auxin transport gradient (Swarup et al. 2001) and causes loss of root gravitropism. Function as auxin import carrier has been demonstrated via functional expression of the AUX1 protein in *Xenopus* oocytes and subsequent uptake studies with radiolabeled auxin (Yang et al. 2006). It could be shown that AUX1 is a high-affinity auxin transporter, which favors the anionic form of auxin as transport substrate.

AUX1, however, is only one member of the family of AUX/LAX (like AUX1) transporters which all belong to the AAAP superfamily of permeases (Parry et al. 2001). The family consists of the AUX1 protein and LAX1-3. LAX3 has also been shown via heterologous expression in oocytes to be an auxin importer. It is involved in lateral root emergence. LAX3 mutant seedling forms about 40% less lateral roots than wild type, a similar reduction in lateral root number than in AUX1 mutant seedlings. However, as aux1 mutants form less lateral root primordial, lax3 plants initiate three times more lateral root primordial than wild type, indicating that the mechanism for lateral root suppression differs from aux1 to lax3 (Marchant et al. 2002; Swarup et al. 2008).

8.2.3 Lysine/Histidine Transporters

The founding member of this group of ten ATFs in *Arabidopsis* is AtLHT1, which was initially described as a mainly lysine and histidine transporting PM transporter (Chen and Bush 1997). Subsequent analysis of AtLHT1 and AtLHT2 in heterologous expression studies allowed addressing the LHTs as high-affinity transporters for neutral and acidic amino acids. LHTs are involved in uptake of amino acids from the rhizospere and import into tapetum and mesophyll cells (Lee and Tegeder 2004; Hirner et al. 2006; Svennerstam et al. 2007).

8.2.4 Proline Transporters

Proline transporters (ProTs) have been described and characterized from a number of plant species including *Arabidopsis*, tomato, mangrove, rice, orach, and barley (see chapter "Organic Carbon and Nitrogen Transporters" by Tegeder et al.; Grallath et al. 2005). ProTs are a small ATF subfamily in *Arabidopsis* with just three members. GFP-fusion protein studies revealed that all ProTs are localized to the PM. ProTs have been shown to act as low-affinity transporters for proline, glycine betaine, and quarternary ammonium compounds such as γ-aminobutyric acid (GABA) (Rentsch et al. 1996; Breitkreuz et al. 1999; Schwacke et al. 1999; Grallath et al. 2005).

8.2.5 GABA Transporters

GABA transporters (GATs), which transport GABA and GABA-related substrates, make up the smallest subfamily of ATFs in *Arabidopsis* with only two members. A high-affinity GAT (AtGAT1) has been found in *Arabidopsis* and functionally characterized via expression in GABA uptake-deficient yeast strains and *Xenopus* oocytes (Meyer et al. 2006). According to the role of GABA as stress-related compound, AtGAT1 is induced by wounding and during senescence.

8.2.6 Aromatic and Neutral Amino Acid Transporters

From this subfamily of 19 predicted transporters in *Arabidopsis*, only AtANT1 is functionally characterized as transporter for aromatic and neutral amino acids with moderate affinity to its substrates (Chen et al. 2001). In a study to analyze the interaction of aphids and their feeding behavior on *Arabidopsis* mutants, a T-DNA insertion mutant in *AtANT1* has been used, which shows a change in amino acid composition and an increase in essential amino acids in phloem sieve elements (Hunt et al. 2006).

8.3 Peptide Transporters

Peptide transport in plants seems to serve roles in the uptake of protein degradation products as well as nutritional functions to supply developing seeds and embryos with necessary peptides and during seed germination and subsequent seedling development. Proven plant peptide transporters can be found in the peptide transport/nitrate transporter 1 (PTR/NRT1) family and in the oligopeptide transporter (OPT) family (Tsay et al. 2007). PTR/NRT1 and OPTs can be distinguished by the length of their transport substrates. As PTR/NRT1 transports di- and tripeptides, tetra- and pentapeptides are transported by the OPT family (see chapter "Organic Carbon and Nitrogen Transporters" by Tegeder et al.).

8.3.1 PTR/NRT1 Peptide Transporters

Uptake of small peptides is an important aspect of nutrition in prokaryotes, fungi, and animals. This function of uptake transporters is less defined in plants. The PTR/TRR1 protein family in plants is surprisingly large, whereas one and six proteins – all of them are peptide transporters – are found in yeast and humans respectively. The *Arabidopsis* genome comprises 53 PTR/NRT1 genes and rice has 80 (Tsay et al. 2007). The plant protein family of PTR/NRT1 contains peptide transporters, as well as nitrate transporters such as CHL1 (AtNRT1.1), AtNRT2.1, and AtNRT2.2. Activity as peptide transporters for PTR/NRT1s has been shown by complementation of peptide transporter mutants in yeast as well as through expression and transport studies in *Xenopus* oocytes. Substrate specificity in PTR/NRT1 is not very high. A number of di- and tripeptides are transported with different affinities (Dietrich et al. 2004). As PTR/NRT1s are localized to PM (PTR1, PTR5) (Dietrich et al. 2004; Komarova et al. 2008) as well as tonoplast (PTR2) (Shimaoka et al. 2004), a role for peptide uptake as well as intracellular peptide transport can be assumed. Cotransport of protons and peptides has been found for PTR2 in electrophysiological studies using *Xenopus* oocytes (Chiang et al. 2004). Expression studies and mutant analysis of PTR/NRT1s suggest a nutritional role in seedling development and germination processes.

8.3.2 Oligopeptide Transporter

The OPT protein family is present in prokaryotes, fungi, and plants but cannot be found in animals and humans. In plants, their role as uptake transporters for proton-coupled transport of phytosiderophores and nicotineamine-chelated metals [Fe(III)-complexes] has been shown in the subfamily of yellow stripe proteins. Its founding member is characterized by the yellow stripe phenotype in maize (Curie et al. 2001; Yen et al. 2001; Schaaf et al. 2004). Most OTPs have 14 TMs and can be found in small protein families in *Arabidopsis* (nine genes) and maize (eight genes) (Schwacke et al. 2003). Oligopeptide transport has been demonstrated for five *Arabidopsis* OPTs in yeast complementation assays (Koh et al. 2002); however, AtOPT3 seems to function as iron transporter in developing seeds and is not involved in oligopeptide transport (Stacey et al. 2008). Substrate specificity in yeast assay has been found for KLLLG peptides, whereas AtOPT4 transported the tetrapeptide KLGL with high affinity in *Xenopus* oocytes (Osawa et al. 2006). Glutathione and its conjugates are transported by AtOPT6, expanding the transport spectrum of OPTs in plants (Cagnac et al. 2004).

8.4 Ammonium Transporters

Apart from nitrate itself, ammonium (as NH_4^+ and NH_3) is a very important nitrogen source that is taken up by plants from the soil. The most prominent family of ammonium transporters is the AMT protein family, which can be found in all classes of organisms. Related to plant, fungal, and bacterial AMTs are the rhesus-associated glycoproteins (RhAG), which are found in erythrocytes, kidney, and liver. They represent a separate branch in the AMT phylogeny and have been shown to facilitate growth on low ammonium level. AMTs facilitate electrogenic transport coupled to the electrochemical gradient of NH_4^+, whereas RhAGs transport along the concentration gradient of NH_3 without generating electrical currents (Mayer et al. 2006a). With the cloning and analysis of the first plant AMT, the structure of these transporters could be determined as 11–12 TMs with C- and N-terminal extensions (Ninnemann et al. 1994). Small gene families encode AMTs in *Arabidopsis* (six genes) and rice (ten genes). The AMT genes are expressed in most tissues indicating that the uptake of ammonium from the soil is not the sole function of these transporters (von Wiren and Merrick 2004). Mutant studies in *Arabidopsis* suggest a high level of redundancy of ATMs as the phenotypic effects of mutations are not severe or inconclusive. Expression and localization studies of AMT2 in *Lotus* showed a role of LjAMT2 in the PM of symbiotic nodule tissues. Transport of ammonium from nodules has therefore been suggested as function for LjAMT2 (Simon-Rosin et al. 2003). Mutational studies in AMTs from *Aspergillus nidulans* identified glycine residues in a conserved GAVAERxK/R motif, which is essential for transport function. A second glycine residue in the C terminus of AMTs from *E. coli*, yeast, and *A. nidulans* has been found to also render LeAMT1;1

from tomato inactive after expression in oocytes or yeast (Ludewig et al. 2003). Coexpression of mutant and wild-type version of AMTs indicates oligomerization of these transporters. This assumption is also corroborated by studies with *E. coli* AMTB protein, which was isolated as a trimer (Blakey et al. 2002). Structural modeling of LeAMT1;1 onto EcAMTB showed that most relevant residues for NH_3 channeling in EcAMTB are identical in LeAMT1;1, which might suggest that LeAMT1;1 should also be able to transport NH_3, which seems to be not the case (Mayer et al. 2006a, b). However, it cannot be excluded that NH_4^+ is deprotonated within the pore and re-protonated after being transported throughout the pore channel as NH_3. Proton cotransport occurs during the NH_4^+ transport. The external NH_4^+ recruitment site is highly conserved in all AMTs. Surprisingly, mutation of the site in LeAMT1;1 (Y133I) increased the affinity for NH_4^+, which indicates additional regions that are necessary for NH_4^+ recruitment (Ludewig et al. 2007).

9 Sugar Transporters

Sugars are the main product of photosynthesis in plants and are used as cellular energy compounds, nutrients, and building blocks for complex carbohydrates and cell signaling. Sugar transporters can be best separated into monosaccharide and disaccharide transporters (sucrose transporters).

9.1 Monosaccharide Transporters

The monosaccharide transporter (MST)-like family contains 53 genes in *Arabidopsis* and 65 genes in rice, which can be grouped into seven subfamilies by their phylogenetic relation (Büttner 2007). All MST-like proteins are membrane proteins with 12 TMs, which show overall sequence conservation – especially between proteins of the same subfamily. All MST-like proteins also exhibit highly conserved domain motifs including sugar transport signatures (Büttner 2007). Several subfamilies are localized to specific cellular endo-compartments as AtVGTs (vacuolar glucose transporter-like proteins) and AtTMTs (tonoplast monosaccharide transporters) are localized to the vacuolar membrane. AtGlcT/AtSGB1 (plastidic glucose transporter/suppressor of G protein beta 1) is known as glucose exporter from the chloroplast (Weber and Fischer 2007) and was localized in the Golgi system (Wang et al. 2006). Despite their sequence homology with other MSTs, the AtERD6-like (early response to dehydration) proteins could not yet be characterized as sugar transporters. Their cellular localization is also mostly in the tonoplast (Endler et al. 2006). Only the three subfamilies of AtSTPs, AtPLYs, and AtINTs are localized to the PM.

9.1.1 Sugar Transport Protein

The best-characterized subfamily of MSTs are the sugar transport proteins (STPs), which are encoded by a gene family with 14 members in *Arabidopsis* and 28 members in rice (Schwacke et al. 2003). Eight STPs from *Arabidopsis* were functionally characterized. All function as PM-localized proton symporters with high affinity (K_m: 10–100 μM glucose) or medium affinity (K_m: 2 mM glucose) for glucose. Most STPs accept a number of D-hexoses and D-pentoses as transport substrates. Only AtSTP6 has a strong preference for hexoses (Scholz-Starke et al. 2003). Most STPs are strongly expressed in sink tissues, whereas the expression patterns of single genes overlap in many cases.

9.1.2 Inositol Transporter

Inositol is an important signaling substance in its phosphorylated form IP_3. It can also occur in biomembranes as phosphatidylinositol and phytate as seed storage molecule (Büttner 2007). Inositol transporters (INTs) are high-affinity proton symporters at the PM and the tonoplast, which accept inositol and its derivatives as transport substrates. INTs from *Mesembryanthemum* function as symporters for sodium and myo-inositol in the tonoplast (Chauhan et al. 2000).

9.1.3 Polyol Transporter

The osmotically active group of polyols functions in plants as protectant against salt, water, or colt stress (Noiraud et al. 2001a). This gene family comprises six members in *Arabidopsis*. In rice, gene duplication events increased the number of family members for this family to 15 (Schwacke et al. 2003). The first polyol transporter (PLT) in plants was cloned by yeast complementation as mannitol transporter AgMaT1 from celery (Noiraud et al. 2001b). Subsequent approaches resulted in cloning of several PLTs from a broad variety of plant species (see chapter "Organic Carbon and Nitrogen Transporters" by Tegeder et al.). The PM-localized AtPLT5 from *Arabidopsis* was the first *Arabidopsis* PLT that was functionally characterized. It acts as a proton symporter for quite a number of transport substrates including sorbitol, xylitol, erythritol, glycerol, myo-inositol, sugar alcohol, hexoses, and pentoses (Reinders et al. 2005; Klepek et al. 2005).

9.2 Sucrose Transporter

The major transport form for long-distance transport of soluble carbohydrates in plants is the nonreducing disaccharide sucrose, which serves as energy supply, building block for metabolic processes, and carbon storage. All sucrose transporter

(SUT) proteins are PM-localized, energy-dependent proton symport carriers, which play important roles in symplastic sucrose transport from cell to cell and in phloem loading and unloading during long-distance transport. SUTs are encoded by small gene families of five genes in rice and nine genes in *Arabidopsis* (Sauer 2007). Phylogenetic analysis of the SUT protein families led to a nomenclature proposed by Lalonde et al. (2004) that divides the plant SUT family into three different clades. Clade I contains high-affinity SUTs with an apparent K_m of 0.07–2 mM sucrose. Clade II and III contain low-affinity SUTs whose apparent K_m is between 4 and 20 mM sucrose. All SUTs have 12 PMs with an elongated N terminus of ca. 20 amino acids for dicot SUTs in clade III. These SUTs are distinguishable through an enlarged cytoplasmic loop area, which cannot be found in monocot species for members of this clade (Lalonde et al. 2004). All other analyzed SUTs function as proton symporters, with the exception of PvSUF1, PsSUF1, and PsSUF4 from legumes, common bean and pea, which act as proton-independent sucrose facilitators (Zhou et al. 2007; Sauer 2007). Clade I – which is made up exclusively by dicot SUTs – contains AtSUC1, 2, 5, 6, 7, 8, and 9 from *Arabidopsis*. The transport substrates of these transporters are not restricted to sucrose alone but also include α- and β-glucosides (SUC2; SUC9) and biotin (SUC5) (Chandran et al. 2003; Sivitz et al. 2008; Ludwig et al. 2000). SUTs are localized in PM of sieve elements in Solanacaea and in companion cells of *Plantago major* and *Arabidopsis* (Sauer 2007). Localization in both cell types has been reported for *Alonsoa meridionalis* (Knop et al. 2004). Mutant analysis indicates a role for phloem-localized SUTs in phloem loading in source leaves and unloading in sink tissues (Sauer 2007). In clade II, we find AtSUT4 from *Arabidopsis*, which is localized to the tonoplast like SUT4 homologs from barley (Endler et al. 2006) and *Lotus japonicus* (Reinders et al. 2008). However, SUT4 homologs from Solanaceae are localized to the PM of sieve elements (Weise et al. 2000). The AtSUT member in clade III is AtSUC3, which is a specific sucrose transporter. The sucrose affinity in AtSUC3 is determined by the N terminus of the protein, whereas the long central loop has no influence on sucrose transport kinetics (Schulze et al. 2000). The AtSUC3 homolog from rice (OsSUT1) can be found in PM of sieve elements and companion cells (Scofield et al. 2007), whereas SUC3 from tomato, *Arabidopsis*, *Plantago*, and wheat localizes to the PM of sieve elements only (see chapter "Organic Carbon and Nitrogen Transporters" by Tegeder et al.; Sauer 2007). The maize SUC3 homolog ZmSUT1 shows reverse transport kinetics of sucrose if the free energy of the sucrose concentration gradient is greater than proton motive force (Carpaneto et al. 2005). This ability would allow apoplasmic phloem loading and unloading in vegetative tissue and seed sinks (Zhang et al. 2007).

10 Purine Permease

Purine permeases (PUPs) are plant-specific membrane transporters that belong to a gene family with 21 predicted genes in *Arabidopsis* and 12 predicted genes in rice (Schwacke et al. 2003). PUPs are high-affinity transporters for adenine, cytosine,

and purine derivatives such as alkaloids, which mediate energy-dependent transport of purines against a concentration gradient in a proton-dependent fashion. PUP proteins have ten TMs and an N-terminal sequence extension. The first PUP was AtPUP1 from *Arabidopsis*, which was cloned via molecular complementation in adenine transport-deficient *fcy2*/*ura3* yeast mutants (Gillissen et al. 2000). Expression pattern of AtPUP1, 2, and 3 indicates different roles for these transporters in plants. Long-distance transport of adenine, cytokinins, and alkaloids is a likely role for members of the PUP protein family. AtPUP1 is expressed in hydathodes and stigmatic tissue of the silique, indicating retrieval of nucleobases and purine derivatives. AtPUP2 may play a role in phloem transport and the pollen-specific AtPUP3 may be involved in supply of pollen with nucleobases (Bürkle et al. 2003). It would be justified to say that what is known about transport and transporters of all other phytohormones combined cannot rival what is known to date about auxin transport. In most cases, a need for transporters that allow the release of the hormones from the cell or uptake into the cell is formulated based on signaling or long-distance transport data. Yet many of the players in plant hormone business are still elusive. Candidates for cytokinin transport have been suggested from the plant-specific PUP family (Gillissen et al. 2000; Cedzich et al. 2008). Heterologous expression of AtPUP1 and AtPUP2 in yeast resulted in the import of cytokinin into yeast cells (Bürkle et al. 2003; Gillissen et al. 2000). Uptake studies using radiolabeled *trans*-zeatin in *Arabidopsis* microcallus cultures showed that AtPUP1 is a good candidate for a low-affinity cytokinin transporter in plants (Cedzich et al. 2008). In rice, and subsequently in *Arabidopsis*, members of the equilibrative nucleoside transporter (ENT) family have been implicated in cytokinin transport (Hirose et al. 2008).

11 Aquaporins

For a long time, biological textbook knowledge stated that water is able to diffuse freely through biomembranes. Biophysical studies have shown something different: water fluxes into and out of cells cannot be explained by simple diffusion. The surprising discovery of water-transporting membrane proteins [aquaporins (AQP)] changed the view at water transport trough membranes and the regulation of water relations in plants (Agre 2004). The sequenced plant genomes of *Arabidopsis* and rice contain 35 and 33 predicted AQP genes, respectively (Quigley et al. 2002; Sakurai et al. 2005). Four subclasses of AQPs can be distinguished in phylogenetic analysis. Small basic intrinsic proteins (SIPs) are the smallest subclass of AQPs with three genes in the genome and are mainly localized in the ER (Ishikawa et al. 2005). Nodulin26-like intrinsic proteins (NIPs) have the founding protein of this subclass in the AQP GmNod26 from soybean. This AQP is localized to the peribacteroid membrane in nitrogen-fixing nodules in soybean roots (Wallace et al. 2006). NIPs are PM- and endomembrane-localized AQP that can be found in nonlegumes. Tonoplast intrinsic proteins (TIPs) are localized to the vacuole. Plasma membrane intrinsic proteins (PIPs) are mainly PM- and endomembrane-localized AQP, which

can be divided into two subclasses, PIP1 (five *Arabidopsis* genes) and PIP2 (eight *Arabidopsis* genes) (Tyerman et al. 2002). In *Physcomitrella patens*, a single PIP3 gene – lacking a Ca^{2+}-binding site and a conserved helix2 cysteine – has been found as a novel subgroup of PIPs (Danielson and Johanson 2008). PIP1s transport water only with low efficiency, whereas they have permeability for glycerol and urea (Maurel et al. 2008). PIP2 proteins are considered to act exclusively as water permeases. To increase efficiency of water transport of PIP1 proteins, heterotetramers need to be formed between both types of PIPs (Fetter et al. 2004). PIP1s have like all other AQPs six TMs and are characterized by a long N-terminal extension, which is PIP1 specific. The C terminus ends close at the intracellular end of the last TM (Kaldenhoff and Fischer 2006). PIP2 proteins have a short N terminus and an elongated C terminus. They are mostly but not exclusively involved in water transport (Chaumont et al. 2000; Johanson et al. 2001) as they can transport glycerol (PIP1; Biela et al. 1999; Moshelion et al. 2002), urea (Gaspar et al. 2003), and CO_2 (Uehlein et al. 2003). Permeation of NH_3/NH_4^+ indicates a role of PIPs in nitrogen metabolism (Holm et al. 2005). Transport of H_2O_2 – structurally closely related to water – by AQPs shows that not only water relations in plants but also integration of stress signals and stress responses are functions that AQPs are involved (Bienert et al. 2007). NIP5;1 has been shown to act as boron uptake facilitator under limiting boron conditions (Takano et al. 2006). Posttranslational modification of PIPs has structural and regulatory functions. Phosphorylation at multiple serine residues in the C- and N terminus or in the cytoplasmic loop between TM2 and TM3 (ser119) has been found in different plant species (see chapter "Physiological Roles for the PIP Family of Plant Aquaporins" by Vera-Estrella and Bohnert; van Wilder et al. 2008). Mutant analysis showed that phosphorylation of PIP AQPs through Mg^{2+}- and Ca^{2+}-dependent protein kinases plays an important role in pore opening and closing and water permeability (Sjovall-Larsen et al. 2006). Modifications at the N terminus of PIP2s have been found in the form of mono- or dimethylation (Santoni et al. 2006). Aquaporins form tetramers with each monomer forming an individual pore for transport. Heteromerization can affect the activity and localization of AQP in the target membrane systems. ZmPIP1;2, an inactive AQP, is localized to the ER when expressed transiently in maize mesophyll protoplasts. The active ZmPIP2;1 is localized to the PM. Coexpression of both proteins in mesophyll protoplasts resulted in molecular interaction of ZmPIP1;2 and ZmPIP2;1 and localization of the complex to the PM as active water-transporting channel. Immunoprecipitation in roots and suspension cultures showed this complex formation between both AQPs *in planta* (Zelazny et al. 2007). Crystal structure analysis using X-ray crystallography of SoPIP2;1 from spinach shows that N- and C terminus as well as the second and fourth interhelix loop are facing the cytosol, whereas the other three loops are oriented toward the apoplasm. The two cytoplasmic loops are involved in forming the pore as they form a transmembrane-like structure in the folded protein (Törnroth-Horsefield et al. 2006). Selectivity and transport are mediated by AQP through size exclusion of substrates and interaction with particular intramolecular domains such as the highly conserved NPA region, which mediates reorientation of water molecules during transfer

through the pore (Tajkhorshid et al. 2002). Protons are kept out of AQPs by the NPA region's strong electrostatic field and the selectivity filter Ar/R formed by four aromatic amino acids and arginine. Arginine acts here as proton repulser (Fujiyoshi et al. 2002).

12 Nitrate Transporter

Nitrate is the most important nitrogen nutrient source for plants and plays an important role as fertilizer in agriculture. Plants have two different types of nitrate transporters, NRT1 and NRT2, which are not similar in sequence. They share, however, the same membrane domain structure with 12 predicted TMs. Most nitrate transporters (NRTs) localize to the PM, but tonoplast localization has also been found (AtNRT2.7; Chopin et al. 2007). The gene family of NRT1 has 53 predicted members in *Arabidopsis* and 80 members in rice (Tsay et al. 2007). The much smaller gene family of NRT2s contains seven members in *Arabidopsis* and four predicted members in rice (Schwacke et al. 2003). NRT2s are high-affinity nitrate transporters (K_m: 7–50 μM), whereas NRT1s can be mostly classified as low-affinity nitrate transporters (K_m in millimolar range). The exception is the first isolated nitrate transporter CHL1 (chlorate resistant 1), which has a dual affinity for nitrate (Tsay et al. 2007). In the phosphorylated stage (phosphorylation of Thr101), CHL1 is a high-affinity transporter with an apparent K_m of 50 μM nitrate. The dephosphorylated version of CHL1 acts as low-affinity nitrate transporter with apparent K_m of 5 mM nitrate (Liu et al. 1999; Liu and Tsay 2003). CHL1 is a plant-specific nitrate transporter as all homologs from other organisms and even some plant homologs of CHL1 act as di- and tripeptide transporters (Tsay et al. 2007; see chapter "Organic Carbon and Nitrogen Transporters" by Tegeder et al.). However, nitrate transporters are unable to transport peptides and none of the peptide transporters transports nitrate. The activity of CHL1 as a nitrate transporter has been shown in transport assays with *Xenopus* oocytes (Tsay et al. 2007). NRT2 transporter has been cloned from plants and tested for their ability of nitrate transport. High-affinity nitrate transport can only be observed in the presence of NAR2 (Tong et al. 2005; Orsel et al. 2006). Only the tonoplast-localized NRT2.7 transporter shows transport activity in oocytes without NAR2 (Chopin et al. 2007). At least four transporters are involved in nitrate uptake in *Arabidopsis* roots: CHL1, NRT1.2, NRT2.1, NRT2.2 (see chapter "The Role of Plasma Membrane Nitrogen Transporters in Nitrogen Acquisition and Utilization" by Tsay and Hsu; Huang et al. 1996, 1999; Little et al. 2005). Nitrate uptake depends on a coordinated system of inducible and constitutive high- and low-affinity uptake systems, respectively. Therefore, expression of the genes involved in response to nitrogen status in the soil and plant as well as developmental stage of plants regulate the efficiency of the entire uptake system. CHL1 has been shown to also function as nitrate sensor regulating transcription of nitrate-induced genes (Ho et al. 2009). Transporters of the NRT1 family can also be involved in nitrate efflux under stress conditions

(Segonzac et al. 2007; Lin et al. 2008). The net uptake of nitrate in plants results from influx and efflux efficiency. AtNRT1.5 is expressed in root pericycle cells adjacent to the protoxylem. Mutant analysis in *Arabidopsis* has shown that atnrt1.5 plants have reduced root to shoot transport of nitrate. Functional studies in oocytes showed that AtNRT1.5 is able to transport nitrate in both directions implicating this transporter in xylem loading as well as xylem unloading processes (Lin et al. 2008).

13 Sulfate Transporter

Sulfur is an important element in cell physiology as many proteins are built with sulfur-containing amino acids. Enzymes, vitamins, chlorophyll formation, and symbiotic nitrogen fixation require sulfur. Sulfur can be found in plant compounds such as glutathions, sulfolipids, phytochelatins, glucosinolates, and others. Sulfate is the form of sulfur from soil, which is taken up by plant roots. This uptake process takes place across the PM as proton-coupled transport against a sulfate concentration gradient. After uptake, sulfate is reduced to sulfite and sulfide before it is channeled into the primary metabolism via cysteine formation from *O*-acetylserine and sulfide. Sulfate can also be temporarily stored in the vacuole after uptake. Uptake through roots involves high-affinity sulfate uptake systems with an apparent K_m of 19 μM (see chapter "Sulfate Transport" by Hawkesford). The specificity for sulfate is high as the transport is not inhibited by any other macronutrient but can be inhibited by the sulfate analog selenate. This pharmacological characteristic was used to clone sulfate transporters (SulPs) from *Stylosanthes hamata* and barley (Smith et al. 1995, 1997). High- and low-affinity plant SulPs have been characterized after heterologous expression in yeast suggesting functions for uptake of sulfate from soil and for transport of sulfate throughout the plant. Low pH optima indicate proton gradient-coupled transport mechanisms (Smith et al. 1995, 1997; Takahashi et al. 2000; Yoshimoto et al. 2002).

13.1 SulP Gene Family

Cloned and characterized sulfate transporters belong to the SulP gene family, which is present in all classes of organisms from bacteria to animals and humans. SulPs are membrane proteins with 12 predicted TMs, which have cytoplasmic-localized long N- and C terminus (Schwacke et al. 2003). A number of conserved amino acid positions and a conserved SulP and a C-terminal anti-sigmafactor antagonist motif (STAS) have been identified in proteins of this transporter family (Shelden et al. 2001, 2003). This gene family consists of 14 genes each in rice and *Arabidopsis*. Most of the SulPs seem to be PM-localized SulPs. Phylogenetic sequence analysis allows clustering all genes in five different clades. PM SulPs are found in clades 1–3 (see chapter "Sulfate Transport" by Hawkesford). The clade 1 and clade 2 SulPs are

high-affinity and medium-affinity SulPs, respectively. Clade 1 transporters are prominently expressed in root tips and roots and can be considered primary sulfate uptake transporters. These genes, however, are also expressed in phloem tissue, which indicates a role in phloem loading (Yoshimoto et al. 2003). Clade 2 SulPs are expressed only in the PM of vascular tissue, which again implicates these transporters in cell-to-cell transport within the plant (Smith et al. 1995; Takahashi et al. 2000). Clade 3 SulPs are also expressed in internal plant tissues. They are transporters with low affinity for sulfate. Examples that indicate heterodimer formation, which enhances transport substrate affinity, can be found in this clade (Kataoka et al. 2004).

14 Metal Transporter

Considering plant mineral nutrition, metal cations belong to the group of micronutrients. However, their role as essential factors and cofactors in countless metabolic processes as well as elements in structural roles make them a group of very important ions in plant growth and development. The most essential processes of plant viability such as photosynthesis and respiration would be impossible without metal cations. Typical metal cations such as Fe, Mn, Zn, and Cu can be found in concentrations from a few to 100 ppm in the dry matter of plants (Epstein 1965). However, metal cations need like other charged and hydrated ions to be imported via active and energized transport processes (see chapter "Metal Transport" by Atkinson and Guerinot). As metals are very reactive due to their high redox potential, oxidative damage and radical formation can be induced by high metal concentrations in cells. To avoid these toxic effects, plants need to regulate the homeostasis of cellular metals either by chelation and binding of metals or by efflux of unwanted concentrations of metals from the cells.

14.1 Iron Transporter

Iron is not readily available in soil as its cation is not soluble at neutral pH (Guerinot and Yi 1994). Therefore, different strategies for iron uptake were developed in plants to supply plant metabolism with iron. Reduction-based uptake of iron can be found in all plants besides grasses, which use a chelation-based uptake strategy using phytosiderophores (Kim and Guerinot 2007). The model for reduction-based iron uptake can be found in *Arabidopsis*. Reduction of soil pH in the rhizosphere by H^+-ATPase2 (AHA2) results in acidification and solubilization of Fe^{3+}, which is subsequently reduced to Fe^{2+} by the ferric chelate reductase FRO2 (Kim and Guerinot 2007). Fe^{2+} is subsequently imported into root epidermis cells by PM-localized iron-regulated transporter 1 (IRT1) (Henriques et al. 2002; Varotto et al. 2002; Vert et al. 2002). IRT1 transports besides Fe^{2+} also cadmium,

cobalt, manganese, and zinc (Vert et al. 2002). Null mutants of irt1 are not viable without high concentration nutritional iron supplement (Varotto et al. 2002; Vert et al. 2002). IRT1 homologs in other plant species seem to fulfill a similar function as AtIRT1 (see chapter "Metal Transport" by Atkinson and Guerinot). Interestingly, the grass species rice is also using a reduction-based iron uptake strategy in addition to the grass-specific phytosiderophore-based uptake of iron. Both Fe^{3+} and Fe^{2+} are uptake transport substrates in rice depending on the actual supply of phytosiderophores. OsIRT1 is active in roots under iron deficiency conditions and overexpression of OsIRT1 in transgenic rice plants results in increased iron deficiency tolerance (Kim and Guerinot 2007; Lee and An 2009).

14.1.1 ZIP Transporters

The family of ZIP transporters is a large protein family, which can be found in all organisms and is made up of zinc uptake transporters (ZRT) and IRT1-like iron transport proteins (Zhao and Eide 1996a, b; Eide et al. 1996). In *Arabidopsis*, the majority of ZIP transporters belongs to the ZIP I subfamily; only AtIAR1 belongs to the LZT (LIV-1-like ZIP zinc transporter) subfamily (Taylor and Nicholson 2003). AtIAR1 – a seven-membrane TM protein – seems to be involved in auxin homeostasis in plants (Lasswell et al. 2000). Aside from IRT1, IRT2, and IRT3 (*Arabidopsis*) (Vert et al. 2009; Lin et al. 2009) and OsIRT1 and OsIRT2 (rice), which have been shown to transport mainly iron and zinc and are implicated in iron uptake and compartmentalization, the ZIP transporters are characterized as zinc and copper transporters (Grotz et al. 1998; Wintz et al. 2003), which are also suggested to transport an even broader range of metal cations.

14.1.2 Natural Resistance-Associated Macrophage Protein

This large ubiquitous protein family has been shown to transport manganese and iron, but some family members are also involved in transport of nickel, zinc, copper, cobalt, and cadmium. Four of the six natural resistance-associated macrophage protein (NRAMP) genes have been functionally characterized in *Arabidopsis* and only AtNRAMP1 was localized to the PM (Cailliatte et al. 2010). *AtNRAMP1* encodes a high-affinity uptake system to acquire Mn from the soil under Mn deficiency conditions. The *Atnramp1-1* mutant shows limited growth, reduced Mn levels, and fails to take up Mn under limiting Mn concentrations. Consistent with its function in Mn acquisition from the soil, *NRAMP1* expression is restricted to the root and stimulated by Mn deficiency. NRAMP1 also restores the iron and cobalt uptake capacity of *irt1* mutants, indicating that NRAMP1 has a broad transport substrate range.

14.1.3 Copper Transporter

Copper is an essential micronutrient in plants as it is involved in metabolic processes as cofactor in many redox-regulated enzyme reactions or electron transport systems as well as hormone perception (Burkhead 2009). Copper cation transporters (COPTs) are three TM proteins that form trimers which act as high-affinity copper uptake transport systems with several copper-binding sites on each monomer (Penarrubia et al. 2010). The expression of COPT genes is induced by copper deficiency. *Arabidopsis* has a small gene family with six COPT members (Schwacke et al. 2003), which are likely localized to the PM and other cellular membrane systems. COPT1, which is highly expressed in roots, is negatively regulated by Cu (Sancenon et al. 2003). Downregulation of COPT1 in transgenic *Arabidopsis* plants results in lower Cu levels and defects in pollen development and root elongation. Supplement of Cu reversed these Cu deficiency phenotypes back to wild type (Sancenon et al. 2004). COPT1 and COPT2 restore copper uptake in uptake-deficient ctr1ctr3 double mutants in yeast (Kampfenkel et al. 1995; Sancenon et al. 2003), whereas COPT3 and COPT5 show only weak copper transport efficiency when heterologously expressed.

14.1.4 SLC40 Transporters

Ferroportin/iron-regulated mRNA1 (FPN/IREG1) is the only iron efflux transporter identified in animals. Two closely related orthologs in *Arabidopsis* – IRON REGULATED1 (IREG1/FPN1) and IREG2/FPN2 – with 12 TMs each have been identified. FPN1 localizes to the PM and is expressed in roots (stele), root–shoot junction, and major veins of cotyledons, which suggests a role in vascular loading. Expression of *AtFPN1* is induced under iron deficiency conditions. AtFPN2 localizes to the vacuole and is not able to complement iron transport-deficient yeast mutants (Schaaf et al. 2006). Ferroportins also play a role in cobalt homeostasis; a survey of *Arabidopsis* accessions for ionomic phenotypes showed that truncation of FPN2 results in elevated shoot cobalt levels and results in increased sensitivity to cobalt. Loss-of-function mutants in *FPN1* show greatly reduced cobalt accumulation in shoots. In fpn1/fpn2 double mutants, cobalt cannot move to the shoot via FPN1 and does not accumulate in root vacuoles through FPN2 (Morrissey et al. 2009).

14.2 Chelation-Based Strategy of Iron Uptake

Grasses such as maize and rice developed an alternative high-efficient iron uptake system using the ability of chelators to bind iron with high affinity. The chelators used in this system are phytosiderophores, which are mugineic acids derived from L-methionine. Genes required for biosynthesis of phytosiderophores and its precursors are coordinated and upregulated by iron deficiency (Kim and Guerinot 2007).

Each grass species produces its own set of phytosiderophores, which are regulated by the availability of iron in the soil (Marschner 1995).

14.2.1 Yellow Stripe1-Like Transporter

After Fe^{3+} chelation by phytosiderophores, high-affinity uptake of the complexes takes place. The transposon-tagged mutant yellow stripe1, which is deficient in Fe-siderophore uptake, has been used for cloning of the YS1 gene, which encodes a proton-coupled symporter for siderophore complexes. This gene belongs to the above-mentioned family of oligopeptide transporters (OPTs) (Curie et al. 2001). Heterologous expression of YS1 in Fe-uptake yeast mutants in the presence of Fe-DMA as siderophore restored the iron-deficient phenotype in this mutant. The rice genome contains 18 different genes of the yellow stripe1-like (YSL) family. Several of them have been shown to be involved in iron uptake. Surprisingly, also the *Arabidopsis* genome encodes a small gene family of eight predicted YSL proteins (Curie et al. 2009). Intact phytosiderophores are not synthesized in *Arabidopsis*, but the metal-binding phytosiderophore precursor nicotinamine (NA) can be found in *Arabidopsis*. Mutation analysis of YSLs in *Arabidopsis* indicates a role of these proteins in mobilization of iron during leaf senescence, loading of metal ions in developing seeds, vascular loading of metal ions, and long-distance metal transport (Le Jean et al. 2005; Waters et al. 2006; Klatte et al. 2009; DiDonato et al. 2004).

15 Phosphate Transporter

Four families of Pi transporters can be found in plants: Pht1, Pht2, Pht3, and Pht4. The only subfamily, which is localized to the PM, is the Pht1 family. Pht1 phosphate transporters are phosphate/proton symporter from the major facilitator superfamily (MFS). Following the typical membrane protein structure of MFS proteins, the Pht1 proteins have 12 TMs, which are arranged in two sets of six TMs connected by a large hydrophilic loop region. They share highly conserved sites with related transporters including the conserved Pht1 motif GGDYPLSA-TIXSE (Karandashov and Bucher 2005). The first Pht1 transporters (AtPT1 and AtPT2) from plants were cloned and characterized by complementation of phosphate uptake-deficient pho84 mutant in NS219 yeast. Phosphate starvation induces expression of AtPT1 and AtPT2, which are expressed only in roots (Muchhal et al. 1996). Subsequent cloning and functional expression in yeast revealed Pht1 transporters with variable affinity for Pi ranging from 3 to 700 μM in plants (Bucher 2007). The Pht1 family in *Arabidopsis* has nine members (Schwacke et al. 2003), and the rice genome contains 13 Pht1 genes (Paszkowski et al. 2002). Most Pht1 genes are expressed in roots and some are also expressed in leaves and pollen, which indicates functions for Pht1s other than only Pi uptake from the soil.

Mutant analysis in *Arabidopsis* showed important roles for the highly expressed Pht1;1 and Pht1;4 transporters, whose double-knockout mutant displayed reduced Pi uptake under high and low Pi concentrations (Shin et al. 2004). Developmental changes in ph11;1pht1;4 were increased formation of root hairs and lateral roots, anthocyanin accumulation, and reduced Pi levels in shoots.

16 Conclusion

Transport systems on the PM enable the cell to communicate with its exterior environment. The functions that can be found in PM transporters are manifold, from nutrient uptake to cellular detoxification and cell-to-cell communication. Owing to their nature as membrane proteins, PM transporters are often less amenable to functional analysis than cytoplasmic proteins and enzymes. Structural analyses, using crystallographic methods, are still limited for membrane transporters. Functions that are revealed using biochemical and biophysical methods in vitro or in heterologous cell systems are often not analyzed *in planta* because mutants in the respective transporters are not available or the genes for the observed transport function are still unknown. In cases of large gene families, functional redundancy has been observed. This does not always allow one to clearly address the function of a particular transport protein. In other cases, only subsets of transporter families have been analyzed, which leaves many transporters without a functional context after they have been categorized upon sequence similarities with other genes. Novel methodology using intracellular biosensors will allow investigating transporter activity in their genuine cellular environment and therefore open up novel avenues for membrane transport research.

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Plasma Membrane ATPases

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Abstract The plasma membrane separates the cellular contents from the surrounding environment. Nutrients must enter through the plasma membrane in order to reach the cell interior, and toxic metabolites and several ions leave the cell by traveling across the same barrier. Biological pumps in the plasma membrane include ABC transporters, vacuolar (V-type) H^+ pumps, and P-type pumps. These pumps all utilize ATP as a fuel for energizing pumping. This review focuses on the physiological roles of plasma membrane P-type pumps, as they represent the major ATP hydrolytic activity in this membrane.

1 Introduction

The plasma membrane is an extremely important cellular barrier as it separates the cellular contents from the surrounding environment. Mineral nutrients that are essential for growth must pass the plasma membrane (PM) in order to reach the cell interior, and other ions and metabolites have to leave the cell across the same barrier. In nature, biological pumps catalyze active transport of matter from one compartment to another. PM transporters that could be described as pumps include ABC transporters, vacuolar (V-type) H^+ transporters, and P-type transporters (Fig. 1). These pumps all utilize ATP as an energy source to mobilize their respective substrates.

ABC transporters, which have been localized to the PM and all endomembranes, form a large superfamily of transporters that mediate the efflux of organic compounds including organic acids, hormones, alkaloids, toxic breakdown products, and xenobiotics (Rea 2007; Verrier et al. 2008). V-type H^+ pumps are present in the

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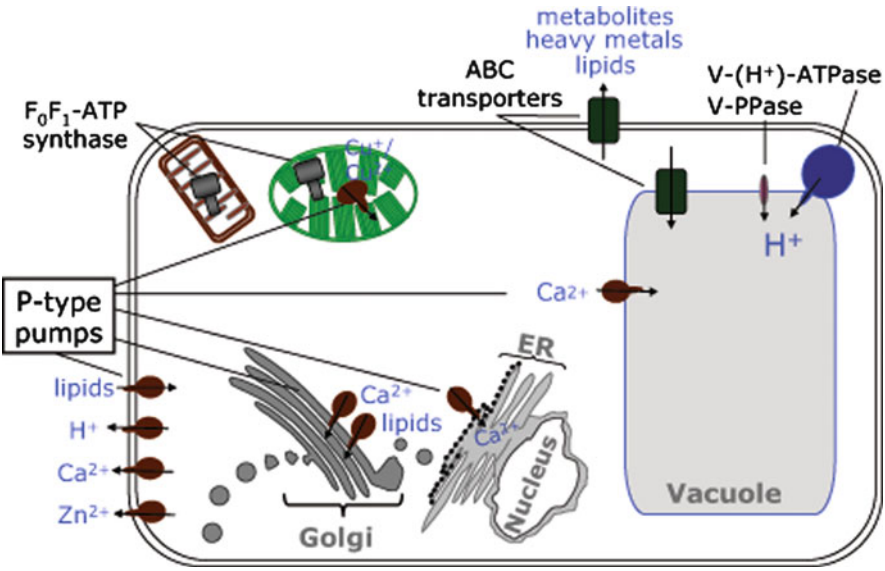

Fig. 1 Main pumps in plant cells. Mitochondria and chloroplasts contain the F_OF_1-synthase involved in ATP synthesis during respiration and photosynthesis, respectively. In the vacuole, a vacuolar H^+-ATPase and a vacuolar pyrophosphatase (V-PPase) are in charge of generating H^+-gradients required for secondary transport processes across the vacuolar membrane. P-type pumps are also located to chloroplast (heavy metals, type P_{1B}) and to the vacuole (Ca^{2+}, type P_2). Along the secretory pathway (ER and Golgi), several other P-type ATPases can be found including P_2, P_3, P_4, and P_5. At the plasma membrane level, P-type pumps dominate but ABC transporters and V-type H^+-ATPases are also present

vacuolar membrane and endomembrane compartments within the secretory pathway, where they acidify secretory vesicles, and, as such, terminate at and are recycled from the PM (Schumacher 2006). However, this review focuses on the physiological roles of plasma membrane P-type pumps, as they represent the major ATP hydrolytic activity in this membrane.

P-type pumps are cation transporters that derive their name from the fact that they are phosphorylated during the catalytic cycle (Pedersen and Carafoli 1987). This phosphorylation does not involve regulatory phosphorylation mediated by protein kinases, which typically modifies serine, threonine, and tyrosine residues of target proteins. Rather, catalytic phosphorylation in P-type pumps is autocatalytic and takes place at an aspartate residue. This phosphorylation event takes place during each catalytic cycle and results in a conformational change of the pump, which is required for transport function (Møller et al. 1996). Subsequently, the pump is dephosphorylated, again by an autocatalytic process, and returns to its original conformation. P-type ATPases can have additional subunits, but, in all P-type pumps, ATP hydrolysis and cation transport are carried out by a single catalytic polypeptide. Well characterized members of this family comprise the plasma membrane H^+-ATPase, the animal Na^+/K^+-ATPase and plasma membrane,

and the sarco/endoplasmatic reticulum Ca^{2+}-ATPases (Axelsen and Palmgren 1998).

P-type ATPases have been divided phylogenetically into five major subfamilies, P_1 to P_5, each transporting a specific set of ions (Axelsen and Palmgren 1998). In the model plant *Arabidopsis thaliana*, P-type ATPases form a family of 46 members representing all five subfamilies. Members from three of these (P_{1B}, P_{2B}, and P_{3A} ATPases) have been identified in the PM. A characteristic of all of these ATPases is that they transport cations out of the cell.

P-type ATPases are differentially regulated, but regulatory components share some common characteristics that suggest a general mechanism for pump regulation. Thus, at least P_{3A} H^+-ATPases and P_{2B} Ca^{2+}-ATPases, respectively, are autoinhibited by C-terminal and N-terminal regulatory (R) domains that require neutralization by cellular factors for pump activation.

2 Plasma Membrane H^+-ATPases (P_3-ATPases)

Plasma membrane H^+-ATPases (P_{3A} pumps) are found in plants, fungi, protistae, and archaea, but not in animals. PM proton pump genes were first cloned from the fungus *Saccharomyces cerevisiae* (Serrano et al. 1986) and plants (Harper et al. 1989; Pardo and Serrano 1989; Boutry et al. 1989). Subsequently, related genes have been identified in archaebacteria and protists (Axelsen and Palmgren 1998). The crystal structure of a plant plasma membrane H^+-ATPase has also recently been solved (Pedersen et al. 2007) (Fig. 2). In higher plants, H^+-ATPases are encoded by families of 9–12 gene members (Palmgren 2001; Arango et al. 2003).

Plasma membrane H^+-pumps have been localized in all plant tissues and organs by immunolabeling and reporter gene analysis. However, some cell types have a much higher concentration of pumps than others, particularly epidermal, root hair, phloem, stomatal guard cells, and other cell types specialized for transport of solutes across membranes. All plant cells harbor at least one, and often multiple, PM P-type H^+-ATPase isoforms. The isoform diversity of these proteins has raised the question whether the different gene products exhibit different kinetics or regulatory properties. This has been investigated by characterization of Arabidopsis and tobacco H^+-ATPase isoforms expressed individually in yeast (*S. cerevisiae*). However, the validity of this approach has recently been questioned following the finding that plant H^+-ATPases heterologously expressed in yeast are phosphorylated by their host at important regulatory sites (Jahn et al. 2002).

A key function of PM H^+-ATPases in all kingdoms except animals is the generation of a proton electrochemical gradient that provides the driving force for the uptake and efflux of ions and metabolites across the plasma membrane (reviewed in Sondergaard et al. 2004; Boutry and Duby 2008). They control the electrochemical gradient across the PM and thus provide the electrochemical gradient that motivates the activity of a broad range of secondary transporters. PM H^+-ATPases extrude H^+ from the cell to generate a transmembrane proton gradient

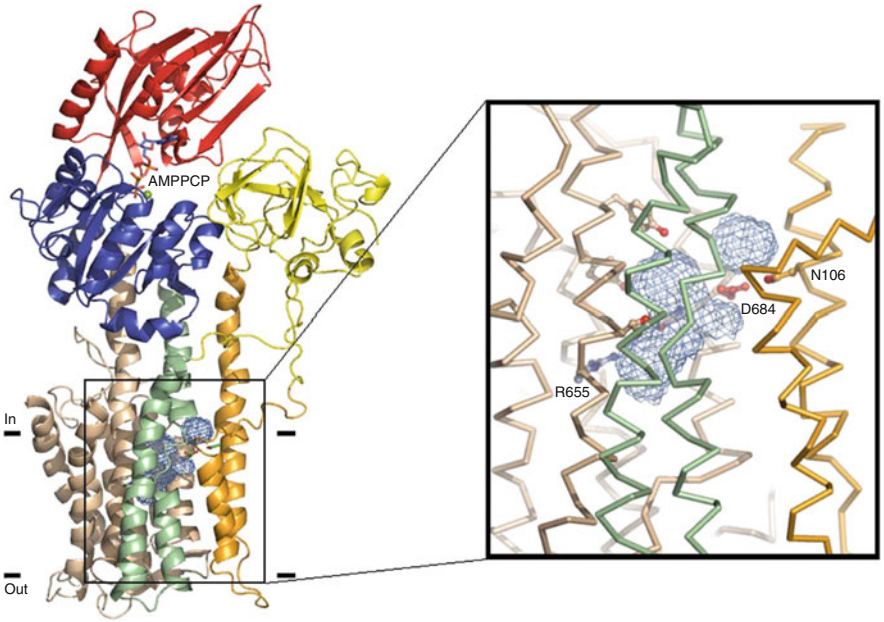

Fig. 2 Structure of a plant plasma membrane H^+-ATPase at 3.4 Å resolution (Pedersen et al. 2007). The structure represents an active form of the AHA2 proton pump, without its autoinhibitory C-terminus, in complex with Mg-AMPPCP, a nonhydrolyzable ATP homolog. Ten transmembrane helices (*orange*, *green*, and *brown*), nucleotide-binding domain (*red*), the phosphorylation domain (*blue*), and the actuator domain (*yellow*). Mg-AMPPCP is found at the interface between the N and P domains. *Black lines* depict the expected location of the plasma membrane. The *inset* is an enlarged view of the transmembrane part showing the central cavity of the pump, which is likely to be filled with water. Key residues mentioned in the text are shown as sticks. In, cytosolic side. Out, extracellular side. Reprinted from Buch-Pedersen et al. (2009) with permission

and a membrane potential. This pH gradient is typically 1.5–2.0 pH units (acid outside) whereas the membrane potential usually lies between −120 and −220 mV (negative on the inside). Thus, H^+-ATPases play the same crucial physiological role as Na^+/K^+-ATPases (P_{2C} pumps) found only in animal cells (Glynn 2002).

Different genetic strategies have been undertaken to investigate the diverse roles of PM H^+-ATPases in plant physiology, but they are often hampered by apparent genetic redundancy. These include gene knockout studies, gene silencing, overexpression of genes expressing H^+-ATPases, and analysis of constitutively active mutants. In the model plant Arabidopsis, the closely related AHA1 and AHA2 are the major H^+-ATPases and are present in many different cell types (Palmgren 2001). AHA1 is predominantly expressed in shoots and AHA2 in roots. It is likely that AHA1 and AHA2 serve as "housekeeping" proteins that provide the cells with the basic electrochemical gradient required for nutrient uptake, but this hypothesis has yet to be genetically tested.

PM H^+-ATPases in the root endodermis are thought to be required for active loading of solutes into the xylem of the vascular cylinder. In Arabidopsis, *AHA4* is

expressed in root pericycle cells surrounding the xylem. A mutation generated by a T-DNA insertion in the mid portion of the *AHA4* open reading frame results in the production of truncated transcripts and a semidominant phenotype with increased sensitivity to high salt (Vitart et al. 2001). It is possible that the truncated pump suppresses the activity of normal full-length AHA4 molecules or other pumps in the root endodermis that play a role in loading of cations into the root xylem.

Photosynthetic assimilates and other organic compounds produced by the plant are translocated into phloem tissues for delivery throughout the plant to sink tissues. In the case of amino acids and sucrose, loading requires the presence of H^+-coupled symporters (Lalonde et al. 2004). *AHA3* is expressed in the vascular tissue (especially in phloem companion cells) and in pollen. Arabidopsis plants expressing *AHA3* with an altered C-terminus were found to be more resistant to acid medium when grown in vitro (Young et al. 1998), suggesting a role for this plasma membrane H^+-ATPase in cytoplasmic pH homeostasis. No effect was reported for plants grown under normal conditions, but *aha3* insertion mutations have been reported to result in male gametophyte lethality (Robertson et al. 2004).

In pollen, the male gametophyte of plants, plasma membrane H^+-pumps (AHA9 in Arabidopsis), are abundant and are thought to be essential for pollen maturation and for germination and growth of the pollen tube. In a recent study by Certal et al. (2008), it is demonstrated that a *Nicotiana tabacum* AHA is subject to spatial regulation and thereby associated with cell polarity and tip growth of the pollen tube.

Vessel-associated cells adjacent to dead xylem vessels are enriched in PM H^+-ATPases (Alves et al. 2004). These cells are very similar to phloem companion cells in the phloem as they are small cells with a dense cytoplasm and many mitochondria, except that their primary role is in the reabsorption of minerals and nutrients from the xylem sap. In trees, PM H^+-ATPases in vessel-associated cells are thought to drive uptake of sucrose from the vessels in the spring and reallocate metabolites to bursting buds (Alves et al. 2004).

Plasma membrane H^+-pumps in guard cells are believed to be involved in controlling the size of the stomatal aperture. Swelling of guard cells following the uptake of K^+ and water is a prerequisite for opening of the stomatal pore. The expression of all 11 H^+-ATPase isogenes can be detected in Arabidopsis guard cell protoplasts, with *AHA1*, *AHA2* and *AHA5* being predominant (Ueno et al. 2005).

AHA10, which is relatively distantly related to the other Arabidopsis PM H^+-ATPases (Axelsen and Palmgren 2001), is active primarily in endothelial cells in the developing seed integument. Gene disruption of *AHA10* resulted in seed coats with a transparent testa, a marked reduction in proanthocyanidin levels, and fragmentation of the vacuole in seed coat endothelial cells (Baxter et al. 2005). The relationship of *aha10* phenotypes to PM H^+-ATPase disruption is unknown. However, most unlike other PM H^+-ATPases, *Petunia* PH5, an ortholog of AHA10, is expressed in the vacuolar membrane (Verweij et al. 2008). Here, it enhances anthocyanin coloration by acidification of the vacuolar lumen and, in addition, may drive loading of proanthocyanidin precursors into the vacuole. It remains to be investigated whether AHA10 is expressed in the vacuolar membrane as well.

Plants that express *Nicotiana plumbaginifolia* PMA4 in a form constitutively activated by deletion of a C-terminal autoinhibitory region exhibit an increase in proton pumping activity, improved salt tolerance, and altered plant development, which is possibly related to cell expansion (Gevaudant et al. 2007). Overexpression of wild type PMA4 does not induce any phenotypic modification. Molecular genetic cosuppression of *PMA4* results in pleiotropic effects on plant growth, retarded development, male sterile flowers, and guard cells with a reduced stomatal aperture (Zhao et al. 2000). An open stomata mutation (*ost2*) in Arabidopsis renders plants unable to close their stomata. The gene has been found to encode the AHA1, which is constitutively active in both *ost2* alleles (Merlot et al. 2007). This suggests that AHA1 functions in regulation of stomata closure and that AHA1 is a principle target of inhibition by ABA during drought responses. Since *ost2* mutations cause constitutive and ABA-insensitive AHA1 activity, it is likely that, in the wild type, ABA reverses the membrane potential by inactivation of the proton pump. Additional targets are likely to be ABA-activated anion channels.

3 Regulation of PM P-Type H^+-ATPases

A key to understanding enzymatic regulation of plasma membrane H^+-ATPase is the elucidation of the function of the C-terminal regulatory (R) domain. The R-domain of plant PM H^+-ATPase consists of approximately 100 amino acid residues. Removal or mutagenesis of the C-terminal domain results in pump activation (Palmgren 1991; Morsomme et al. 1996, 1998; Baunsgaard et al. 1996). Two regions (I and II) are important for the autoinhibitory role of the C-terminus (Axelsen et al. 1999). In the Arabidopsis proton pump, AHA2, these domains are located between Lys-863 to Leu-885 (Region I) and Ser-904 to Leu-919 (Region II). Mutations within the central part of the pump can abolish the autoinhibitory effect of the C-terminus, but the intramolecular receptor for the C-terminal R-domain has not yet been identified.

In vivo, the PM H^+ pump is regulated by activating 14-3-3 proteins at a unique site in the extreme end of the C-terminus. Phosphorylation of a penultimate threonine residue in the C-terminus (Thr-947 in AHA2) is required in order to stabilize 14-3-3 protein binding (Fuglsang et al. 1999; 2003; Maudoux et al. 2000; Svennelid et al. 1999). Binding of a14-3-3 protein is believed to result in displacement of the autoinhibitory domain of the plasma membrane H^+-ATPase resulting in pump activation. A crystal structure of the complex formed by a plant 14-3-3 protein and the last 52 amino acid residues of PMA2 revealed a mode of interaction in which a 14-3-3 dimer simultaneously binds two H^+-ATPase C termini (Ottmann et al. 2007). How this regulatory system functions *in planta* is best documented by the finding that an H^+-ATPase/14-3-3 complex is formed in guard cells upon blue light activation (Kinoshita and Shimazaki 1999, 2001). Two Arabidopsis PHOTOTROPIN blue light photoreceptors (PHOT1 and PHOT2) are serine/threonine kinases that have been shown to activate the H^+ pump via phosphorylation of

a yet unidentified intermediate (Kinoshita et al. 2001). ABA induces closure of stomatal aperture by utilizing the same signal cascade as blue light, since the blue light-stimulated activation can be inhibited by ABA (Zhang et al. 2004).

Several lines of evidence suggest that pathogens short-circuit plasma membrane H^+-ATPase regulation to gain entry into leaves via open stomatal pores. The fungal pathogen *Fusicoccum amygdali* secretes fusicoccin (FC) in order to constitutively activate guard cell plasma membrane H^+-ATPases. As a result, the stomatal pore opens and the fungus gets access to the leaf interior. FC-treated cells acidify the external medium (Schaller and Oecking 1999) and have constitutive expression of a subset of pathogen-inducible genes (Frick and Schaller 2002; Higgins et al. 2007). FC has been shown to function by irreversibly preventing dissociation of the blue light-responsive H^+-ATPase/14-3-3 complex. Conversely, H^+-ATPase inhibitors alkalinize the exterior of plant cells and induce wound response genes in whole plants. These results have been interpreted to suggest a general role for H^+-ATPases as targets in defense signaling (Schaller and Oecking 1999). It has recently been demonstrated that the *Arabidopsis* protein RIN4, which is a negative regulator of plant immunity, interacts in vivo with both AHA1 and AHA2. *RIN4* overexpression and knockout lines exhibit differential PM H^+-ATPase activity (Liu et al. 2009). The *rin4* knockout line has reduced PM H^+-ATPase activity, and its stomata cannot be reopened by virulent *Pseudomonas syringae*. These results indicate that the *Arabidopsis* protein RIN4 functions with the PM H^+-ATPase to regulate stomatal apertures, inhibiting the entry of bacterial pathogens into the plant leaf during infection (Liu et al. 2009).

The protein kinase responsible for phosphorylation of the penultimate threonine residue in PM H^+-ATPases has not yet been identified. However, the involvement of another kinase in regulation of the H^+ pump has recently been demonstrated (Fuglsang et al. 2007). This kinase, PKS5, belongs to a family of kinases (PKS/CIPK) that are regulated by a Ca^{2+}-binding protein (CBL/SCaBP). PKS5 phosphorylates a highly conserved serine residue, Ser-931, situated in the 14-3-3-binding site (Fuglsang et al. 2003). Phosphorylation of Ser-931 in AHA2 results in a decrease of 14-3-3 binding to the H^+ pump shifting the pump into its low-activity state. A mass spectrometric analysis of PMA2 (PM H^+-ATPase isoform 2) isolated from *N. tabacum* suspension cells identified Ser-938 (corresponding to Ser-931 in AtAHA2) as in in vivo phosphorylated residue in the enzyme, thus confirming that this site is a common site of regulation for PM H^+-ATPases (Duby et al. 2009).

In vitro, phosphatases are found to interfere with the stability of the H^+ pump/14-3-3 protein complex (Fuglsang et al. 1999, 2006; Camoni et al. 2000) and reduce H^+ pump activity (Marin-Manzano et al. 2004). However, due to the relative broad spectra of phosphatases, it is difficult to determine their role in planta, and there is still no genetic evidence for a specific phosphatase involved in PM H^+-ATPase regulation.

Phosphoproteomic studies have revealed that several residues within the C-terminal domain are phosphorylated in vivo (Nuhse et al. 2004; Niittyla et al. 2007; Duby and Boutry 2008), but only a few of these have been assigned with a physiological role. As described above, blue-light induces phosphorylation of the penultimate threonine residue, and resupply of sucrose to Arabidopsis seedlings activate the

H^+-ATPase by phosphorylation of binding of 14-3-3 protein (Niittyla et al. 2007) as well as aluminium stress, where the response and secretion of citrate is dependent on the activity of the H^+ pump (Shen et al. 2005). Heavy metal stress lowers the activity of the H^+ pump by reducing the phosphorylation level (Janicka-Russak et al. 2008). Thus, in order fully to understand the regulation of the H^+-ATPase, it is of importance to identify the protein kinases and phosphatases involved.

4 Plasma Membrane Ca^{2+}-ATPases (P_{2B}-ATPases)

The P-type Ca^{2+} pumps have been classified as ER-type (group P_{2A}), PM-type (group P_{2B}), and secretory pathway-type, based on amino acid sequences. In *A. thaliana*, four pumps have been classified as P_{2A} (ECAs, endoplasmic reticulum type Ca^{2+}-ATPases) and ten as P_{2B} Ca^{2+} pumps (ACAs, autoinhibited Ca^{2+}-ATPases). Secretory pathway Ca^{2+} pumps have so far not been identified in plants (Axelsen and Palmgren 1998, 2001; Boursiac and Harper 2007).

Plant P_{2B} pumps are distinct from P_{2A} pumps, in that they are equipped with a calmodulin (CaM)-regulated autoinhibitory domain at the N-terminal end (Sze et al. 2000). In contrast, all animal P_{2B} pumps (PMCAs) have their CaM-binding domain situated in their C-terminus (Carafoli and Brini 2000). Further, P_{2B} pumps only have a single Ca^{2+} ion-binding site whereas P_{2A} pumps bind two Ca^{2+} ions in their transmembrane region (Guerini et al. 2000; Toyoshima et al. 2000).

Isoform expression in the plant – ACA8, ACA9, and ACA10 are closely related P_{2B} pumps, and between these, ACA8 and ACA9 have been identified in the plasma membrane (Bonza et al. 2000; Schiott et al. 2004). An aca10 mutation can be complemented by ACA8, and ACA10 is therefore also likely to be plasma membrane localized as well (George et al. 2008). Among these pumps, ACA8 and ACA10 are distributed throughout the plant body whereas ACA9 is located in pollen and pollen tubes only (George et al. 2008; Schiott et al. 2004).

Physiological role – In all eukaryotes, Ca^{2+}-ATPases are important for cellular signaling by maintaining a low cytosolic Ca^{2+} concentration. Together with Ca^{2+}/H^+ antiporters, they transport Ca^{2+} out of the cell to the apoplasm across the plasma membrane or into intracellular compartments (White and Broadley 2003). The large difference in concentration of Ca^{2+} between the cytoplasm on the one hand and the apoplasm and intracellular compartments on the other provides the basis for the use of Ca^{2+} signals as a link between extracellular stimuli and intracellular responses in many different physiological pathways (Reddy 2001; Sanders et al. 2002).

Only very few P_{2B} ATPase mutations has been characterized so far. Knockout mutants of ACA9 are found to have reduced growth of pollen tubes than wild type pollen tubes, and the mutant pollen tubes fail to reach the ovules in the lower part of the pistil. Furthermore, about half of the pollen tubes that reach an ovule fail to rupture and release the sperm cells (Schiott et al. 2004). Ca^{2+} is known to be

important for pollen tube growth (Holdaway-Clarke et al. 2003), and ACA9 might play an important role in controlling cytoplasmic Ca^{2+} during this process.

Recently, a *CIF1* gene has been identified as *ACA10* (George et al. 2008). An *A. thaliana cif* (*compact inflorescence*) mutant shows a severe lack of elongation of inflorescence internodes, resulting in the formation of tightly bunched clusters of flowers either at the ends of very short inflorescence shoots or within the center of the rosette (Goosey and Sharrock 2001). This finding suggests that internode elongation is regulated by calcium signaling via a plasma membrane Ca^{2+} pump.

In the moss *Physcomitrella patens*, a Ca^{2+}-ATPase gene (*PCA1*) was identified as induced by stress treatments. *PCA1* loss-of-function mutants (*ΔPCA1*) exhibit an enhanced susceptibility to salt stress. The *ΔPCA1* lines show sustained elevated $[Ca^{2+}]_{cyt}$ in response to salt treatment in contrast to wildtype moss that shows transient Ca^{2+} elevations, indicating a direct role for PCA1 in the restoration of prestimulus $[Ca^{2+}]_{cyt}$ (Qudeimat et al. 2008).

Regulation – Upon binding of Ca^{2+} ions, calmodulin (CaM) binds to and activates P_{2B} Ca^{2+} pumps. Although there does not exist a consensus CaM-binding domain (CaMBD), they are usually 15–30 amino acids long and have a tendency to form an α-helix containing two bulky hydrophobic residues that function as anchors for CaM binding (Crivici and Ikura 1995; Yap et al. 2000). N-terminal CaMBDs have so far been identified in all investigated plant P_{2B} Ca^{2+} pumps (Baekgaard et al. 2006).

An alanine mutagenesis scanning analysis has revealed that the CaMBD of ACA8 comprises a stretch of approximately 25 amino acid residues in the N-terminus (Arg-43 to Lys-68) (Baekgaard et al. 2005). This region appears to have a dual function in both autoinhibition and CaM recognition as a number of conserved residues in the 25 acimo acid stretch are required both for CaM binding and pump autoinhibition (Baekgaard et al. 2005). A simple working model for regulation of P_{2B} Ca^{2+} pumps is that the autoinhibitory domain (R-domain) interacts with another region of the pump and either blocks conformational changes required for catalysis, or access of Ca^{2+} to the pore domain, or both. Activation occurs when CaM binds to the autoinhibitor and thereby disrupts its blocking interaction (Baekgaard et al. 2006; Boursiac and Harper 2007).

Phosphorylation and lipids may also affect the activity of plant plasma membrane Ca^{2+} pumps. Acidic phospholipids activate a plasma membrane Ca^{2+} pump from radish by a mechanism different from CaM stimulation (Bonza et al. 2001), although it appears that the phospholipid-binding site overlaps the CaM-binding site (Meneghelli et al. 2008). ACA8 is phosphorylated in vivo but the physiological impact of phosphorylation is not known (Niittyla et al. 2007).

5 Plasma Membrane Zn^{2+}-ATPases (P_{1B}-ATPases)

P_{1B}-ATPases are heavy metal pumps found in all life forms. In plants, P_{1B} heavy metal ATPases transport not only mainly Zn^{2+} and Cu^{+} but also other heavy metals such as Cd^{2+} and Co^{2+}. Most other of these pumps has been identified in

intracellular membranes, such as those of the chloroplast that need pumps in order to provide their photosystems with sufficient amounts of Cu (Williams and Mills 2005). In plants, PM Zn^{2+} pumps belong to this category (Arguello et al. 2007; Williams and Mills 2005). In Arabidopsis, two P_{1B} ATPases (HMA2 and HMA4), both Zn^{2+} pumps, have been found exclusively in the PM of root xylem parenchyma and pericycle cells (Hussain et al. 2004; Verret et al. 2004; Sinclair et al. 2007). A number of studies have indicated that HMA2 and HMA4 are involved in Zn homeostasis (Williams and Mills 2005). These HMAs are thought to transport Zn^{2+}across the plasma membrane of root vascular cells into the xylem for transport to the shoot (Hussain et al. 2004; Verret et al. 2004). Heterologous expression in yeast has suggested that Cd^{2+} ions might be a second substrate for HMA2 and HMA4 (Eren and Arguello 2004; Mills et al. 2005; Verret et al. 2005),

An *hma2*, *hma4* double mutant accumulates Zn in root tissue and exhibits a Zn-deficient growth phenotype because of insufficient Zn translocation from root to shoot. This deficiency can be rescued by increasing the level of Zn in the growth medium or soil. Thus, an *hma2*, *hma4* double mutant suffers from inadequate Zn supply to the leaves, resulting in stunted growth and chlorosis (Hussain et al. 2004). This suggests that active Zn pumps are required for Zn to exit xylem parenchyma cells.

The metallophyte *Arabidopsis halleri* accumulates and tolerates extremely high levels of Zn in the leaf and is classified as Zn hyperaccumulator (Mitchell-Olds 2001). Silencing of *A. hallerii HMA4* by RNA interference completely suppresses Zn hyperaccumulation and results in metal partitioning between roots and shoots characteristic of the nonaccumulator *A. thaliana*. In wild type plants, promoter activity is substantially higher for the promoters of three tandem *A. halleri HMA4* gene copies than for the *A. thaliana HMA4* promoter (Hanikenne et al. 2008). Consequently, efforts to enhance Zn translocation from roots to shoots for biofortification purposes are often focused on increasing the expression of *HMA4* or a closely related gene.

Like P_{2B} Ca^{2+}-ATPases and P_{3A} H^+-ATPases, the P_{1B} ATPases have extended N- and/or C-terminal domains (Arguello et al. 2007); however, the potential role in regulation of these terminal domains of P_{1B} ATPases is less clear. HMA2 and HMA4 have extended N- and C-terminal ends containing metal-binding domains rich in cysteines and histidines. Although not essential for pump function, the N- and C-terminus are important for catalytic activity besides being involved in metal binding (Eren and Arguello 2004; Eren et al. 2006; 2007; Mills et al. 2005).

6 Additional Plasma Membrane P-Type ATPases

In addition to the pumps described above, plants harbor a number of other cation pumps, which all belong to the P-type ATPase family. Apart from the H^+, Ca^{2+} and heavy metal pumps discussed above, P_4 and P_5 ATPases are also present in plants. The substrate specificities of the P_4 and P_5 ATPases have not yet been determined,

and their physiological functions are poorly understood. The pumps seem to be widespread among eukaryotes but are absent from prokaryotes (Axelsen and Palmgren 1998). In Arabidopsis, the P_4 subfamily contains 12 members while the P_5 subfamily comprises only one protein (Axelsen and Palmgren 2001).

The yeast PM contains two P_4-ATPases, Dnf1p and Dnf2p, which appear to function in endocytosis (Pomorski et al. 2003). Whether plants have PM P_4-ATPases in the PM remain to be tested.

Evidence from reverse genetics experiments suggests that a member of the P_4-ATPase subfamily in Arabidopsis, aminophospholipid ATPase1 (ALA1), could be important for cold tolerance (Gomes et al. 2000). Another P_4-ATPase, ALA3, localizes to the Golgi apparatus of peripheral columella cells of the root tip. An *ala3* mutation results in impaired ability of the root tip *trans*-Golgi network to produce secretory vesicles destined for the PM. In yeast complementation experiments, ALA3 function requires interaction with members of a novel family of plant membrane-bound proteins, ALIS1 to ALIS5 (for ALA-Interacting Subunit), and in this host, ALA3 and ALIS1 show strong affinity for each other. The ALIS1 protein is a beta-subunit of ALA3, and the protein complex forms an important part of the Golgi machinery required for secretory processes during root development (Poulsen et al. 2008).

In conclusion, a battery of P-type pumps operates in the plant plasma membrane. They all consume ATP to pump cations out of the cell. In this way, they serve essential physiological roles such as generating the electrochemical gradient required for nutrient uptake (H^+ pumps), maintaining Ca^{2+} homeostasis (Ca^{2+} pumps), and providing the basis for long-distance transport of Zn^{2+} (heavy metal pumps). An interesting hypothesis that remains to the tested is whether the plasma membrane harbors P_4 type ATPases involved in cellular endocytosis.

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Physiological Roles for the PIP Family of Plant Aquaporins

Rosario Vera-Estrella and Hans J. Bohnert

Abstract Aquaporins are a class of intrinsic membrane proteins that are primarily associated with water movement across membranes. In plants, biophysical studies indicated that water fluxes could not be explained by simple diffusive movement. The discovery of aquaporins that facilitate water movement across membranes in other organisms rapidly led to the identification of this class of proteins in plants. The large number of aquaporin genes identified in plant genomes compared to those from other organisms suggests that they play a major role in plant water relations. However, plant aquaporins also facilitate the transport of small solutes such as glycerol, silicon, ammonium, urea, boric acid, CO_2, arsenite, and hydrogen peroxide within and between cells. Here, the aquaporin-like proteins that function on the plant plasma membrane are reviewed in the context of their apparent role in plant adaptive evolution.

1 Introduction

The fundamental role played by water movement in the biology of plants established an early interest in the mechanisms that mediate water transport across the plasma membrane (PM). Biophysical studies indicated that water fluxes could not be explained by simple diffusive movement, and the discovery of the intrinsic membrane proteins known as aquaporins (AQPs) that facilitate water movement across membranes attracted much interest in the plant community. The large

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number of AQP genes identified in plant genomes compared to those from other organisms emphasized their importance in the physiology of plant water relations. It has now become clear that plant AQPs also facilitate the transport of small solutes such as glycerol, silicon, ammonium, urea, boric acid, CO_2, arsenite, and hydrogen peroxide within and between cells. AQPs are characterized by a high degree of amino acid sequence similarity, and all conform to one topological model: six transmembrane domains (1–6) connected by five loops (A–E) with two highly, but not absolutely, conserved asparagine-proline-alanine (NPA) signature sequences in the partially membrane-embedded loops B and E (Fig. 1). Biochemical and structural studies have demonstrated that AQP channel function depends on a tetrameric structure, although monomeric forms have also been shown to facilitate water transport.

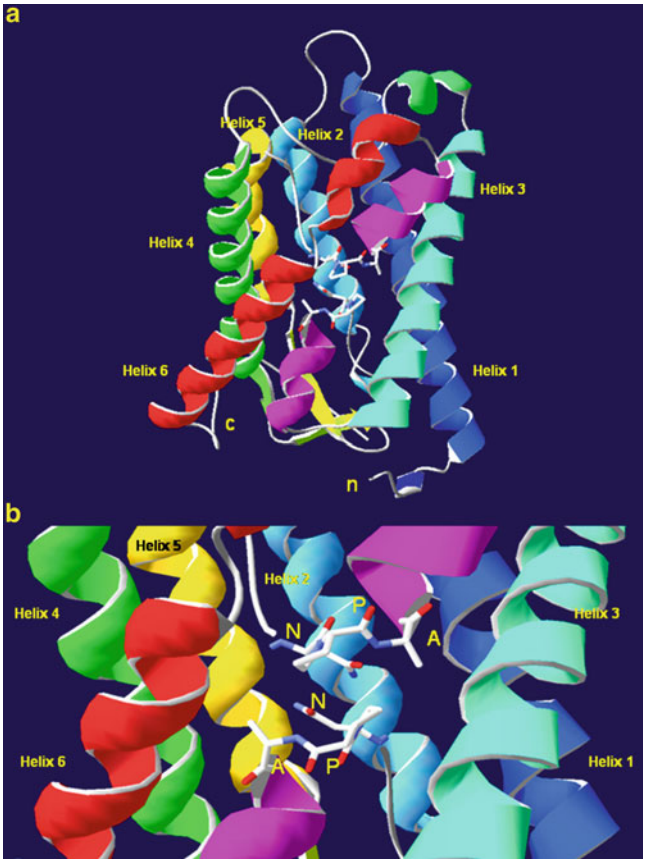

Fig. 1 Structure of the closed conformation of *Mc*TIP2;1.Stereo model conformation overlaid on that of *So*PIP2;1. The structure was designed using the Swiss-Pdb Viewer program from the Swiss Institute of Bioinformatics (Guex and Peitsch 1997)

The plant AQP-like proteins may be separated into four subfamilies that appear to serve different, possibly multiple, physiological functions. An initial focus on aspects of AQP-facilitated water movement has gradually given rise to a view advocating more general essential roles as facilitators of metabolic integration and possibly as signal transducers (Hill and Shachar-Hill 2006). AQPs appear to exert fine control over the hydraulic conductivity of cells and mediate intra- and intercellular movement of small molecules in response to biotic and abiotic challenges. AQPs also appear to function in the regulation of cellular and/or subcellular pH homeostasis, maintenance of proton gradients, calcium signaling, bulk carbon transport, and ROS or redox signaling (Vanderleur et al. 2005; Kaldenhoff and Fischer 2006; Katsuhara et al. 2008; Maurel et al. 2008). As such, it appears that the expansion of genes encoding AQP-like proteins has been an important component of plant adaptive evolution.

Importantly, AQP regulation and function are strongly associated with physiological responses to environmental signals that generate water, ion, and metabolite imbalances. Plant AQPs appear to be regulated by multiple mechanisms including genes, reversible posttranslational protein modifications, dynamic subcellular localization, hetero-tetramerization, and protein:protein interactions.

Plant aquaporins have been discussed in a number of recent reviews (Quigley et al. 2002; Vander Willigen et al. 2004; Luu and Maurel 2005; Chaumont et al. 2005; Vanderleur et al. 2005; Kaldenhoff and Fischer 2006; Kruse et al. 2006; Maurel 2007; Katsuhara and Hanba 2008; Maurel et al. 2008; Kaldenhoff et al. 2008; Neumann 2008). These reviews have focused on complexity, structure, function, and regulation of plant AQPs. Here, after a general discussion of AQP attributes, we will concentrate on AQPs associated with the PM, also known as PM intrinsic proteins (PIPs). We will discuss AQP structure, function at the PM, Transcriptional and posttranscriptional regulation of AQP abundance, as well as the dynamics of intracellular targeting. We will comment on transport specificity and the function of these solute and water facilitator proteins in a system that requires the balancing and integration of multiple biochemistries.

2 Aquaporin Substrates

In animals, AQPs have been separated into true aquaporins that conduct only water and aquaglyceroporins, which conduct other small molecules, such as glycerol (Heller et al. 1980; Borgnia and Agre 2001), urea (Borgnia et al. 1999), nitrate (Ikeda et al. 2002), and arsenite (Liu et al. 2002). Relatively minor amino acid variations appear to confer differences in substrate specificity. Although most molecules conducted by AQPs are neutral, AQP1, which bears some structural resemblance to K^+ and cyclic nucleotide-gated channels, it has been suggested that transports ions (Yool and Weinstein 2002; Yool et al. 1996). In addition, mammalian AQP6 has been shown to act as a chloride channel that selectively excludes Na^+ (Yasui et al. 1999; Hazama et al. 2002; Yu et al. (2006).

Table 1 List of small, charged or uncharged, molecules that permeate through plant AQPs

Solute	AQP and plant species
Glycerol and/or urea	*Gm*NOD26 (*Glycine max*); NLM1 and NLM2 (*Lotus japonicus*; *Nt*TIPa, *Nt*AQP1; NIP (*Nicotiana tabacuum*); *Pt*NIP6;1 (*Populus trichocarpa*); AtTIP1;1, *At*TIP1;2 (*A. thaliana*); OsTIP1;2, *Os*TIP3;2, *Os*TIP4;1 (*Oriza sativa*)
Formamide	*Pt*NIP6;1 (*P. thrichocarpa*); *Ta*TIP2;1 (*Tritcum aestivum*); *At*TIP2;1 (*A. thaliana*)
Boric acid	*Zm*PIP1 (*Zea mays*); *At*NIP5;1 (*A. thaliana*)
Silicon	*Os*NIP2;1 (*Oriza sativa*) Compound
Arsenite	*At*NIP5;1, *At*NIP6;1 (*A. thaliana*), *Os*NIP2;1, *Os*NIP3;2 (*O. sativa*); *Lj*NIP5;1 and *Lj*NIP6;1(*Lotus japonicus*)
CO_2	*Nt*AQP1 (*N. Tabacuum*); *Hv*PIP2;1 (*Hordeum vulgare*)
H_2O_2	*At*TIP1;1 and *At*TIP1;2 (*A. thaliana*)
NH_3^+	*Gm*NOD26 (*Glycine max*); *Ta*TIP2;1 (*T. aestivum*), *At*TIP2;1, (*A. thaliana*)
NH_4	*Ta*TIP2:1, *Ta*TIp2;1 (*T. aestivum*); *At*TIP2;1 (*A. thaliana*)

Ample experimental evidence suggests that a subset of plant AQPs can transport small molecules in addition to H_2O; especially plant AQPs exhibit differing levels of water facilitation when expressed in Xenopus oocytes. The list of small, charged, or uncharged molecules that pass through AQPs include NH_3^+ and NH_4 (Niemietz and Tyerman 2000; Tyerman et al. 2002; Jahn et al. 2004; Loque et al. 2005), boric acid (Dordas et al. 2000 and Dordas and Brown 2000; Takano et al. 2006; Bastias et al. 2004; Vander Willigen et al. 2006), hydrogen peroxide (Henzler and Steudle 2000; Bienert et al. 2006, 2007), urea and/or glycerol (Schutz and Tyerman 1997; Hertel and Steudle 1997; Biela et al. 1999; Dean et al. 1999; Gerbeau et al. 1999; Guenther and Roberts 2000; Weig and Jakob 2000; Ciavatta et al. 2001; Liu et al. 2003; Klebl et al. 2003; Fetter et al. 2004; Wallace and Roberts 2006; Li et al. 2008), formamide (Jahn et al. 2004; Holm et al. 2005; Wallace and Roberts 2005), silicon (Ma et al. 2006; Yamaji et al. 2008), CO_2 (Terashima and Ono 2002; Uehlein et al. 2003; Hanba et al. 2004; Katsuhara and Hanba 2008), and arsenite (Ma et al. 2008) (Table 1). The diversity of cargo molecules that can pass through subsets of plant AQPs is consistent with the necessity of mechanisms for cell-to-cell movement of metabolites in a kingdom of organisms lacking a circulatory system.

2.1 *PIPs: Mostly Water Channels and Mostly on the PM and Trafficking Vesicles*

The PIP gene family includes 13 genes in *Arabidopsis thaliana*, 13 in *Zea mays*, 11 in *O. sativa*, and 14 in *Populus trichocarpa*. Phylogenetic analysis initially supported the separation of the PIPs into two subgroups, PIP1 and PIP2 (Kjellbom et al. 1999). While both PIP1 and PIP2 subgroups show high overall identity, their deep origin indicates that the division already existed before the monocot/dicot

divergence and is present in gymnosperms and bryophytes (Zardona 2005). However, in the *Physcomitrella patens* genome, as in *Arabidopsis* and *Brassica*, a further subgroup of PIP3 sequences is found (Danielson and Johanson 2008). In *P. patens*, the single PIP3 gene lacks a Ca^{2+}-binding site in the N-terminal region and an otherwise conserved cysteine in helix 2 (Danielson and Johanson 2008).

Maurel et al. (2008) have suggested that the PIP1 proteins are restricted as water pores to the PM, while the PIP2 proteins were viewed as water pores in endocytotic vesicles. However, the PIP1 subgroup has been reported to have low water permeability (and shows glycerol and urea permeability) when compared to the high and almost exclusive water permeability of the PIP2 subgroup (Chaumont et al. 2000; Moshelion et al. 2002; Fetter et al. 2004; Suga and Maeshima 2004; Sakurai et al. 2005). Also, PIP1 proteins have to form heterotetramers with some PIP2 monomers to be able to facilitate water permeation (Fetter et al. 2004; Zelazny et al. 2007) Finally, water permeability can be increased in PIP1 by site-directed mutagenesis (Suga and Maeshima 2004), which suggests that their primary role in the PM may not be water facilitation. Clearly, to distinguish between hypotheses, more attention should be focused on the presence and function of PIP1s in endosomal vesicles, and the functions of PIP3 proteins need to be established. As genomic sequences increase in number, it should be possible to determine whether divergence of the PIP subfamily coincides with the emergence of increasingly complex phototrophic organisms.

All PIP1 proteins are characterized by a long N-terminal extension relative to other AQPs and a C-terminus that ends close to the intracellular end of transmembrane region 6 (Kaldenhoff and Fischer 2006). Some members in the PIP1 subfamily are capable of transporting glycerol (Biela et al. 1999; Moshelion et al. 2002), urea (Gaspar et al. 2003), and CO_2 (Uehlein et al. 2003). Proteins in the PIP2 group generally show high water permeability, when expressed in *Xenopus* oocytes. Their N-terminal region is short, while the C-terminus is longer than that in the PIP1 subgroup (Chaumont et al. 2000, 2001; Johanson et al. 2000, 2001). *H. vulgare* PIP2;1 and tobacco PIP1 (AQP1) appear to transport CO_2 in addition to water (Uehlein et al. 2003; Flexas et al. 2006; Hanba et al. 2004). The possibility that AQPs can facilitate CO_2 flux across a membrane is intriguing, as biological membranes show greater permeability to gases than deproteinated membranes. However, it is difficult to see how a molecule as large as CO_2 can pass through the single-file pore monomer, and the data showing localization of AQP1 to guard cells is controversial (see Katsuhara et al. 2008, for discussion).

2.2 Regulation of PIP AQP Expression and Activity

AQPs are regulated by a variety of mechanisms (reviewed by Vander Willigen et al. 2004; Luu and Maurel 2005; Chaumont et al. 2005; Maurel et al. 2008). Evidence points towards regulation by changes in gene expression, protein translation and protein turnover (abundance), posttranslational, reversible modifications,

intracellular changes in pH, subcellular protein targeting, recycling within membranes, and heterotetramerization (Fortin et al. 1987; Morrison et al. 1988; Daniels et al. 1994; Kammerloher et al. 1994; Robinson et al. 1996; Fleurat-Lessard et al. 1997; Chaumont et al. 1998, 2000; Barkla et al. 1999; Barrieu et al. 1999; Cutler et al. 2000; Kirch et al. 2000; Verkman and Mitra 2000; Reisen et al. 2003; Vera-Estrella et al. 2004; Ma et al. 2004; Ishikawa et al. 2005; Boursiac et al. 2005; Prak et al. 2008; Maeshina and Ishikawa 2008). By directly affecting the gating of the channel, a basis for short-term regulation exists that would allow fast fine-tuning of water or metabolite permeability. The divergent regulation of plant AQPs – ranging from environmental signals to the activation of signal transducers and altered vesicular trafficking – could be attributable to the activation of signals upon stimulation or activation of pools of signal transducers that involve phosphorylation, glycosylation, or ubiquitination. We now know that glycosylation of, for example, TIP1;2 from *Mesembryanthemum crystallinum* is fundamental for its internalization to multivesicular bodies. Trafficking of this AQP only occurs in the presence of osmotic stress, while the actual signal that induces this response remains unclear (Vera-Estrella et al. unpublished).

2.2.1 Phosphorylation

In vivo and in vitro phosphorylation of PIP AQPs has been reported in spinach leaves (PIP2;1), soybean roots, *Mimosa pudica* (*Mp*PIP1;1), and tc *Z. mays* (*Zm*PIP2;1; Van Wilder et al. 2008). In all cases, the modification targets serine residues located either in the carboxy-terminal or amino-terminal regions, or a serine located in loops close to the NPA motif (Maurel et al. 1995; Johansson et al. 1998). The evidence suggests that these AQPs are phosphorylated by calcium-dependent protein kinases (Weaver and Roberts 1991, 1992; Johnson and Chrispeels 1992; Miao et al. 1992; Johansson et al. 2000). Replacing the serine, S-274, residue by alanine in *So*PIP2;1 resulted in a loss of activity when expressed in oocytes (Maurel et al. 1995). In *M. pudica* (*Mp*PIP1;1), residue Ser-131 was phosphorylated by a PKA (Temmei et al. 2005). Opening of the pore in *So*PIP2;1 is achieved by phosphorylation of two serine residues: Ser115 in the cytoplasmic loop B and Ser274 in the C-terminal region (conserved in all members of PIP2 subgroup; Sjovall-Larsen et al. 2006) by Mg^{2+} and Ca^{2+}-dependent protein kinases. In maize, the mutations of the phosphorylation sites S129 and S203 to alanine or glutamate decreased water permeability by 30–50% when expressed in oocytes (Van Wilder et al. 2008). In combination, the high degree of conservation of serine residues appears to indicate structural as well as regulatory functions. Nevertheless, in vivo experiments have demonstrated the role of phosphorylation in regulating AQP activity as, for example, it has been reported that phosphorylation of an AQP from tulip (*Tulipa gesneriana*) regulated the protein's activity and controlled opening of petals at 20°C and closing at 5°C (Azad et al. 2004). The results from these studies are important indications for the seminal role of controlled AQP phosphorylation in, most likely, multiple positions. Conceivably, protein

modification at different sites regulates AQP gating and transport activity, subcellular distribution, complex assembly, and possibly also the cargo transported through the channel. These numerous functions of AQPs, which still need more testing and confirmation, implicate the importance and essentiality of these proteins for the general physiology of plant cells.

2.2.2 Quaternary Structure

AQP activity can be affected by the oligomerization state. In membranes, AQPs are present as tetramers, even though they are functionally active as monomers as well (Fetter et al. 2004; Zelazny et al. 2007). Tetramerization involves the interaction of α-helices and the partially membrane-embedded loops, which appear to stabilize the tetramer (Murata et al. 2000). The coexpression of *Zm*PIP1;2-uc from *Z. mays* with different members of the *Zm*PIP2 subfamily in *Xenopus* oocytes demonstrated a positive cooperative effect in the activity of *Zm*PIP1;2 as a result of heterodimerization (Fetter et al. 2004). Further support emerged from comparisons of structural models of PIP1 and PIP2 isoforms and dynamic simulations utilizing single amino acid substitutions, which identified loop E as important for the formation of oligomeric/multimeric structures (Fetter et al. 2004; Chaumont et al. 2005). In *M. pudica*, *Mp*PIP1;1 is not permeable to water; however, when this AQP was coexpressed with *Mp*PIP2;1, water permeability increased significantly over the permeability of oocytes injected with *Mp*PIP2;1 alone. This cooperative effect was dependent on phosphorylation. Point mutations of *Mp*PIP1;1 showed that Ser-131 is phosphorylated by a PKA, and that cooperative regulation of assembly with *Mp*PIP2;1 is regulated by this residue (Temmei, et al. 2005). Using immunoprecipitation assays, *Mp*PIP1;1 was shown to be bound directly to *Mp*PIP2;1 in a phosphorylation-independent manner (Temmei, et al. 2005). These results suggest that PKA-dependent phosphorylation of Ser-131 may influence the structure of the complex when *Mc*PIP1;1 and *Mc*PIP2;1 form heterodimers or heterotetramers and, as a consequence, may also affect the activity and possibly also the specificity of the (water) channel. The regulation of plant AQPs by heterotetramerization is an intriguing regulatory process that requires further investigation. Mixing and matching of different AQPS monomers into protein complexes with different functions in distinct tissues or developmental stages could increase the number and complexity of these channels beyond what is indicated by the number of AQP genes in the respective genomes.

2.2.3 Regulation by the Cellular Environment

The regulation of AQPs by Ca^{2+} and intracellular pH has been documented for AQP0, AQP3, and AQP6 from mammals and for some plant PIPs (Yasui et al. 1999; Zeuthen and Klaerke 1999; Nemeth-Cahalan and Hall 2000; Gerbeau et al. 2002; Tournaire-Roux et al. 2003; Nemeth-Cahalan et al. 2004). In cell suspensions

and the root PM of *A. thaliana*, water permeability is reduced in the presence of Ca^{2+} and/or low cytosolic pH (Gerbeau et al. 2002; Tournaire-Roux et al. 2003). Water permeability of *At*PIP2;1, *At*PIP2;2, *At*PIP2;3, and *At*PIP1;2 expressed in *Xenopus* oocytes was reduced by a change in cytosolic pH from 7 to 6. Further, red beet PM vesicles, used in shrinking assays to assess PIP activity, showed their highest water conductance at neutral or basic pH, and the increased water conductance was repressed by a drop to cytoplasmic (slightly acidic) pH values or by increased cytosolic Ca^{2+} (Alleva et al. 2006).

A histidine residue (H197) located in loop D has been shown to be required for this response, as an alanin substitution of this residue reduced the effect of acidification (Tournaire-Roux et al. 2003; Chaumont et al. 2005). A structural model of *At*PIP2;2 using the atomic resolution structure established for bovine and human AQP1 proteins indicates that the cytoplasmic face of *At*PIP2;2 is made up of a large number of basic amino acids that would be presented as a negatively charged surface consistent with pH-dependent gating. A three-dimensional structure of *So*PIP2;1 in a closed state at a resolution of 2.1 and 3.9 Å in open configuration (Törnroth-Horsefield et al. 2006) shows that, in the closed configuration, loop D of *So*PIP2;1 is folded underneath the structure and blocks the pore. A leucine residue conserved in all PIPs (Leu197) is inserted inside the cavity close to the entrance of the channel and combines with His99, Val104, and Leu108 of loop D to form a hydrophobic barrier that blocks the pore. In the open configuration, loop D is extended and separated to a distance of 16 Å allowing water flux through the channel by removing Leu197 from the vicinity of the pore (Törnroth-Horsefield et al. 2006). The results can be readily extrapolated to explain gating of other PIPs and their interactions with different cations, such as calcium, copper, nickel, lead, and protons. However, other proteins, lipids, and metabolites might also function in the regulation of plant AQPs.

3 Subcellular Distribution and Protein Trafficking

Although only a subset of plant AQPs encoded in a genome have been studied in detail, the subcellular distribution of those AQPs that have been investigated appears to be more complex than what is implied by their labeling as PM PIPs or tonoplast TIPs (Barkla et al. 1999; Vera-Estrella et al. 2004; Uehlein et al. 2008; Prak et al. 2008). Plant AQPs have been localized to membranes of multiple compartments (Fortin et al. 1987; Morrison et al. 1988; Daniels et al. 1994; Kammerloher et al. 1994; Robinson et al. 1996; Fleurat-Lessard et al. 1997; Chaumont et al. 1998, 2000; Barkla et al. 1999; Barrieu et al. 1999; Cutler et al. 2000; Kirch et al. 2000; Reisen et al. 2003; Vera-Estrella et al. 2004; Ma et al. 2004; Ishikawa et al. 2005; Boursiac et al. 2005; Prak et al. 2008; Maeshina and Ishikawa 2008). For instance, *A. thaliana* PIPs have been found in "plasmalemmasomes" (invaginations of the PM), whose function is unknown, where they could be instrumental in enhancing exchange of water between the apoplast and the central

vacuole (Robinson et al. 1996). Different types of vacuoles may, themselves, be distinguished by the type of TIP residing in their membrane (Paris et al. 1996; Jahn et al. 1999). GFP fusions of *Zm*PIP1;2 and *Zm*PIP2;5 have been detected in the PM, endomembrane compartments, and in the perinuclear membrane, and it has been proposed that PIPs have a functional role in the secretory pathway (Chaumont et al. 2000).

M. crystallinum AQPs exhibit an equally complex distribution pattern (Kirch et al. 2000; Vera-Estrella et al. 2004). *Mc*PIP1;4 was localized to fractions corresponding to the tonoplast and lighter membranes. *Mc*PIP2;1 is expressed both at the PM in several other cellular compartments. Changes in phosphorylation state of specific residues of *A. thaliana*. PIP2;1 have been shown to control trafficking from the PM to an intracellular location under salt stress (Prak et al. 2008). The dynamic changes in PIP distribution observed in response to osmotic perturbation support a proposed role for PIP AQPs in vesicular trafficking.

4 Multiple Physiological Roles for PIP Aquaporins

PIP AQPs participate in developmental and environmental response mechanisms including general osmotic homeostasis, water uptake from the soil, transpiration, stomatal aperture control, cytosolic osmotic homeostasis, leaf movement, floral expansion, and postmeristematic elongation (Maurel et al. 2002; Tyerman et al. 2002). Their participation in these numerous physiological processes suggests that their expansion in plant genomes (e.g., Quigley et al. 2002) and additional complexity resulting from heterotetramerization coevolved with plant diversity (Wallace and Roberts 2004; Luu and Maurel 2005; Chaumont et al. 2005). Further, meta analyses of AQP gene expression data from *A. thaliana* microarrays suggest discrete tissue- and organ-specific functions of subsets of AQPs at various developmental stages (Fig. 2). However, the transport specificity and capacity of most plant AQPs remainunknown. Detailed reviews cataloging AQPs' physiological functions have recently been published by Chaumont et al. (2005), Luu and Maurel (2005), Kaldenhoff and Fischer (2006), and Maurel et al. (2008).

If any generality about PIP AQP function is to be drawn, it is that these proteins do play a major role in regulating water balances, particularly during cell elongation and expansion during normal development or under conditions that necessitate modifications in water flux, including drought or salt stresses, pathogen interactions, and chilling. That AQPs play a role in plant water transport can be implied from their distribution in tissues with high water conductance (Kaldenhoff et al. 1995, 1998; Yamada et al. 1995; Sarda et al. 1997; Barrieu et al. 1998; Grote et al. 1998) and their increased expression in expanding/elongating cells during development (Ludevid et al. 1992; Yamada et al. 1997; Fukuhara et al. 1999). Environmental stimuli, resulting in osmotic stress, have

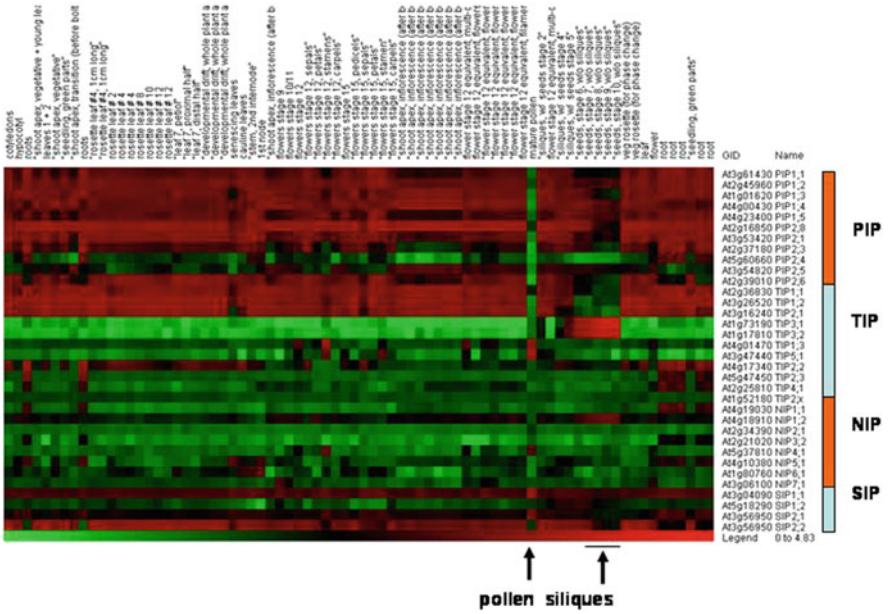

Fig. 2 Expression of the AQP gene family during developmentand in different tissues of *Arabidopsis thaliana* .Expression data for the AQP gene family have been taken from the AtGenExpress dataset (Schmid et al. 2005). Values are represented as ranging from no expression (*blue*) to low expression (*white*), and strong expression (*red*) over >4 orders of magnitude (Ma 2006). The *last row* shows the color scheme for the log2 expression value, with *deep blue* as 2 and *deep red* as 14. ATGE_X identify the tissues (see the Table 2)

also been shown to regulate AQP expression. Transcripts and promotor-reporter gene expression, for both putative tonoplast and PM AQP, have been shown to be either induced or downregulated by drought, dehydration, dessication, and/or salinity stress (Daniels et al. 1994; Fray et al. 1994; Yamada et al. 1995; Mariaux et al. 1998; Barrieu et al. 1998; Pih et al. 1999; Kirch et al. 2000; Aharon et al. 2003; Lian et al. 2004; Vera-Estrella et al. 2004; Yu et al. 2005; Boursiac et al. 2005; Zhu et al. 2005; Guo et al. 2006; Prak et al. 2008). Direct evidence of AQP's involvement in plant water transport comes from studies in which AQP activity has been demonstrated by the evidence that heterologous expression of several plant AQP mRNAs in *Xenopus laveis* oocytes resulted in increased osmotic water permeability (P_f; Maurel et al. 1993, Daniels et al. 1994; Yamada et al. 1995; Johansson et al. 1998). Further, protoplasts form *A. thaliana* that constitutively expressed an antisense construct of the *H2* MIP showed a reduced rate of protoplast osmotic swelling that also correlated with a reduction in H2 protein levels (Kaldenhoff et al. 1995). Finally, reconstitution of the peribacteroid membrane-specific MIP NOD26 in liposomes resulted in a higher P_f than that measured in control liposomes (Dean et al. 1999).

Table 2 Developmental series of gene expression for the AQP family in *Arabidopsis thaliana* (Col-0)

#	Sample ID	Experiment description	Genotype	Tissue	Age	Photoperiod	Substrate
1	ATGE_1	Development baseline	Wt	Cotyledons	7 days	Continuous light	Soil
2	ATGE_2	Development baseline	Wt	Hypocotyls	7 days	Continuous light	Soil
3	ATGE_3	Development baseline	Wt	Roots	7 days	Continuous light	Soil
4	ATGE_4	Development baseline	Wt	Shoot apex, vegetative + young leaves	7 days	Continuous light	Soil
5	ATGE_5	Development baseline	Wt	Leaves 1 + 2	7 days	Continuous light	Soil
6	ATGE_6	Development baseline	Wt	Shoot apex, vegetative	7 days	Continuous light	Soil
7	ATGE_7	Development baseline	Wt	Seedling, green parts	7 days	Continuous light	Soil
8	ATGE_8	Development baseline	Wt	Shoot apex, transition (before bolting)	14 days	Continuous light	Soil
9	ATGE_9	Development baseline	Wt	Roots	17 days	Continuous light	Soil
10	ATGE_10	Development baseline	Wt	Rosette leaf # 4, 1 cm long	10 days	Continuous light	Soil
11	ATGE_11	Development baseline	glf-T	Rosette leaf # 4, 1 cm long	10 days	Continuous light	Soil
12	ATGE_12	Development baseline	Wt	Rosette leaf # 2	17 days	Continuous light	Soil
13	ATGE_13	Development baseline	Wt	Rosette leaf # 4	17 days	Continuous light	Soil
14	ATGE_14	Development baseline	Wt	Rosette leaf # 6	17 days	Continuous light	Soil
15	ATGE_15	Development baseline	Wt	Rosette leaf # 8	17 days	Continuous light	Soil
16	ATGE_16	Development baseline	Wt	Rosette leaf # 10	17 days	Continuous light	Soil
17	ATGE_17	Development baseline	Wt	Rosette leaf # 12	17 days	Continuous light	Soil
18	ATGE_18	Development baseline	glf-T	Rosette leaf # 12	17 days	Continuous light	Soil
19	ATGE_19	Development baseline	Wt	Leaf 7, petiole	17 days	Continuous light	Soil
20	ATGE_20	Development baseline	Wt	Leaf 7, proximal half	17 days	Continuous light	Soil
21	ATGE_21	Development baseline	Wt	Leaf 7, distal half	17 days	Continuous light	Soil
22	ATGE_22	Development baseline	Wt	Developmental drift, entire rosette after transition to flowering, but before bolting	21 days	Continuous light	Soil
23	ATGE_23	Development baseline	Wt	As above	21 days	Continuous light	Soil
24	ATGE_24	Development baseline	Wt	As above	22 days	Continuous light	Soil
25	ATGE_25	Development baseline	Wt	Senescing leaves	23 days	Continuous light	Soil
26	ATGE_26	Development baseline	Wt	Cauline leaves	35 days	Continuous light	Soil
27	ATGE_27	Development baseline	Wt	Stem, 2nd internode	21+ days	Continuous light	Soil

(*continued*)

Table 2 (continued)

#	Sample ID	Experiment description	Genotype	Tissue	Age	Photoperiod	Substrate
28	ATGE_28	Development baseline	Wt	1st internode	21+ days	Continuous light	Soil
29	ATGE_29	Development baseline	Wt	Shoot apex, inflorescence (after bolting)	21+ days	Continuous light	Soil
30	ATGE_31	Development baseline	Wt	Flowers stage 9	21+ days	Continuous light	Soil
31	ATGE_32	Development baseline	Wt	Flowers stage 10/11	21+ days	Continuous light	Soil
32	ATGE_33	Development baseline	Wt	Flowers stage 12	21+ days	Continuous light	Soil
33	ATGE_34	Development baseline	Wt	Flowers stage 12, sepals	21+ days	Continuous light	Soil
34	ATGE_35	Development baseline	Wt	Flowers stage 12, petals	21+ days	Continuous light	Soil
35	ATGE_36	Development baseline	Wt	Flowers stage 12, stamens	21+ days	Continuous light	Soil
36	ATGE_37	Development baseline	Wt	Flowers stage 12, carpels	21+ days	Continuous light	Soil
37	ATGE_39	Development baseline	Wt	Flowers stage 15	21+ days	Continuous light	Soil
38	ATGE_40	Development baseline	Wt	Flowers stage 15, pedicels	21+ days	Continuous light	Soil
39	ATGE_41	Development baseline	Wt	Flowers stage 15, sepals	21+ days	Continuous light	Soil
40	ATGE_42	Development baseline	Wt	Flowers stage 15, petals	21+ days	Continuous light	Soil
41	ATGE_43	Development baseline	Wt	Flowers stage 15, stamen	21+ days	Continuous light	Soil
42	ATGE_45	Development baseline	Wt	Flowers stage 15, carpels	21+ days	Continuous light	soil
43	ATGE_46	Development baseline	*clv*3-7	Shoot apex, inflorescence (after boiling)	21+ days	Continuous light	Soil
44	ATGE_47	Development baseline	*Lfy*-12	Shoot apex, inflorescence (after bolting)	21+ days	Continuous light	Soil
45	ATGE_48	Development baseline	*ap*1-15	Shoot apex, inflorescence (after bolting)	21+ days	Continuous light	Soil
46	ATGE_49	Development baseline	*ap*2-6	Shoot apex, inflorescence (after bolting)	21+ days	Continuous light	Soil
47	ATGE_50	Development baseline	*ap*3-6	Shoot apex, inflorescence (after bolting)	21+ days	Continuous light	Soil
48	ATGE_51	Development baseline	*ag*-12	Shoot apex, inflorescence (after bolting)	21+ days	Continuous light	Soil
49	ATGE_52	Development baseline	*ufo*-1	Shoot apex, inflorescence (after bolting)	21+ days	Continuous light	Soil

50	ATGE_53	Development baseline	*Clv3-7*	Shoot apex, inflorescence (after bolting); multi-carpel gynoeceum; enlarged meristem; increased organ number	21+ days	Continuous light	Soil
51	ATGE_54	Development baseline	*Lfy*-12	Flower stage 12; shoot characteristics; most organs lead-like	21+ days	Continuous light	Soil
52	ATGE_55	Development baseline	*ap*1-15	Flower stage 12; sepals replaces by leaf-like organs, petals mostly lacking, 2^{o} flowers	21+ days	Continuous light	Soil
53	ATGE_56	Development baseline	*ap*2-6	Flower stage 12; no sepals or petals	21+ days	Continuous light	Soil
54	ATGE_57	Development baseline	*ap*3-6	Flower stage 12; no petals or stamens	21+ days	Continuous light	Soil
55	ATGE_58	Development baseline	*ap*- 12	Flower stage 12; no stamens or carpels	21+ days	Continuous light	Soil
56	ATGE_59	Development baseline	*ufo*-1	Flower stage 12; filamentous organs in whorls two and three	21+ days	Continuous light	Soil
57	ATGE_73	Pollen	Wt	Mature pollen	6 wk	Continuous light	Soil
58	ATGE_76	Seed & silique development	Wt	Siliques, wf seeds stage 3; mid globular to early heart embryos	8 wk	Long day (16/8)	Soil
59	ATGE_77	Seed & silique development	Wt	Siliques, wf seeds stage 4; early to late heart embryos	8 wk	Long day (16/8)	Soil
60	ATGE_78	Seed & silique development	Wt	Siliques, wf seeds stage 5; late heart to mid torpedo embryos	8 wk	Long day (16/8)	Soil
61	ATGE_79	Seed & silique development	Wt	Seeds, stage 6, w/o siliques; mid to late torpedo embryos	8 wk	Long day (16/8)	Soil
62	ATGE_81	Seed & silique development	Wt	Seeds, stage 7, w/o siliques; late torpedo to early walking-stick embryos	8 wk	Long day (16/8)	Soil

(*continued*)

Table 2 (continued)

#	Sample ID	Experiment description	Genotype	Tissue	Age	Photoperiod	Substrate
63	ATGE_82	Seed & silique development	Wt	Seeds, stage 8, w/o siliques; walking-stick to early curled cotyledons embryos	8 wk	Long day (16/8)	Soil
64	ATGE_83	Seed & silique development	Wt	Seeds, stage 9, w/o siliques; curled cotyledons to early green cotyledons embryos	8 wk	Long day (16/8)	Soil
65	ATGE_84	Seed & silique development	Wt	Seeds, stage 10, w/o siliques; green cotyledons embryos	8 wk	Long day (16/8)	Soil
66	ATGE_87	Phase change	Wt	Vegetative rosette	7 days	Short day (10/14)	Soil
67	ATGE_89	Phase change	Wt	Vegetative rosette	14 days	Short day (10/14)	Soil
68	ATGE_90	Phase change	Wt	Vegetative rosette	21 days	Short day (10/14)	Soil
69	ATGE_91	Comparison with CAGE	Wt	Leaf	15 days	Long day (16/8)	1 MS agar, 1% sucrose
70	ATGE_92	Comparison with CAGE	Wt	Flower	8 days	Long day (16/8)	soil
71	ATGE_93	Comparison with CAGE	Wt	Root	8 days	Long day (16/8)	1 MS agar, 1% sucrose
72	ATGE_94	Development on MS agar	Wt	Root	8 days	Continuous light	1 MS agar
73	ATGE_95	Development on MS agar	Wt	Root	8 days	Continuous light	1 MS agar, 1% sucrose
74	ATGE_96	Development on MS agar	Wt	Seedling, green parts	21 days	Continuous light	1 MS agar
75	ATGE_97	Development on MS agar	Wt	Seedling, green parts	21 days	Continuous light	1 MS agar, 1% sucrose
76	ATGE_98	Development on MS agar	Wt	Root	21 days	Continuous light	1 MS agar
77	ATGE_99	Development on MS agar	Wt	Root	21 days	Continuous light	1 MS agar, 1% sucrose
78	ATGE_100	Development on MS agar	Wt	Seedling, green parts	21 days	Continuous light	1 MS agar
79	ATGE_101	Development on MS agar	Wt	Seedling, green parts	21 days	Continuous light	1 MS agar, 1% sucrose

The results are based on triplicate analysis and have been deposited in ATGenExpress. Numbers refer to numbers in Fig. 2
RNAs provided by MPI Tübingen (Schmid, Lohmann; development baseline), Uni. Leicester (Twell; pollen). MIP Cologne (Weisshaar; seed and silique development), UNi. Pennsylvania (Poething; phase change), CAGE sonsortium (Kuiper; common reference samples) and Univ. Utrecht (Scheres; development on MS agar). Probes prepared and hybridized by Markus Schnid, Jan Lohmann and Monika Demar at the MPI Tübingen (Dept. Weigel)

4.1 The Role of PIPs in Seed Germination

In order to germinate, seeds require optimal moisture, acceptable temperature, and oxygen. In general, seed germination follows three phases. Imbibition of water occurs during phase I and is followed by the activation or the synthesis of enzymes. These enzymes break down storage material into simpler compounds and generate the energy required for seedling growth and development. Towards the end of this phase, cell elongation and radicle emergence occur. During phase II, the shoot (plumule) and root (radicle) axes grow as cells elongate and divide to establish the autotrophic seedling (phase III). The function of AQPs in water exchange during seedling germination and establishment has been studied in Arabidopsis, rice, canola, pea, spruce, and tobacco. Early evidence of involvement of AQPs in seed germination came from studies showing that mercury, a general blocker of aquaporins delayed seed germination and induced a delay in maternal seed coat (testa) rupture and radicle emergence (Toole et al. 1956). Mercury's influence is, however, not connected to seed imbibition, suggesting that AQPs are not required for phase I functions, but are instead associated with a delayed initiation of phase III, the uptake of water accompanying expansion and growth of the embryo. However, it is to be remembered that not all AQPs are sensitive to mercury, and mercury acts on other targets, particularly those containing thiols, in the plant.

A number of subsequent studies indicated a possible involvement of AQPs during the first phase of seed germination. For instance, pea (*Pisum sativum*) PIP1;1 is expressed in mature dry seeds and, during germination, plays a key role in water absorption during imbibition (Schuurmans et al. 2003). Macroarrays using *A. thaliana* AQP gene-specific tags and antibodies raised against PIP AQPs revealed low expression of all 13 PM aquaporin (PIP) isoforms in dry and germinating seeds (Vander Willigen et al. 2006). In contrast, expression of AQPs of the *TIP1*, *TIP2*, and *PIP* subgroups was induced in *A. thaliana* during seedling establishment. Transcripts of the genes encoding canola (*B. napus*) PIP1 and γ-TIP2 were also found to be present earlier during germination of primed seeds compared to nonprimed seed, and priming induced the expression of *PIP1* but had no effect on γ-*TIP2* expression. These results suggested that PIP1 functions in water transport required for enzymatic metabolism of storage nutrients at early stages of seed germination, whereas γ-*TIP2* expression is related to cell growth associated with cell elongation during radicle protrusion. In *Ricinus communis*, a cell pressure probe and in situ hybridization were used to show that *PIP2;1* expression correlated with the increased hydraulic conductivity of cortical cells in elongated etiolated hypocotyls compared to nonelongated ones (Eisenbarth and Weig 2005). Taken together, the data suggest that individual AQPs play important roles in discrete processes during seedling germination and establishment.

4.2 PIPs in Elongation Growth and Differentiation

AQP function in cell growth, differentiation, and elongation is suggested by the abundance of AQPs in growing tissues. Studies of *PIP* gene expression implicate AQPs in the elongation of rice internodes, *PIP2* from *Solanum chacoense* is predominantly expressed in the pistil and anther tissues of flowers and in developing fruits (O'Brien et al. 2002), and a strong correlation was found between the maximal rate of fruit growth and the expression of TIP genes in pea (O'Brien et al. 2002; Schuurmans et al. 2003). Similarly, the gene encoding PIP2;1, a water-permeable PM AQP in rose, is highly expressed in expanding petals. Expression of *PIP2;1* was reduced when the petals' expansion was inhibited by ethylene treatment of the flowers, and silencing of *PIP2;1* in the flowers resulted in floral phenotypes that could be phenocopied by ethylene treatment (Shao et al. 2008). In roots, *SIP1* and *SIP2* are expressed in differentiated and elongating regions mainly in the pericycle, cambium, metaxylem, protoxylem, and xylem parenchyma cells, suggesting a similar role of AQPs in the expansion of root cells (Ishikawa et al. 2005). Finally, low and largely invariant transcript abundance of *PIP1a* and *PIP1b* at different developmental stages in *Z. mays* suggests a function of these AQPs in addition to leaf expansion (Chaumont et al. 2000).

During sexual reproduction in higher plants, water movement between cells and tissues has been measured during dehiscence of anthers and the hydration of the pollen grain immediately after its deposition on the stigma. At least 15 AQPs are expressed in reproductive organs, indicating that water flow is important during reproduction. In, tobacco, *NtPIP1;1* and *PIP2;1* are differentially expressed in reproductive organs, *PIP1;1* is highly expressed in the stigma, and both AQPs are expressed in the anther (Sakurai et al. 2005). *Nt*PIP1;2 protein abundance is modulated during anther development, and *Nt*PIP2 RNA interference resulted in slower anther dehydration, and later dehiscence compared with control plants(Bots et al. 2005a, b). This result clearly showed that AQPs of the PIP2 class are required for efficient anther dehydration prior to dehiscence (Bots et al. 2005a, b). Evidence from studies of PIP2a from *S. chacoense*, which is highly expressed in pistil and anther tissues, indicate that PIP2a is developmentally regulated during anthesis (O'Brien et al. 2002), and *OsPIP1;1* and *OsPIP4;1* are highly expressed in rice anthers (Sakurai et al. 2005).

In flowers, SIPs are expressed in the stigma of the carpel and pollen, suggesting a role in seed maturation and fertilization. After expression of ArabidopsisPIP2;1 and *At*PIP2;2 in lilly pollen, signals were observed in the PM of pollen grains but not in pollen tubes, suggesting that the regulation of these two AQPs are important to prevent pollen tube bursting during pollen germination (Sommer et al. 2008). Strong evidence exists for the control of cell expansion by AQPs; for instance, Siefritz et al. (2004) reported diurnal changes of aquaporins during epinastic leaf movements and growth in tobacco. From the studies, it is clear that AQPs have a major role during cell and organ growth, differentiation, elongation, and reproduction.

4.3 A Role for PIPs in Programmed Cell Death and Plant Microbe Interactions

Cells activate multiple mechanisms when their integrity becomes compromised to eliminate damages that threaten survival of the organism. In plants, cell death is a part of many processes, ranging from developmental programs that lead to the generation of xylem elements (Fukuda 2000) to the hypersensitive response (HR) by which pathogen attack and wounding injuries are countered (Greenberg 1997). These processes activate localized mechanisms that then form protective barriers via cell wall lignification, cross-linking of cell wall proteins, or other modifications of the extracellular matrix (Bostock and Stermer 1989; Lamb and Dixon 1997). These mechanisms are thought to limit pathogen access while activating lipid peroxidative chain reactions that then signal neighboring cells. Accompanying plant defense mechanisms are dynamic secretory and relocalization mechanisms, which involve movement of proteins and cell wall materials through subcellular compartments uniquely marked by some AQPs (Kirch et al. 2000; Barkla et al. 1999; Vera-Estrella et al. 2004) Although AQP function in each compartment is still unknown, a number of reports have implicated them in maintenance of the homeostasis of each compartment (Fortin et al. 1987; Morrison et al. 1988; Daniels et al. 1994; Kammerloher et al. 1994; Robinson et al. 1996; Fleurat-Lessard et al. 1997; Chaumont et al. 1998, 2000; Barkla et al. 1999; Barrieu et al. 1999; Cutler et al. 2000; Kirch et al. 2000; Reisen et al. 2003; Vera-Estrella et al. 2004; Ma et al. 2004; Ishikawa et al. 2005; Boursiac et al. 2005; Guo et al. 2006; Prak et al. 2008). Production of (ROS) and reactive oxygen species is thought to induce cell death; a correlation between ROS production, oxidative stress, and *PIP2;1* has been observed in *A. thaliana*. An increase in the amount of PIP1 and PIP2 family membranes as well as TIP1;1 and TIP2;1 has been associated with cell death in cell suspensions from *A. thaliana*. These increments were also accompanied by a gradual decrease of*t*SIP1 and*t*SIP2;1 (Takano et al. 2006) and an increase of several members of PIP2 and TIP1 subfamilies (Kobae et al. 2006) The possibility of increased damage to cells by H_2O_2 resulting from overexpression of AQPs may indicate an important role of AQPs in the detoxification of ROS. This may not mean that H_2O_2 can diffuse through AQPs but may result from altered cellular water status and subsequent increases in the accumulation of ROS.

One of the first responses observed during plant microbial interactions was the change in the PM electrical potential (e.g., Vera-Estrella et al. 1994). Such a change and altered turgor would indicate that AQP-mediated changes in water fluxes across the PM may be one of these initial responses. Evidence in support of an AQP involvement in plant microbial interactions comes from transcript profiling of cotton hypocotyl and root tissues infected with the fungal pathogen *Fusarium oxysporum* f. sp. Vasinfectum (Dowd et al. 2004). Apart from increased expression of defense-related genes, a decline of aquaporin gene expression (*MipH*, Δ-*TIP*, *MipC* γ-*TIP* and *SIMIP* (PM intrinsic protein 3) was observed (Dowd et al. 2004). Further studies are needed to determine if these AQPs have a specific role in the

plant response to pathogen attack, or whether downregulation of these AQPs is induced by damage to cells.

During the foundation of symbiosis between soybean roots and nitrogen-fixing rhizobia, expression of plant nodule-specific genes, including the gene encoding protein Nodulin 26, the major symbiosome, is observed. In functional tests in the heterologous *Xenopus* oocyte expression system, Nodulin 26 was shown to be permeable to water, glycerol, and ammonia (Dean et al. 1999; Rivers et al. 1997; Tyerman et al. 2002; Niemietz and Tyerman 2000). These data suggest a role of this AQP in the physiology of symbiosis, although the precise role of Nod26 in ammonia permeation has yet to be demonstrated. PIP and TIP proteins are abundant in both the PM and tonoplast of the inner cortical cells (IC-cells) of *Glycine max* root nodules (Fleurat-Lessard et al. 2005). This distribution is consistent with their putative role of water flux facilitation associated with the regulation of nodule conductance to O_2 diffusion and the subsequent ATP-dependent nitrogenase activity. In the endodermis, these aquaporins might also be involved in nutrient transport between the infected zone and vascular traces (Fleurat-Lessard et al. 2005).

Arbuscular mycorrhizal (AM) fungi are important components of ecosystems in the symbiotic interactions with most vascular plants (Harley and Smith 1983). The benefits of mycorrhizal symbionts to plants can be attributed to increased mobilization and uptake of nutrients, most particularly phosphorus. Increased uptake is based in part on the increased surface area provided by the fungal hyphae, increased solubilization of nutrients, a modification of the root environment, and increased deposition into the plant body (Bolan 1991). Mycorrhizae have also been shown to provide water to their hosts under drought conditions (Marulanda et al. 2003; Khalvati et al. 2005). The expression of AQP genes in *G. max* and *Lactuca sativa* roots was found to be altered in response to the presence of the arbuscular mycorrhizal fungus *Glomus mosseae*, which apparently provided a mechanism that enhanced host plant tolerance to water deficit (Porcel et al. 2005). Also, inoculation with a mixture of AM fungi decreased the expression of one *PIP* gene in the roots of tomato plants grown under saline conditions (Ouziad et al. 2005). In bean (*Phaseolus vulgaris*), mycorrhizal formation reduced the expression of *PIP1;1* but increased the expression of the PIP1;2. In *Medicago truncatula*, several PM proteins were differentially regulated after inoculation with *Glomus intraradices* (Valot et al. 2005). Presence of the ectomycorrhizal fungus *Amanita muscaria* both down- and upregulated the expression of several *PIP* genes in *Populus tremula* × *tremuloides* roots under optimal conditions (Marjanovic et al. 2005a). Four out of seven genes studied were preferentially expressed in roots. Mycorrhiza formation resulted in increased transcript levels for three of these genes, two of which were the most highly expressed root aquaporins. Finally, measurements of the hydraulic conductance of intact root systems revealed increased water transport capacity of poplar roots growing in association with mycorrhizal fungi. While we focus on AQP expression and activity here, ectomycorrhizal symbioses include other changes of plants, including properties of the PM and the apoplast contributing to changes in root hydraulic conductance (Marjanovic et al. 2005a, b).

4.4 Roles of PIPs in Adaptation to Environmental Challenges

Salinity and osmotic stress are major factors that affect plant growth and development. For plants to be able to maintain growth under high salt conditions, the expression of a number of proteins either increase or decline to allow for maintenance of low cytosolic Na^+ levels and sequestration of Na^+ to vacuoles. In the ice plant, regulation of AQP protein amount by salinity stress was observed using peptide-specific antibodies. During salt stress levels of *Mc*PIP2;1 in the root, PM, ER, and tonoplast fractions increased, while *Mc*PIP1;4 in prevacuolar compartments showed no change (Kirch et al. 2000; Vera-Estrella et al. 2000; Vera-Estrella et al. 2004). Similar results were found in a microarray analysis of AQPs in the roots of *A. thaliana* treated with NaCl (Boursiac et al. 2005; Prak et al. 2008). In these studies, salt stress induced a transcriptional downregulation of PIPs and TIPs. Interestingly, the authors also observed subcellular relocalization of PIP1 and PIP2 proteins to intravesicular invaginations within hours after salt treatment (Boursiac et al. 2005). AtPIP2;1 relocalization was due to phosphorylation at serine 283 (Prak et al. 2008).

Zhu et al. (2005) recently studied the response of the entire set of maize TIPs and PIPs from roots to salinity (100–200 mM NaCl). The expression profiles varied among the genes with responses observed within 2 h of stress. Transgenic *A. thaliana* plants overexpressing rice *Os*PIP1;1 or *Os*PIP2;2 developed enhanced tolerance to 100 mM NaCl and 200 mM mannitol but not to higher salt concentrations (150 mM; Guo et al. 2006). Barley *Hv*PIP1;6 showed increased expression in response to salinity (Fricke et al. 2006). Considering that this AQP is strongly expressed in the elongation zone of the root, it may suggest that this AQP plays a major role in maintaining residual growth during salinity stress. A microarray analysis of the 35 *AQP* genes of *A. thaliana* showed that most PIP transcripts are downregulated by osmotic stress in leaves, with the exception of *PIP1;4* and *PIP2;5* (upregulated) and *PIP2;6* and *SIP1;1* (constitutively expressed) (Alexandersson et al. 2005). Maize plants treated with 100 mM NaCl exhibited a loss of cell water content and accumulation of ABA in roots. After 2 h of salt treatment, the plants regained osmotic potential and showed increased expression of *PIP1;1*, *PIP1;5*, and *PIP2;4* in the root epidermis (Zhu et al. 2005). Application of 1 mM exogenous ABA induced a transient increase of *PIP1;2* and *PIP2;4*, suggesting that the induction of expression of these two genes may be regulated by ABA. Plants treated with 200 mM NaCl or 100 μM ABA showed decreased expression of *PIP*s and *TIP*s after 24 h of treatment. Similar results were found in a microarray analysis of the *AQP*s from *A. thaliana* roots treated with NaCl (Boursiac et al. 2005). Treatment of *A. thaliana* cell suspensions with NaCl induced PIP2;1, PIP2;2, and PIP2;3 15-fold in protein amount.

Competitive reverse transcription (RT)-PCR was used to distinguish and quantify transcripts for individual genes in barley during salt stress (Katsuhara et al. 2002). The most highly expressed *AQP* gene, *PIP2;1*, was one of the three

root-expressed transcripts. Salt stress (200 mM NaCl) downregulated PIP2;1 at the transcript and protein levels, but it had almost no effect on the expression of *PIP1;3* or *PIP1;5*. Approximately equal amounts of the transcripts of the three were detected in shoots, and salt stress enhanced the expression of *PIP2;1* but not of *PIP1;3*, or *PIP1;5*. The PIP2;1 protein was localized in the PM.

In rice and tobacco, the overexpression of *PIP1* genes increased the tolerance of the plants to drought (Aharon et al. 2003; Lian et al. 2004; Yu et al. 2005). This effect appeared to indicate a specific reaction to water deficit. Overexpressing *PIP1* did not change salt tolerance in tobacco plants (Aharon et al. 2003). However, overexpression of *PIP1;1* or *PIP2;2* aquaporins in *A. thaliana* was reported to enhance salt tolerance up to 100 mM NaCl (Guo et al. 2006).

In *A. thaliana*, transcriptional downregulation of *PIP* genes was observed upon drought stress with the exception of *PIP1;4* and *PIP2;5*, which were upregulated, while *PIP2;6* and *SIP2;3* were constitutively expressed, responses that could also be observed at the protein level. In short-time experiments of salt stress (<48 h), it has been found that almost all *PIP* genes decreased expression in roots (Martinez-Ballesta et al. 2003; Boursiac et al. 2005; Guo et al. 2006). However, the response depended on stress severity, because individual *PIPs* showed increased expression at low NaCl concentrations (Boursiac et al. 2005; Zhu et al. 2005). Kawasaki et al. (2001) found that the expression of rice root aquaporins that were initially downregulated during a moderate salt stress recovered to wild-type levels after 7 days.

Responsiveness to ABA distinguishes individual aquaporins, implying that the regulation of aquaporin expression involves both ABA-dependent and ABA-independent signaling pathways. A comprehensive expression profile of the 13 members of the *PIP* gene family of *A. thaliana* has provided a basis for allocation of stress-related biological function to each PIP (Jang et al. 2004). The upregulation of the AQP gene, *rice water channel 3* (*RWC3*), in upland rice fits well, with drought adaptations seen in upland rice. The physiological significance of *RWC3* upregulation was then explored with the overexpression of *RWC3* in transgenic lowland rice (*O. sativa* L. spp japonica cv. Zhonghua 11) controlled by a drought-inducible *SWPA2* promoter. Compared to wild type, the transgenic lowland rice exhibited higher root osmotic hydraulic conductivity (Lp), leaf water potential, and relative cumulative transpiration at the end of a 10 h PEG treatment. The results indicated that RWC3 functions in drought avoidance in rice (Lian et al. 2004).

AQPs have been implicated in chilling responses. Chilling of rice decreased expression of *PIP2;4* and *PIP2;5* and was followed by recovery after rewarming (Sakurai et al. 2005). The changes in expression during and after chilling were correlated with water uptake. However, in maize, the transcript levels of eight *AQPs* from mature roots after 24 h of chilling were not significantly upregulated, suggesting that an increase in root hydraulic conductance during chilling was not due to regulation of these aquaporins at the transcriptional level (Melkonian et al. 2004). All *PIP* genes in maize roots decreased in expression after 1 or 3 days at 5°C (Aroca et al. 2005). However, no differences in *PIP* expression or protein

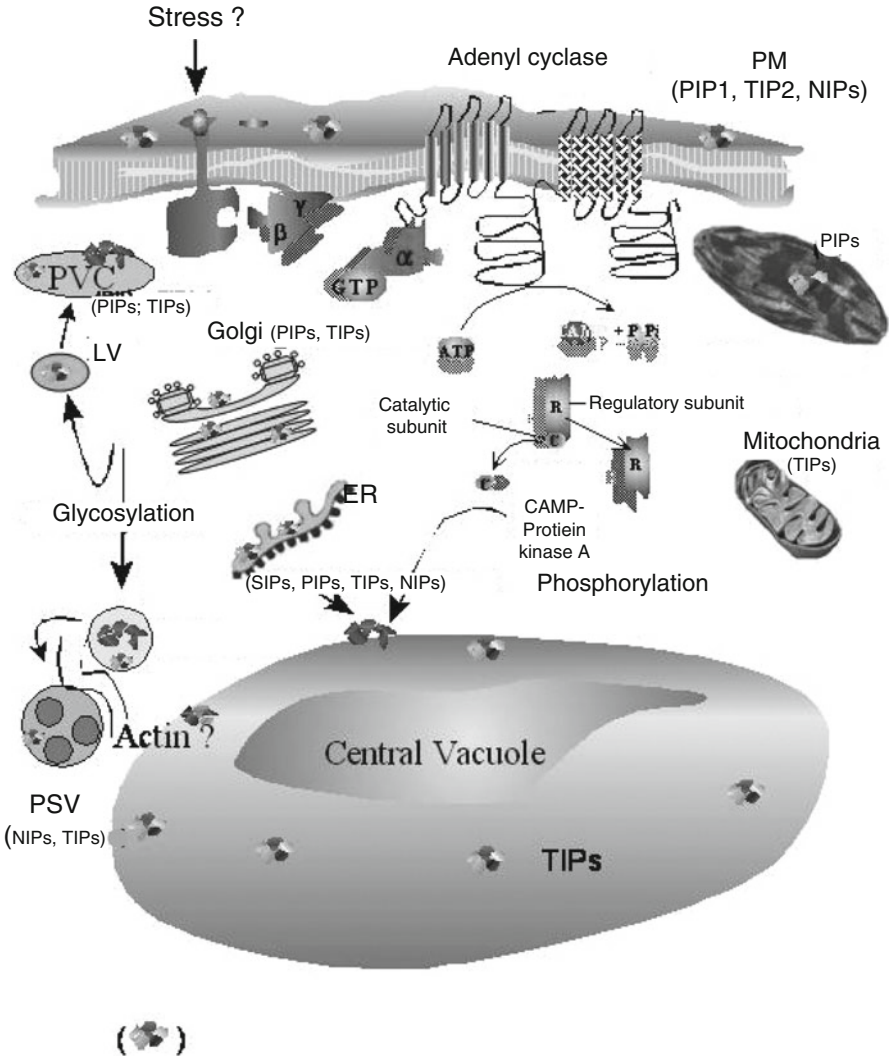

Fig. 3 Multiple subcellular localization of plant aquaporins. Localization of plant AQP types in diverse subcellular compartments. AQPs have been found in every subcellular compartment of plant cells, including: prevacuolar compartments (PVC), multivesicular bodies (MVB), lytic vacuoles; protein storage vacuoles (PSV), Central vacuole, Plasma membrane (PM), endoplasmic reticulum (ER), Golgi, chloroplast, and mitochondrial membranes. It seems that glycosylation and phosphorylation are important protein modifications that determine the subcellular distribution and localization of a particular AQP, and that these changes are triggered by environmental signals

abundance were found when comparing two maize varieties differing in cold tolerance (Aroca et al. 2005). Furthermore, in rice roots, all *PIP* genes decreased in expression after cold exposure, except for *PIP1;3* (Sakurai et al. 2005). However, no correlation between decreases in *PIP* gene expression and cold

tolerance could be observed (Aroca et al. 2005). Quantitative real-time reverse transcription-PCR analyses revealed that among 13 *PIP* genes analyzed, only *PIP*2;5 was upregulated by cold treatment, while most other *PIP* genes were downregulated (Jang et al. 2004).

An interesting fact is that the amounts of PIP1 sub-family proteins did not change during salt stress (Kobae et al. 2006). In transgenic plants overexpressing either *Os*PIP1 (*Os*PIP1-1) or *Os*PIP2 (*Os*PIP2-2) in wild-type, Arabidopsis showed enhanced tolerance to salt (100 mM of NaCl) and drought (200 mM of mannitol) but not to salt treatment at higher concentration (150 mM of NaCl) (Guo et al. 2006). Further research will be required with particular focus on mechanisms that control the discriminatory reaction of plant AQPs to different environmental stress conditions. Also required will be a focus on what the regulatory circuits reveal about physiological functions and cargo selectivity, other than water, of AQPs in a particular response.

5 Perspectives

Although research on PIP aquaporin structure and function has advanced rapidly, major unresolved issues remain. Little is known about the intramolecular dynamics of the diverse aquaporin proteins. It is not known whether aquaporins undergo conformational changes or gating, although indications exist for both control processes. Improved resolution in electron crystallographic analysis of structure and high-resolution X-ray crystallography is needed, particularly of the aquaglyceroporins and aquaporins that are capable of transporting other solutes. Comparative studies of structure and substrate specificity are also required. The possibility that aquaporins facilitate the transport of gases and (many) other solutes requires further investigation because of the physiological relevance, as does the possibility that intrinsic aquaporin function may be subject to specific biochemical regulation. More precise information about the specific functions of plant aquaporins in intracellular compartments and their possible functions outside of bulk small molecule movement is also required (Fig. 3). However, the dynamic nature of *AQP* expression and posttranscriptional regulation of AQP protein modification, interaction, and abundance make the AQPs prime candidates to function as integrators of metabolic signaling. Finally, investigations are needed aimed at identifying functionally significant interactions between aquaporins and other membrane and cytoplasmic proteins.

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The Role of Plasma Membrane Nitrogen Transporters in Nitrogen Acquisition and Utilization

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Abstract Efficient nitrogen acquisition and allocation are important to enable plants to successfully compete for limited and fluctuating nitrogen sources in the soil and sustain vigorous growth. Over the past two decades, several types of nitrogen transporters, such as nitrate transporters, ammonium transporters, and amino acid transporters (Frommer et al. Proc Natl Acad Sci USA 90:5944–5948, 1993; Tsay et al. Cell 72:705–713, 1993; Ninnemann et al. EMBO J 13:3464–3471, 1994), have been identified using genetic approaches or functional complementation cloning in yeast. In this review, we focus on the physiological functions and influence of plasma membrane nitrate, ammonium, and amino acid transporters as revealed by the powerful molecular genetic tools available for investigating Arabidopsis.

1 Introduction

Nitrate and ammonium are two major nitrogen sources for nonlegume plants. For assimilation, nitrate is converted into nitrite by nitrate reductase in cytosol, and then transported into plastids/chloroplasts, and converted into ammonium by nitrite reductase. Nitrate taken up by roots can be assimilated immediately, stored in vacuoles, transported to shoots to be assimilated, or stored in the aboveground parts. To be distributed into these different tissues, nitrate must be transported across the plasma membrane of several different types of cells.

There are several sources of ammonium in higher plants: (1) acquisition from soil solution, (2) generation from nitrate assimilation, (3) generation by photorespiration, and (4) export from a N^2-fxing bacteroid to the plant. Despite being

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essential for their survival, excess ammonium is toxic to plants. Therefore, ammonium acquisition needs to be stringently regulated, and ammonium taken into plant cells needs to be assimilated efficiently by glutamine synthetase/glutamine-oxoglutarate aminotransferase (GS/GOGAT) or sequestered into vacuoles. Little is known about how ammonium is translocated among different tissues, and most molecular studies of plasma membrane ammonium transport to-date have focused on the root acquisition step.

Amino acids are considered the principle nitrogen (N) forms for long distance transport. Nitrate or ammonium acquired from soil can be assimilated and converted to amino acids in roots, and these amino acids can be transported to shoots via xylem. In addition, newly synthesized amino acid in source leaves, or amino acids derived from protein degradation in senescing leaves can be allocated to sink tissues (developing leaves, flowers, and seeds) via phloem. In addition, organic nitrogen can also be transported as ureides and peptides. As ureide and peptide transporters have been reviewed comprehensively in recent articles (Rentsch et al. 2007; Tsay et al. 2007), we will not discuss them further here. This review will focus on the *in plant* function of the plasma membrane transporters of nitrate, ammonium, and amino acids.

2 Nitrate Transporters

Two types of plasma membrane *nitrate transporters*, NRT1 and NRT2, have been identified in higher plants. Both of them are electrogenic and proton-coupled symporters. In Arabidopsis, there are 53 NRT1 genes and 7 NRT2 genes. Not all of the NRT1 and NRT2 transporters are located in the plasma membrane. For example, AtNRT2.7 was located in the tonoplast (Chopin et al. 2007).

The first member of the NRT1 family, CHL1 (NRT1.1), was isolated by a genetic approach, using a T-DNA-tagged chlorate resistant mutant (Tsay et al. 1993). Chlorate, an herbicide, and nitrate analog can be taken up by nitrate uptake systems and then converted by nitrate reductase into chlorite, which is toxic to plants. Mutants resistant to chlorate treatment are defective in nitrate uptake or nitrate reduction. Nitrate uptake mutant *chl1* was isolated and characterized about 40 years ago (Oostindier-Braaksma and Feenstra 1973), while the CHL1 gene was identified about 20 years ago when molecular genetic tools became readily available for Arabidopsis (Tsay et al. 1993). All of the CHL1 homologs identified in bacteria, yeast, and animals are di- and tripeptide transporters (Tsay et al. 2007); further, some CHL1 homologs in higher plants are also peptide transporters (Rentsch et al. 1995; Song et al. 1996; Komarova et al. 2008). Therefore, all together, such nitrate transporters and peptide transporters are referred to as the NRT1(PTR) family. In higher plants, CHL1, expressed in roots, is responsible for nitrogen acquisition by means of nitrate uptake (Tsay et al. 1993). Similarly, in humans and rat, pepT1, a peptide transporter from the NRT family expressed in intestine, is also responsible for nitrogen acquisition by means of peptide absorption

(Liang et al. 1995). Therefore, despite their distinct substrate specificity, plant CHL1 and animal pepT1 have an identical function: nitrogen acquisition.

NRT1 and NRT2 share no sequence similarity, but both were predicted to have 12 transmembrane (TM) domains. Similar to CHL1, the first NRT2 transporter (*crnA* from *Aspergillus*) was also identified by chlorate selection (Unkles et al. 1991). When expressed in Xenopus oocytes, CRNA alone displays high-affinity nitrate transport with a K_m of about 20 μM (Zhou et al. 2000). However, in *Chlamydomonas* and plants, generally an additional component NAR2 (NRT3) is required for the high-affinity nitrate transport of NRT2 (Quesada et al. 1994; Tong et al. 2005; Orsel et al. 2006). The only exception is Arabidopsis NRT2.7. AtNRT2.7 alone injected into Xenopus oocytes without coinjection of any NAR2 revealed nitrate transport activity, but the nitrate concentration tested was 5 mM, in the low-affinity range(Chopin et al. 2007). In Arabidopsis, there are seven *NRT2* genes and two *NAR2* (*NRT3*) genes. Some studies have suggested that NAR2, a two-transmembrane-domain protein, might be involved in targeting NRT2 to the plasma membrane (Quesada et al. 1994) or preventing NRT2 degradation (Wirth et al. 2007).

2.1 Nitrate Uptake

Under many conditions, nitrate is the predominant form of nitrogen in the soil. However, negatively charged nitrate is not well preserved by negatively charged soil particles and can be easily flushed out after rain fall. Therefore, nitrate concentrations in soil can vary by four orders of magnitude. To survive under such fluctuating conditions, plants have evolved two nitrate uptake systems, a high-affinity system and a low-affinity system. The K_m values for the high-affinity uptake systems detected in various plant species are in the range 7–50 μM, and the K_m values for the low-affinity uptake systems are in the range of 1–10 mM.

Physiological studies have shown that plants grown on medium without nitrate still exhibit some basal level of nitrate transport activity. This activity has been referred to as constitutive nitrate uptake. When these plants were exposed to nitrate, nitrate uptake activities increased several-fold, which has been referred to as inducible uptake. Therefore, nitrate uptake can reasonably be classified into four systems: the constitutive high-affinity system (cHATS), the nitrate-inducible high-affinity system (iHATS), the constitutive low-affinity system (cLATS), and the nitrate-inducible low-affinity system (iLATS) (see Fig. 1).

Molecular genetic studies revealed that in Arabidopsis, there are at least four transporter genes, *NRT1.1* (*CHL1*), *NRT1.2*, *NRT2.1*, and *NRT2.2*, involved in nitrate uptake (Huang et al. 1996, 1999; Cerezo et al. 2001; Filleur et al. 2001; Little et al. 2005). But there is no simple one-to-one correlation between the nitrate uptake system and the transporters involved. For example, Arabidopsis NRT2.1, NRT2.2, and NRT1.1 (CHL1) are all involved in iHATS (Wang et al. 1998;

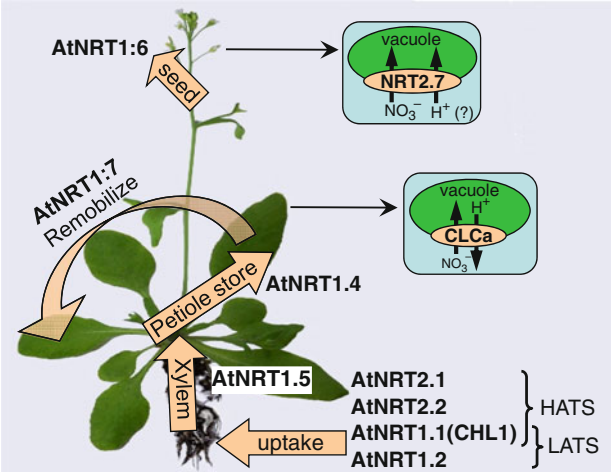

Fig. 1 Physiological roles of Arabidopsis nitrate transporters in nitrate uptake and allocation. HATS: high-affinity nitrate uptake system. LATS: low-affinity nitrate uptake system. Using phosphorylation switch at threonine 101 residue, AtNRT1.1 functions as a dual-affinity nitrate transporter involved both in HATS and LATS

Liu et al. 1999; Cerezo et al. 2001; Filleur et al. 2001; Little et al. 2005; Li et al. 2007). Expression of *NRT1.1* (*CHL1*) and *NRT2.1* is coordinately regulated by nitrate and circadian rhythms but is differentially regulated by developmental stages of roots, N starvation, and N metabolites (Lejay et al. 1999; Guo et al. 2001; Nazoa et al. 2003). Therefore, the relative contributions of NRT2.1, NRT2.2, and NRT1.1 (CHL1) in high-affinity nitrate uptake depend on external and internal N status, as well as the ages of plants.

In contrast to all the nitrate transporters in NRT1 family characterized to-date, which are low-affinity nitrate transporters, AtNRT1.1 is a dual-affinity nitrate transporter with a K_m of about 50 μm during the high-affinity phase and 5 mM during the low affinity phase (Wang et al. 1998; Liu et al. 1999). The two action modes of CHL1 are regulated by phosphorylation status of the threonine residue 101 (Liu and Tsay 2003). When T101 is phosphorylated, CHL1 functions as a high-affinity nitrate transporter, and when dephosphorylated, it functions as a low-affinity transporter. A recent study showed that in addition to being a dual-affinity nitrate transporter, CHL1 also functions as a nitrate sensor regulating nitrate-induced transcriptional response (Ho et al. 2009). Phosphorylation status of T101 is regulated by external nitrate concentration and modulates transcriptional response levels of nitrate response genes. CIPK23 is responsible for T101 phosphorylation when exposed to low concentration of nitrate, and it represses the transcriptional response at low level. These studies indicate that plants use nitrate transporters for acquisition, to sense the soil nitrate concentration changes, and to regulate nitrate signaling. In addition to transcriptional responses, CHL1 was also shown to be involved in nitrate-regulated stomata opening (Guo et al. 2003),

nascent organ development (Guo et al. 2001), root architecture (Remans et al. 2006), and seed dormancy (Alboresi et al. 2005).

In contrast to CHL1 (NRT1.1), NRT1.2 is a pure low-affinity nitrate transporter with a K_m value of about 6 mM. Different from all the other three transporters involved in nitrate uptake whose expression is upregulated by nitrate, NRT1.2 is a constitutively expressed gene (Huang et al. 1999). Indeed, study of nrt1.2 mutant showed that it is involved in constitutive low-affinity nitrate uptake. However, in addition to constitutively expressed nitrate transporters such as NRT1.2, nitrate-inducible nitrate transporters such as NRT1.1 (CHL1) and NRT2.1 also contribute to constitutive nitrate uptake activity. For example, consistent with the sustainable amount of *CHL1* and *NRT2.1* transcripts detected in plants grown in ammonium, cHATS is reduced in *chl1* (Liu et al. 1999) and *nrt2.1* (Li et al. 2007).

Net nitrate uptake actually results from the balance between active influx and passive efflux. The effluence of efflux to net nitrate uptake could be high under stressed conditions, for example, under mechanical shock or medium acidification. Using a biochemical approach, the transporter NAXT1 (At3g45650), which is expressed in root cortex, was found to be responsible for nitrate efflux upon acid load (Segonzac et al. 2007). NAXT1 is one of the seven tandem-linked NRT1 genes (At3g45650, At3g45660, At3g45680, At3g45690, At3g45700, At3g45710, and At3g45720) (Tsay et al. 2007). This efflux activity is not restricted to this cluster however. Another NRT1 transporter, NRT1.5, was also found to be able to mediate nitrate efflux (Lin et al. 2008). The regulation and structure requirement of NRT1 transporters for efflux activity is an open and interesting question.

2.2 Nitrate Xylem Loading

Nitrate taken into plants can be assimilated in the roots or transported to the shoots to be assimilated there. Partition of nitrate assimilation between root and shoot depends on plant species, light intensity, temperature, and external nitrate concentration. To be transported up to shoot, nitrate has to be loaded into xylem. One of the 53 Arabidopsis CHL1 homologs, AtNRT1.5 (NTL2), is involved in xylem nitrate loading (Lin et al. 2008). In situ hybridization indicated that *AtNRT1.5* is expressed in root pericycle cells next to protoxylem. Knockdown or knockout mutations of *NRT1.5* reduced the amount of nitrate transported from root to shoot, suggesting that NRT1.5 participates in root xylem loading of nitrate. For uptake, nitrate has to be imported into plant cells, but for xylem loading, nitrate has to be exported out of plant cells. Oocyte functional analysis indicated that AtNRT1.5 can transport nitrate in both directions. It is generally believed that the channel is responsible for the xylem loading of nitrate; therefore, it is quite surprising that a proton-coupled nitrate transporter AtNRT1.5 is involved in this process. However, the root-to-shoot nitrate transport is not completely eliminated in *nrt1.5* mutants, suggesting that there are some other loading systems that might be mediated by channel.

2.3 Nitrate Remobilization

Nitrate can be stored in vacuoles. The amount of nitrate stored during vegetative growth and efficient remobilization of stored nitrogen during reproductive growth is important for grain yield. Many studies have elucidated the remobilization of organic nitrogen, but a recent study of nitrate transporter AtNRT1.7 showed that nitrate itself can be remobilized from older leaves to younger leaves or developing flowers (Fan et al. 2009). AtNRT1.7, a plasma membrane protein found in leaf vascular tissue, is expressed more in older leaves, and less in younger leaves. In the wild type, $^{15}NO_3^-$ spotted on older leaves was remobilized into young leaves. But, in the mutant, $^{15}NO_3^-$ was not transported to the younger leaves, indicating that AtNRT1.7 is involved in moving stored and excess nitrate from old leaves to young leaves. Under nitrogen-limited conditions, *atnrt1.7* mutants showed growth retardation, indicating that nitrate remobilization is important to sustain vigorous growth upon nitrogen shortage. Expression of AtNRT1.7 in the phloem and reduced phloem nitrate content in the mutant indicated that while root-to-shoot nitrate transport is mediated by xylem, nitrate remobilization among leaves and flowers is mediated by phloem.

2.4 Nitrate and Embryo Development

Nitrate is the primary nitrogen source for vegetative growth. Characterization of nitrate transporter AtNRT1.6 revealed that nitrate is also important for reproductive growth in early embryo development (Almagro et al. 2008). AtNRT1.6, a low-affinity nitrate transporter, is expressed in the funiculus. In *atnrt1.6* mutants, seed abortion rate was increased and seed nitrate content reduced. The embryos of aborted seeds were arrested at very early stages with excess cell division and loss of turgidity found in the suspensor cells. It is well known that organic nitrogen, e.g., as amino acids or peptides, is the nitrogen source of embryo development; however, properties of nitrate transporter ATNRT1.6 showed that nitrate, an inorganic form of nitrogen, is also important for the early stage of embryo development. In addition to being required for early embryo development, nitrate can be accumulated in the embryo at later stages of development. Nitrate transporter AtNRT2.7, located in the tonoplast, is responsible for this storage process (Chopin et al. 2007). No embryo development phenotype was found in *nrt2.7* mutant (Chopin et al. 2007), but nitrate stored in the embryo is important to relieve seed dormancy (Alboresi et al. 2005; Chopin et al. 2007).

3 Ammonium Transporters

The first ammonium transporters (the *am*monium *t*ransporter/*me*thylammonium *p*ermease AMT/MEP family) were simultaneously isolated from yeast and plant by functional complementation of a yeast mutant defective in ammonium uptake

(Marini et al. 1994; Ninnemann et al. 1994). Later, it was found that most eukaryotic and prokaryotic organisms have AMT/MEP/Rh genes. But in contrast to prokaryotic AMT transporters and human erythrocyte rhesus-associated glycoprotein Rh, which function as NH_3 channels, higher plant AMT transporters function as NH_4^+ uniporters (Ludewig et al. 2007). AMT/MEP/Rh transporter contains 11 transmembrane domains. The crystal structure of archaeal and *E. coli* ammonium transporters shows that it functions as a trimer with a substrate-conducting channel in each monomer (Khademi et al. 2004; Andrade et al. 2005).

Other than AMT transporters, Arabidopsis tonoplast intrinsic proteins TIP2;1 and TIP2;3 are able to transport NH_3 when expressed in yeast or oocytes (Loque et al. 2005). TIP2;1 and TIP2;3 might be involved in loading NH_3 into the vacuole.

3.1 Ammonium Uptake

Nitrate and ammonium are two major nitrogen sources for nonlegume plants. In acidic or anoxia soils, plants may encounter substantial levels of NH_4^+ ions. Despite being essential for their survival, excess ammonium is toxic to plants. Therefore, when ammonium reaches excessive levels, plants must expend considerable energy to actively extrude it out. To avoid this situation, plants have evolved intricate mechanisms to regulate ammonium uptake. There are two ammonium uptake systems: a saturable high-affinity ammonium uptake system and a nonsaturable low-affinity ammonium uptake system. Ammonium transporters (AMT) are responsible for high-affinity ammonium uptake (Fig. 2). The proteins involved in the low-affinity system have not been identified, but the system might be mediated by the aquaporin or cation channel.

In Arabidopsis, there are six AMT transporters: AMT1;1, AMT1;2, AMT1;3, AMT1;4, and AMT1;5 belong to the same AMT/MET subfamily; and AMT2;1 is the only member that belongs to another subfamily. Four of these AMT transporters, AMT1;1, AMT1;2, AMT1;3, and AMT1;5 are involved in ammonium uptake

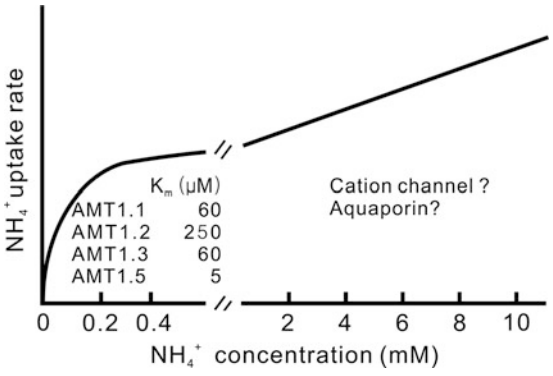

Fig. 2 Transporters involved in ammonium uptake. Four ammonium transporters with different affinities are involved in high-affinity ammonium uptake

(Yuan et al. 2007b). AMT1;1, AMT1;3, and AMT1;5 are mainly expressed in the rhizodermis, while AMT1;2 is expressed in the cortex and endodermis. Analyses of quadruple and triple mutants revealed that AMT1;1, AMT1;2, AMT1;3, and AMT1;5 contribute to ~30%, ~20%, ~30%, and ~10%, respectively, of high-affinity ammonium influx in Arabidopsis with ammonium affinities of 50μM, 234μM, 61μM, and 4.5 μM, respectively. However, the potential among these AMT transporters to form heterotrimers (Ludewig et al. 2003) may add another dimension of complexity to ammonium uptake.

In addition to transcriptional and RNA stability regulation (Yuan et al. 2007a), AMT1.1 and AMT1.2 are regulated by allosteric *trans*-activation among the monomers in the trimer complex to avoid excess ammonium influx (Loque et al. 2007; Neuhauser et al. 2007). Homology models using the crystal structure archaeal and *E. coli* ammonium transporters, suppressor screen, and phosphoproteomic study suggested that the cytosolic C terminal of plant AMT transporters containing two conserved short α-helices can interact with the cytosolic surface of a neighboring subunit, and phosphorylation of a threonine residue (460 in AMT1.1 and 472 in AMT1.2) in the C-terminal of a single monomer leads to a conformational change resulting in the cooperative closure of all three pores in the trimer. This conformational coupling between monomers provides a rapid shot-off mechanism to prevent toxic accumulation of ammonium. The phosphorylation status of T460 is regulated by ammonium concentration in the rhizosphere (Lanquar et al. 2009) suggesting that AMT1;1 may also function as a transceptor (i.e., transporter and receptor). In response to high concentration of ammonium in the rhizosphere, phosphorylation of T460 could serve as an inhibitory feedback loop to shut down its transport activity.

3.2 AtAMT2.1 and AtAMT1.4

Arabidopsis AMT2;1 is expressed in the root in the same way as Arabidopsis AMT1;1, AMT1;2, AMT1;3 and AMT1;5. However, no ammonium uptake defect is evidenced in *amt2.1* mutants (Sohlenkamp et al. 2002; Yuan et al. 2007b). In contrast to all the other Arabidopsis AMT transporters, AMT1;4 with a K_m of 17 μM is specifically expressed in pollen. But, no pollen-related phenotype could be detected in the mutants.

4 Amino Acid Transporters

There are two major superfamilies of plasma membrane amino acid transporters in higher plants: the amino acid transporter superfamily (ATF) and the amino acid-polyamine-choline transporter superfamily (APC), which have 46 and 14 members, respectively, identified in Arabidopsis (Wipf et al. 2002; Liu and Bush 2006; Rentsch et al. 2007). ATF transporters were first identified in plants (Frommer

et al. 1993), but later, homologs were found in yeast and animals as well. The ATF family can further be divided into six subfamilies: amino acid permeases (AAP), lysine/histidine transporters (LHT), proline transporters (ProT), γ-aminobutyric acid transporters (GAT), aromatic and neutral amino acid transporters (ANT1), and auxin-resistant (AUX). Topology analysis revealed that AtAAP1/NAT contains 11 transmembrane domains (Chang and Bush 1997). Most amino acid transporters characterized show broad selectivity. Sometimes, their substrate specificity cannot be inferred from their family names. For example, AtLHT1 and AtLHT2 actually transport neutral and acidic aminos with higher affinity than the basic amino acids lysine and histidine (Lee and Tegeder 2004; Hirner et al. 2006).

The plant APC family contains two subfamilies: cationic amino acid transporters (CAT) and L-type amino acid transporters (LAT). CAT and LAT were predicted to have 14 and 12 transmembrane domains, respectively. Yeast does not have a CAT-type transporter. All yeast APC transporters belong to the LAT subfamily. In animals, LAT required an additional one-transmembrane domain protein (heavy subunit) for targeting and amino acid uptake activity (Wipf et al. 2002). But no homolog of the heavy subunit can be found in yeast and plants. Probably due to the lack of interacting proteins, no plant LAT transporters have been functionally characterized.

Recently, a new type of amino acid transporter, Arabidopsis BAT1, which has 12 transmembrane domains, has been shown to transport amino acids in both directions (Dundar and Bush 2009). Despite sharing highest sequence identity (34%) with *Aspergillus nidulans* GABA permease, BAT1 exhibits no GABA transport activity. In Arabidopsis, CAT1, which has 14 transmembrane domains, shares highest sequence identity (23%) with BAT1.

4.1 Amino Acid Uptake

Arabidopsis can grow on medium with amino acids as the sole nitrogen sources. In Arabidopsis, two amino acid transporters AAP1 and LHT1 with a different spectrum of substrate specificities were involved in amino acid uptake into roots (Fig. 3). T-DNA-inserted mutant *aap1* was isolated by a forward genetic screen for mutants insensitive to high concentration of phenylalanine (Lee et al. 2007). Growth and [^{14}C]-labeled uptake studies of *aap1* mutant showed that glutamate, histidine, and neutral amino acids are the physiological substrates of AAP1, whereas aspartate, lysine, and arginine are not. Similarly, using reverse genetics, it was shown that *lht1* mutant cannot use glutamate and aspartate as its sole nitrogen sources, and glutamine, aspartate, and glutamate uptake activities were reduced by 75–85% in *lht1* mutant (Hirner et al. 2006). In addition to uptake, AAP1 and LHT1 are also involved in the import of amino acids into developing embryos and mesophyll cells, respectively (Hirner et al. 2006; Sanders et al. 2009). Findings such as these suggest that multiple physiological functions may be a common feature of plant amino acid transporters.

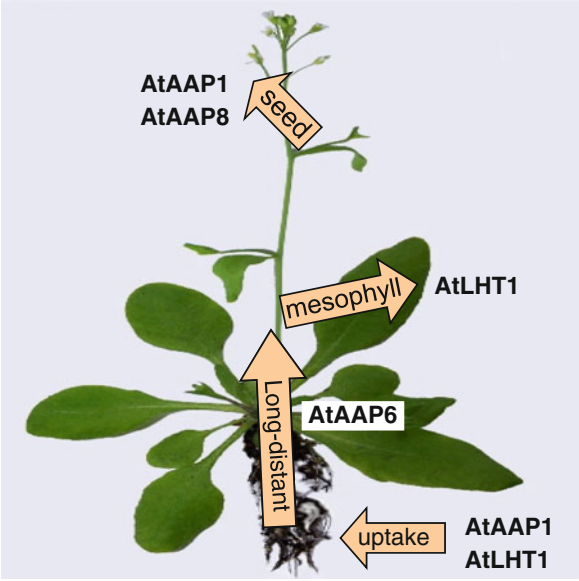

Fig. 3 Physiological functions of Arabidopsis amino acid transporters. AAP1 expressed in embryo and AAP8 expressed in endosperm are involved in import of amino acid into embryo. AAP6 expressed in xylem parenchyma regulate phloem sap amino acid composition. AAP1 and LHT are involved in amino acid uptake

4.2 Long Distance Transport of Amino Acids

AAP6 has a tenfold higher affinity for all substrates than other Arabidopsis AAP transporters characterized. *AAP6* is expressed in the xylem parenchyma cells of sink tissues, roots, sink leaves, and cauline leaves (Okumoto et al. 2002). A recent study showed that the abolition of AAP6 reduced the mean total amino acid concentration of sieve element sap by 30% (Hunt et al. 2010). Consistent with the higher substrate affinity of AAP6 and the lower amino acid content of xylem sap compared to that of phloem sap, phenotype of *aap6* mutant suggested that AAP6 mediates the transfer of amino acids from the xylem to the phloem and, therefore, regulates the sieve element composition. In addition to AAP6, several amino acid transporters are expressed in vasculature or phloem, but their physiological role in long distance transport remains to be further characterized by genetic approaches.

4.3 Amino Acid Transport and the Embryo

Genetic evidence has shown that two Arabidopsis amino acid transporters AAP1 and AAP8 are important for importing amino acids into developing seeds (Schmidt

et al. 2007; Sanders et al. 2009). In *aap1* mutant, more neutral and acidic amino acids were accumulated in the seed coat/endosperm, indicating that AAP1, expressed in embryo, is responsible for the apoplastic import of amino acids into embryo. This import process is important for seed yield because seed protein level, seed weight, silique number, and seed number was reduced in the mutant. Probably through a similar mechanism, in legumes, ectopic expression of VfAAP1 in pea (*Pisum sativum*) and *Vicia narbonensis* increased seed protein content (Rolletschek et al. 2005).

AtAAP8 is expressed in endosperm in young seeds, but at a later stage of development, it is expressed in the veins of the mature silique. Consistent with its higher affinity for acidic amino acid, aspartic acid and glutamic acid are significantly reduced in young siliques of the *aap8* mutants (Schmidt et al. 2007). In contrast with *aap1* mutant, which shows no embryo development phenotype, in *aap8* mutant, 45% of the seeds are aborted at, or before, the globular stage. Since neither AAP1 nor AAP8 was able to transport cationic amino acids, import of cationic amino acids into the embryo might be mediated by AtCAT6 expressed in seeds (Hammes et al. 2006).

5 Conclusions

In Arabidopsis, more than 120 genes are predicted to encode plasma membrane nitrogen (nitrate, ammonium, and amino acid) transporters. Only some of them, less than one-fourth, are functionally characterized in planta or in heteroexpression systems using either yeast or Xenopus oocytes. A lot of nitrogen transporter mutants, e.g., Arabidopsis *nrt1.1 (chl1), nrt2.1, nrt1.6, nrt1.7, lht1, aap8* showed growth phenotypes (Guo et al. 2001; Little et al. 2005; Hirner et al. 2006; Remans et al. 2006; Schmidt et al. 2007; Almagro et al. 2008; Fan et al. 2009), indicating that multiple steps of nitrogen acquisition and allocation play critical roles in regulating plant development. Moreover, several recent studies have shown that nitrogen transporters, *e.g.*, CHL1 (NRT1.1) and AMT1;1, are involved either directly or indirectly in nutrient sensing (Ho et al. 2009; Lanquar et al. 2009). This adds a further dimension of value and complicity to the plasma membrane nitrogen transporters.

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Plant Plasma Membrane and Phosphate Deprivation

Laurent Nussaume, Eric Maréchal, Marie Christine Thibaud, and Maryse A. Block

Abstract Phosphorus is a major plant macronutrient, but it is also one of the less accessible mineral elements for these organisms due to the very low solubility of phosphate and phosphorus incorporation in organic matter in soils. Plants have developed multiple strategies to enhance phosphorus acquisition, and much of that activity takes place at the plasma membrane. Phosphorus remobilization from membrane lipids, regulation of transporter activity, and rhizosphere acidification are the primary mechanisms associated with the plasma membrane that function under phosphorus deficiency conditions. In this review, we summarize both lipid remodeling and adjustments of transporters in the plasma membrane of plant cells following Pi deprivation, and discuss the control and coordination of these major modifications in the global response of plants to this stress.

1 Introduction

Phosphorus is a major plant macronutrient, but it is also one of the less accessible mineral elements for these organisms. Indeed, although abundantly present in soils, it is acquired by roots in the form of phosphate (Pi), a molecule of very low solubility (as a result of its association in soils with cations or organic compounds). In addition, from 20 to 80% of total phosphorus in forest or agricultural soils is in organic matter (such as phytic acid) and requires mineralization steps to be absorbed by plants (Schachtman et al. 1998). In approximately half of the world's

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arable lands, the average Pi concentration is below 10 μM and constitutes a limitation for the growth of crops. Plant physiology is therefore adapted via multiple strategies to cope with low Pi environments by optimizing internal Pi utilization and enhancing external Pi acquisition (for recent review, see (Amtmann et al. 2006; Bucher 2007)). The plasma membrane (PM) of plants facing Pi deficiency has a primary function in both of these processes. As a starting point, the phospholipids are major molecular constituents of the PM and source of phosphorus remobilization under phosphorus deficiency conditions. In *Arabidopsis thaliana* grown in non limiting-Pi medium, phospholipids account for one-third of total lipids and serve as an important reserve of phosphorus for the plant (Dormann and Benning 2002). During Pi deprivation, these intrinsic membrane components are replaced by Pi-free galactolipids (see chapter "Plasma Membrane Protein Trafficking" by Peer), thus allowing the salvage of Pi for use in housekeeping metabolism. Sulfolipids can also replace phospholipids during Pi deficiency, but these modifications are restricted to chloroplast membranes, where sulfolipids comprise <20% of the total lipids. The second major modification affecting the PM during Pi deprivation is a strong variation of expression of several genes encoding transporter proteins that contribute to the absorption of Pi and metals. Transfer experiments from rich to Pi-depleted media have shown that the remodeling of membrane lipids and the modification of transporters takes place rapidly and occurs before substantive decrease of the free Pi pool in the plant cells is evident. This suggests the existence of complex Pi-sensing mechanisms that precede metabolic responses resulting from modulation of Pi content in the cells. Since plants only detect internal Pi concentration (Lai et al. 2007), the plasma membrane appears to play a key role not only as an important pool of available Pi but also as a key component for the sensing and the partitioning of this ion. In this review, we report both lipid remodeling and adjustments of transporters in the plasma membrane of plant cells following Pi deprivation, and discuss the control and coordination of these major modifications in the global response of plants to this stress.

2 Plasma Membrane Components Modulated by Phosphate Availability

2.1 Glycerolipids

2.1.1 Lipid Composition of Plasma Membrane

The plant plasma membrane is highly enriched in phospholipids, sphingolipids, and sterols (Moreau et al. 1998) (see Chapter "Plasma Membrane Protein Trafficking" by Peer). In standard conditions of growth, the plasma membrane bulk lipid composition derives from membrane flow in the endomembranes, a networked

vesicular trafficking system finding its origin in the endoplasmic reticulum, where phospholipids are synthesized. As in most nonplastidial membranes, the most abundant phospholipids are phosphatidylcholine (PC, 25–45%) and phosphatidylethanolamine (PE, 30–40%). The plasma membrane differs from other membranes by a relatively high content of some usually low-abundance phospholipids such as phosphatidic acid (PA, 5–20%), phosphatidylserine (PS, 3–12%), and phosphatidylinositol (PI, 2–8%). The phospholipids are asymmetrically distributed between the two membrane leaflets. Most of the PC is located in the outer leaflet whereas PS and a part of the PE are in the cytosolic leaflet. After treatment with camptothecin, an apoptosis inducer in animal cells, externalization of PS is observed, suggesting a developmental role of plasma membrane lipid asymmetry (O'Brien et al. 1997).

A large body of evidence from the past decade supports the existence of functional microdomains in plasma membranes of animal and yeast cells, which play important roles in protein sorting, signal transduction, or infection by pathogens. Formation of these microdomains is based on the dynamic clustering of sphingolipids and sterol. Similar detergent-resistant membrane (DRM) microdomains also exist in plant plasma membrane (Mongrand et al. 2004; Borner et al. 2005; Lefebvre et al. 2007). The work of Roche and collaborators (2008) supports the role of phytosterols in the lateral structuring of the plant plasma membrane and suggests that they are key compounds for the formation of DRM microdomains. They could create a dynamic scaffold to organize cellular processes and synchronize efficiency and specificity of cellular responses.

Accurate membrane sterol composition is also essential for the formation of plasma membrane macrodomains necessary for the acquisition of cell polarity. A mutant impaired in sterol biosynthesis was thus shown to display an altered PIN2 polarity and a disturbed cell-to-cell auxin transport (Men et al. 2008).

2.1.2 Phosphate Deprivation Promotes Phospholipid Recycling and Digalactolipid Accumulation in Plasma Membrane

Galactolipids are highly represented in plastidial membranes, a membrane compartment disconnected from the endomembrane vesicular trafficking system, and they are detected in only trace amounts in plasma membrane, when plants are cultivated in the presence of phosphate. By contrast, when plants are deprived of Pi, digalactosyldiacylglycerol (DGDG) constitutes a substantial proportion of plasma membrane lipids, in shoots and in roots (Andersson et al. 2003, 2005; Russo et al. 2007). Under severe Pi starvation, DGDG can raise up to 70% of the plasma membrane glycerolipid content (also representing 25% of plasma membrane total lipid content) (Andersson et al. 2005). The accumulation of DGDG in the plasma membrane counterbalances the decrease of phospholipids, mainly PC and PE, and contributes to overall saving of phosphorus for the cell.

Upon Pi deprivation, several other cell membranes usually devoid of DGDG get enriched in DGDG. This is the case of tonoplast (Andersson et al. 2005) and mitochondrial membranes (Jouhet et al. 2004). Fatty acid composition of DGDG

formed under Pi deprivation is strikingly different from chloroplast DGDG, as it is enriched in 16:0 and 18:2 fatty acids, whereas chloroplast DGDG contains a high proportion of 18:3, suggesting that both forms of DGDGs are synthesized by different pathways (Hartel et al. 2000). They are however formed by the same type of galactosylation enzymes (Hartel et al. 2000; Kelly and Dormann 2002; Jouhet et al. 2004). In a first step, monogalactosyldiacylglycerol (MGDG) is formed by galactosylation of diacylglycerol (DAG) by MGD enzymes, and then DGDG is formed by galactosylation of MGDG by DGD enzymes. Three MGD isoforms (MGD1, 2 and 3) and two DGD isoforms (DGD1 and 2) occur in *Arabidopsis*. Mutant analyses indicate that MGD2, MGD3, DGD1, and DGD2 are involved in the synthesis of DGDG induced by Pi deprivation (Hartel et al. 2000; Awai et al. 2001; Kelly and Dormann 2002; Kobayashi et al. 2004, 2009). These enzymes were reported to be located in the outer membrane of plastid envelope. The contribution of MGD1, which is located in the inner membrane of plastid envelope and essential for formation of thylakoid MGDG, is still a matter of debate. The source of DAG results from hydrolysis of phospholipids either by phospholipases C (PLCs) or a combination of phospholipases D (PLDs) and phosphatidate phosphatases (PAPs). The transient accumulation of PC in the early stage of Pi deprivation and the accumulation of DAG exhibiting the same fatty acid composition as that of PC indicates a role of PC in formation of DAG (Jouhet et al. 2003). The recruitment of PLDs and PLCs in the formation of DAG to fuel DGDG synthesis was therefore examined by mutant analyses. One specific PLC, NPC4, has attracted attention as it is present in the plasma membrane and displays a role in PC-hydrolysis under Pi deprivation (Andersson et al. 2005; Nakamura et al. 2005; Tjellstrom et al. 2008). However, formation of galactolipids was apparently not affected in the *npc*4 knockout mutants. By contrast, another PLC, the cytosolic NPC5, was shown to display a role in accumulation of DGDG during Pi limitation in leaves (Gaude et al. 2008).

PLDs and PAPs are the dominant lipases induced by Pi deficiency (Misson et al. 2005; Li et al. 2006b; Tjellstrom et al. 2008), and they might act simultaneously to generate DAG. Deletion of PLDζ2 affects the capacity for DGDG formation in roots (Cruz-Ramirez et al. 2006; Li et al. 2006a, b). PLDζ2 is present in the tonoplast regardless of available Pi levels (Yamaryo et al. 2008). However, Pi deprivation enhances transient formation of PLDζ2-enriched domains in the tonoplast preferentially positioned near mitochondria and chloroplasts (Yamaryo et al. 2008). Although pldζ1 expression is much less stimulated by Pi deprivation than pldζ2 expression, PLDζ1 displays also a role in formation of DGDG in correlation with Pi deprivation (Li et al. 2006a). PLDζ1 is located in vesicles close to the plasma membrane (Qin and Wang 2002). Finally, activity of PLD generates PA, which is considered both a metabolic precursor to produce DAG and an intracellular signal for activation of many plant cell processes, although it is difficult to discriminate between these functions. Generation of PA through PLDζ2 is likely to be important for signaling processes related to both Pi signaling and endosomal membrane trafficking. This signaling appears to involve interactions with other signal transduction pathways, as membrane lipid alteration during Pi deprivation

has been shown to depend on both Pi signaling and auxin/cytokinin cross-talk (Kobayashi et al. 2006), and PLDζ2 has been shown to be involved in root system architecture, auxin-dependent hypocotyl elongation, and vesicle cycling (Li and Xue 2007). In addition, PLDζ proteins differentiate from other PLDs by the presence of a regulatory domain that contains a PX (PHOX) and a PH (Plekstrin homolog) motif classically involved in phosphoinositide and protein interaction (see chapter "Plasma Membrane and Abiotic Stress" by Barkla and Pantoja).

Localization of galactosylation enzymes in the envelope of plastids indicates that DGDG is transported to its final location after synthesis. This was shown for mitochondrial DGDG (Jouhet et al. 2004). Only background levels of galactosyltransferase activity were detected in highly purified mitochondria from Pi-deprived plant cells, and the low activity observed in some mitochondrial fractions was consistent with cross contamination with envelope membranes. Finally, by following the ratio of DGDG vs. MGDG during purification of mitochondria from Pi deprived cells, a selective transfer of DGDG from chloroplast envelope to mitochondria was demonstrated. Electron microscopy suggested that lipid transfer between the two organelles was not conducted through vesicle trafficking but through membrane apposition. In support of this lipid transfer mechanism, an increased number of contact sites between chloroplast and mitochondria were observed transiently after Pi deprivation, whereas formation of vesicles on the plastid surface was never observed (Jouhet et al. 2004; Andersson et al. 2007). How DGDG emerges in the PM is still an open question. It remains possible that DGDG is locally synthesized by delocalized MGD and DGD or that it is transported from plastids. In the latter case, a direct lipid transfer is likely to occur through membrane contact sites between plastids and some extraplastidial membranes to be identified. Figure 1a summarizes the coupling of phospholipid breakdown in plant cell membranes, including the PM, the concomitant increase of DGDG synthesis in the plastid envelope, and the transfer of this galactolipid to mitochondria, tonoplast, and PM.

3 Transporters

3.1 Phosphate Transporters

Acquisition of soil Pi is a puzzle for plants. The essential need for this ion (accumulated up to 25 mM in the vacuole, (Mimura et al. 1990)) has to be fulfilled in spite of its low availability in the environment (often below 10 μM). Among the various strategies used by plants to acquire external Pi there is one that involves the plasma membrane through the induction of a particular subset of Pi transporters.

In *Arabidopsis*, four families of Pi transporters have been identified and named PHT1 to 4, but very little data are available regarding their function. Most of Pi transporters belong to multigenic families. We will here focus on the PHT1 class,

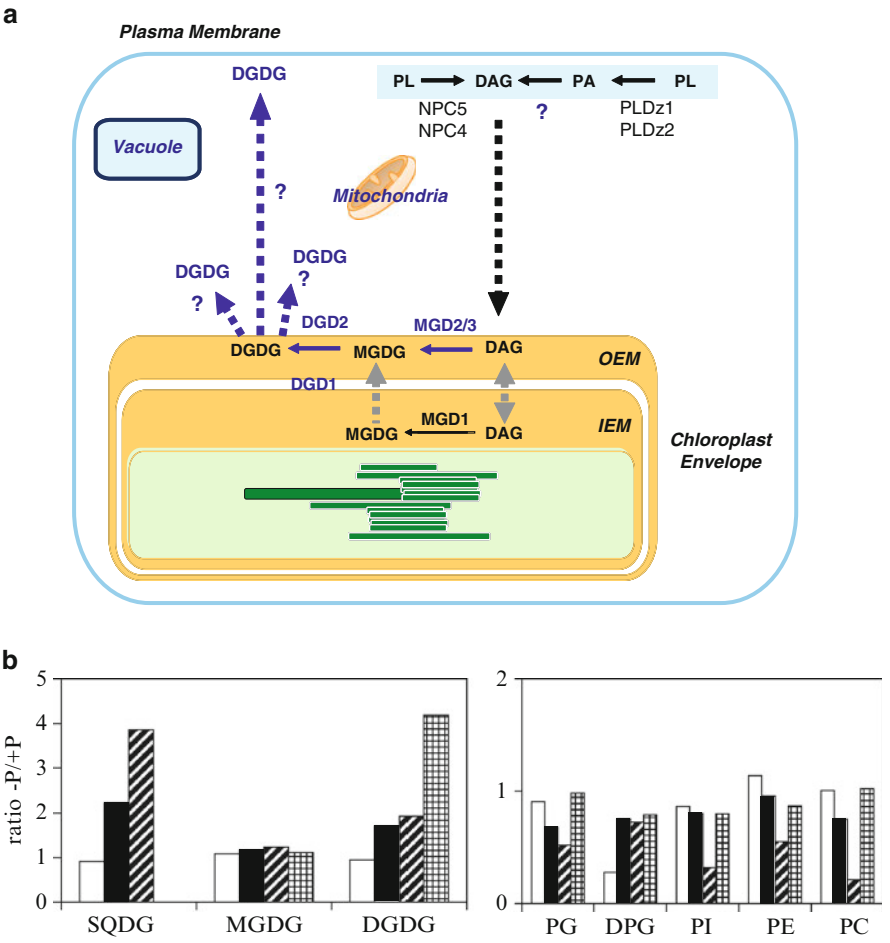

Fig. 1 Lipid remodeling following phosphate deprivation. (**a**) In standard conditions, the plasma membrane is phospholipid-rich. Under phosphate deprivation, phospholipids (PL) are hydrolyzed, the synthesis of phosphate-free galactolipids (MGDG, DGDG) is stimulated, and DGDG is transported outside the plastid, in particular to the plasma membrane, where it replaces hydrolyzed PL. Induced genes and transfers are shown in *blue*. (**b**) Time course and lipid content in Pi-deprived plantlets following transfer from Pi rich medium (results are expressed as ratio of lipid content in −P versus + P plants): less than 12 h (*white bars*), 1–2 days (*black bars*), leaves 10 days (*dark grey bars*), roots 10 days (*light grey bars*)

which is the group restricted to the PM. All three others are located in organellar membranes, like mitochondria (PHT3 (Takabatake et al. 1999)) or plastid (PHT2 (Rausch et al. 2004) and most of the PHT4 transporters) with the exception of PHT4;6 located in the Golgi apparatus (Guo et al. 2008).

The *Arabidopsis* genome contains nine *Pht1* genes named *AtPht1;1* to *AtPht1;9* (Mudge et al. 2002). They encode highly homologous proteins with a size varying

from 520 to 550 amino acids. The PHT1 proteins have 12 transmembrane helices composed of two groups of six helices linked by a large hydrophilic loop. Many phosphorylation sites have been predicted and some were recently identified by mass spectrometry (Nuhse et al. 2004).

Although PHT1 transporters are Pi/protons symporters and belong to the large major facilitator superfamily (MFS) found in all phyla, PHT1 proteins have been isolated and characterized only in plants and fungi. The recent identification of a semidominant mutant of PHT1;1 transporter suggests that they could act as dimers or higher-order oligomers (Catarecha et al. 2007) as previously proposed (Shin et al. 2004). To date, the high level of homology between the PHT1 transporters has hampered the production of specific antibodies necessary to assess their cellular localization. Nevertheless, the use of reporter genes fused to the PHT1 promoters ((Karthikeyan et al. 2002; Mudge et al. 2002; Misson et al. 2004) has allowed the mapping of tissue-specific *PHT1* gene expression. Most are expressed in multiple cell layers of the various plant organs, suggesting different roles for the absorption and/or homeostatic control of Pi in the plant. The identification of the *pht1;1* and *pht1;4* mutants (Misson et al. 2004; Shin et al. 2004; Catarecha et al. 2007) has provided genetic clues regarding the essential role of these transporters for the absorption of Pi from the medium as these two genes contribute approximately 75% of the Pi absorption capacity (Shin et al. 2004).

3.2 Metal Transporters

The ability of Pi to trap cations is a key parameter influencing availability of metals in plants. It is also a crucial parameter for the quality of Pi fertilizers, which can be easily and naturally contaminated by heavy metals (Othman and Al-Masri 2007). This was recently illustrated with the case of Polonium 210-containing apatite used by the US tobacco industry.

In vitro experiments have demonstrated that a reduction of Pi content in growth media resulted in as much as a five- to sixfold increase in plant iron content (Hirsch et al. 2006). A similar effect was seen for zinc (Misson et al. 2005). When Pi is abundant in the growth medium, the availability of the essential micronutrients is reduced and the expression of the genes encoding micronutrient (Zn, Fe, etc) transporters or regulatory factors is strongly induced (Table 1). In low Pi conditions, cations are more available, and Hirsch et al. (2006) have demonstrated a specific modification in the subcellular localization of iron in the leaves, suggesting an adaptation to the Pi homeostasis. It has been suggested that the root growth arrest observed in low Pi-containing medium (Reymond et al. 2006; Desnos 2008; Arnaud et al. 2010) could be a consequence of this accumulation of iron in the plants (Svistoonoff et al. 2007; Ward et al. 2008). Nevertheless, it probably does not result from iron toxicity as we have identified drugs by chemical genetics, which dissociate these two traits (Laurent Nussaume personal communication).

Table 1 Regulation of genes involved in the synthesis of plasma membrane proteins. Ratio of the gene expression −P/+P (underlined: *T*-test significant with a $P < 0.05$). Transcript analysis was performed using Affymetrix chips, for details see Misson et al. (2005)

AGI number		Less than 12 h	1–2 days	Leaf 10 days	Root 10 days
Lipid metabolism					
At5g20410	MGD2	10.37	14.90	24.81	8.32
At2g11810	MGD3	7.25	26.81	55.11	50.38
At3g11670	DGD1	1.36	2.32	3.56	2.00
At4g00550	DGD2	1.63	1.71	2.31	3.57
At4g33030	SQD1	2.59	3.13	4.41	8.03
At5g01220	SQD2	4.88	16.02	14.56	13.01
At3g03540	NPC4 and 5	8.43	10.47	241.91	17.95
At3g05630	PLDz2	9.95	21.83	55.42	20.66
At3g08510	PLC2	1.66	2.47	2.83	2.79
At1g73600	NMT3	0.42	1.35	0.09	0.17
At3g18000	NMT1	1.09	0.80	3.74	1.63
Metal transport-related					
At4g23700	CHXI7	2.92	5.46	7.09	1.32
At2g38460	ferroportin1	0.93	0.97	0.68	0.44
At5g6820	ferroportin2	0.94	0.73	0.85	0.52
At4g19690	IRT1	1.36	1.58	0.13	0.03
At4g19680	IRT2	1.14	2.06	2.85	0.63
At3g46900	COPT2	1.29	0.79	0.46	0.35
At5g59520	ZIP2	0.91	0.95	0.77	0.55
At1g10970	ZIP4	1.14	1.07	0.82	1.28
At1g05300	ZIP5	1.41	2.92	3.83	2.18
At2g30080	ZIP6	0.91	0.89	0.88	1.15
At4g33020	ZIP9	0.79	1.30	1.31	1.19
At4g30110	Cd/Zn P-ATPase	1.20	0.84	1.11	1.10
At4g24120	YSL1	0.79	0.93	2.02	1.16
At1g48370	YSL8	1.56	3.46	4.23	2.95
At1g09930	AtOPT2	1.15	1.51	0.96	1.43
At4g16370	AtOPT3	0.84	0.52	0.45	0.40
At2g46800	AtMTP1	1.01	1.17	0.82	0.89
At2g29410	AtMTPb	3.46	1.76	4.28	0.87
Pi transporters					
At5g43370	Pht1;2	1.0	2.4	4.8	3.4
At5g43360	Pht1;3	1.0	2.5	1.6	5.8
At2g38940	Pht1;4	2.5	10.5	34.0	21.7
At2g32830	Pht1;5	2.4	9.7	25.9	15.7
At5g43340	Pht1;6	1.12	0.9	1	1.12
At1g20860	Pht1;8	1.0	1.7	6.1	8.4
At1g76430	Pht1;9	1.5	4.3	15.5	5.4

4 Timing and Regulation of Phosphate Deficiency-Related Modifications Affecting the Plasma Membrane

The modifications affecting the membranes in response to change in Pi status are rapid processes that are observed within a few hours when plants or cells are transferred from Pi-rich to Pi-depleted medium. When *Arabidopsis* plants are

grown in hydroponic conditions, to favor rapid transfers, the soluble cellular Pi decreases around 15–20% within 3 h and 55% after 12 h (Misson et al. 2005). This reduction is even more rapid in cell culture experiments. Even when the peak of induced gene expression is observed, after a few days of deficiency, the increase in expression of genes involved in phospholipid recycling and Pi/metal transport starts rapidly. In *Arabidopsis* plantlets grown in hydroponic conditions, the increased expression of genes involved in lipid metabolism (Table 1) is induced within 12 h (around seven to ten times for MGD2, MGD3, NPC4 and 5, or PLDZ2). The accumulation of transcripts encoding Pi transporters appears to be more delayed. Aside from Pht1;4, which exhibits an early enhancement of expression, induction of most Pi transporter genes requires an additional 12–24 h.

The time course of lipid remodeling induced by Pi deprivation has been finely dissected by Jouhet et al. (2003), using *Arabidopsis* and *Acer* cells suspensions. Two important phases are evident. First, the proportion of PC unexpectedly peaked 2–4 h after Pi removal. This first phase apparently corresponds to a conversion of phospholipids like PE into PC. This change may be mediated by stimulation of the enzymes responsible for PC neosynthesis, a stimulation of the enzymes involved in the synthesis of PC from PE, and/or an inhibition of PC converting enzymes. Following this striking accumulation of PC, a second phase is observed after Pi stress and is characterized by a breakdown of phospholipids (Fig. 1b), most notably PC. This second phase coincides with a remodeling of gene expression levels and reasonably implicates phospholipases, which are induced by the stress (Table 1). The second phase also corresponds to an increase of galactolipid synthesis, following stimulation of *Mgd2/3* and *Dgd2* expression levels, and DGDG transfer out of plastids, particularly to the plasma membrane.

As mentioned above, induction of the expression of genes encoding Pi transporters is slightly delayed (Table 1), requiring 12–24 additional hours to present high levels of induction (Pht1;6 excepted remaining at a constant level). In parallel, the bulk Pi content in plants grown in liquid medium decreases rapidly after transfer to Pi-deprived medium (around 15–20% within 3 h and 55% after 12 h, Misson et al. 2005). It is still not known how the relative concentrations of vacuolar and cytosolic Pi are involved in this overall cellular Pi collapse. Nevertheless, this rapid depletion of Pi suggests a direct response of the gene expression to cellular, presumably cytosolic, Pi levels. In a recent study, the transcript levels of *PHT1;1* and *MGD3* in plants previously grown under various Pi regimens were compared (Lai et al. 2007). Induction of expression of both genes was delayed in plants previously grown under high Pi regimens compared with plants grown under low Pi conditions. Such results are in agreement with previous data obtained with tomato cell cultures (Köck et al. 1998) following the expression of a ribonuclease gene known to be regulated by Pi deficiency and indicate that internal signals induced by Pi-starvation responses regulate genes encoding proteins that control lipid homeostasis and Pi transporter activity.

Nevertheless, we cannot exclude a role for unidentified signals. Many studies have shown that hormones such as cytokinins (Martin et al. 2000; Franco-Zorrilla et al. 2002), auxins (Kobayashi et al. 2006), ABA (Shin et al. 2006), and sugars

(Franco-Zorrilla et al. 2005) are negative or positive regulators of Pi starvation-responsive gene expression. All these molecules impact cell division activity, which was recently shown to be a process that correlates with the magnitude of the Pi deprivation response (Lai et al. 2007). As such, they are not likely to be considered as part of a specific direct signaling pathway.

It might be interesting to notice that the AVP1 (an Arabidopsis proton-pyrophosphatase) expression is increased by low Pi expression (Yang et al. 2007). It has been previously found that overproduction of AVP1 increased apoplastic acidification (Li et al. 2005), which increased both Pi and auxin transport. Modulation of auxin transport may provide an explanation to the increase of lateral root formation observed in phosphate-deficient condition, as AVP1 overexpression is also associated with an increase of lateral root. Another recent publication also reported effect of phosphate deficiency on increasing auxin sensitivity in pericycle cells (Perez-Torres et al. 2008). As the amount of free auxin is quite similar in plants grown with rich or poor phosphate medium, modulation of auxin transport identified above may provide part of the explanation for the phenotype observed. Another point to take into account is the smaller size of root cells of plant grown in phosphate-deficient conditions, which might increase the concentration of auxin per cell. Phosphate and auxin interaction appears very complex as phosphate deficiency was also found to affect the expression of TIR1, an important component of the ubiquitin machinery involved in the degradation of Aux/IAA transcriptional repressors (Perez-Torres et al. 2008). Nevertheless, we still have no clue if such interaction is direct or indirect.

Response to Pi deprivation is very complex. A myb transcription factor named PHR1 (Rubio et al. 2001) was shown to act downstream of this signaling pathway, using a genetic approach based on the identification of a mutant deregulating Pi starvation markers. This myb factor binds as a dimer to an imperfect palindromic sequence located in the promoter of Pi starvation-responsive structural genes. Only a very limited number of such target genes has been investigated by transcriptomic analysis based on rtPCR. The specificity of the role of PHR1 remains, however, obscure since it belongs to a multigenic family, and functional redundancy is likely to limit the clear identification of its direct targets.

Pi regulation involves local and/or long distance (systemic) regulations. Using "split root" experiments on tomato or Medicago, it has been previously shown that some Pht1 genes were systematically regulated (Liu et al. 1998; Burleigh and Harrison 1999). Using Affymetrix chips technology (Thibaud et al. 2010), it has been possible to extend this study to the whole *Arabidopsis* genome. This work revealed that the PHT1 family and the genes involved in the remodeling of lipids are systemically regulated. Some components of the systemic regulation have been recently identified. Important ones are PHR1 and the microRNA 399, which targets PHO2, a protein involved in ubiquitination processes (Fujii et al. 2005; Aung et al. 2006; Bari et al. 2006; Chiou et al. 2006; Buhtz et al. 2008; Lin et al. 2008; Pant et al. 2008). An additional ribo-regulator, called IPS1 (Franco-Zorrilla et al. 2007), was also found involved in the regulation of the level of microRNA 399 by a novel mechanism called "target mimicry" since it sequesters the miRNA399 in a

sequence-dependent manner. The key role of these components has been recently highlighted in a review (Doerner 2008). All these components are likely parts of a signal transduction cascade and some of them, particularly micro RNAs, might function in systemic signaling.

5 Conclusion

The plant PM functions as the interface between the external medium and the internal constituents of the cell and plays a major role in responses to Pi starvation. Functioning like an active barrier, the PM is involved in the modulation of the ion absorption by the roots, triggering ion movement against the concentration gradient, and the prevention of release of internal ion pools. In addition, the PM behaves as a nutrient reservoir that can release Pi through the degradation of integral phospholipids and replacement of phospholipids with plastidial galactolipids. As it is the case for most ion sensing mechanisms in plants, the primary receptor involved in Pi sensing is still unknown, despite many attempts based on genetic screens. The complexity and redundancy of nutrient uptake and homeostasis in plants suggest that systematic methods based on analyses of natural variation or chemical genetics may be the most effective means of unraveling the regulatory mechanisms involved.

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Biology of Plant Potassium Channels

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Abstract Potassium channels are found in all living organism from bacteria to men, and even in viruses. They were among the first transport proteins to be discovered on the molecular level. Pioneering work on potassium channel structure and function has led to the most comprehensive understanding about the physiology and regulation of potassium transport on cellular and organism levels. Since their discovery in 1984 by the first patch clamp in plants and the identification of genes encoding them in the early 1990s, potassium channels have advanced to the best understood transport proteins in plants. Starting with a brief introduction into the history of plant potassium transport, this review focuses on the molecular physiology of paradigmatic members of the "green" potassium channel family gained from studies in plants with sequenced genomes (the so-called model plants). We will then provide insights into our current knowledge of how channels find the membrane they control. Finally, the reader – in a plant signaling network context – will be introduced which triggers address the channel and how it responds.

1 Introduction

The pivotal steps in the life cycle of a plant, such as growth, movements, and development, are based on or at least associated with osmotic phenomena. Any of these processes requires the formation of turgor pressure using potassium salts as the main osmotically active solute. This force is counteracted by an exoskeleton structure: the plant cell wall. Since the pioneering work by Emanuel Epstein (Epstein et al. 1963), it is known that potassium uptake in plants is comprised of a high-affinity and a low-affinity component. High-affinity transport means

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cytosolic accumulation of potassium from low micromolar sources against steep concentration gradients. To enable uphill transport by often three orders of magnitude, the influx of K^+ is energized by a symport with protons (pH gradient of 2–3 across the plasma membrane), i.e., by proton-driven potassium transporters (the reader is referred to Gierth et al. 2005 and references therein). In contrast to K^+/H^+ symporters, K^+ channels mediate facilitated diffusion of potassium ions across biological membranes. The opening and closing transitions – a process called gating – of most K^+ channels is controlled by changes in membrane voltage. Upon opening of "N" channels, potassium flux is determined by the driving force for potassium, in other words, the chemical and electrical potential difference across the membrane. Plant potassium channels were first identified by the invention of the Patch-Clamp technique on guard cell protoplasts (Schroeder et al. 1984). It took until 1992 when their molecular identity was elucidated upon cloning of the K^+ channel *Arabidopsis thaliana* 1 (KAT1) and the *A*rabidopsis K^+ transporter 1 (AKT1) (Anderson et al. 1992; Sentenac et al. 1992), two K^+ channels sharing structural homology to Drosophila Shaker channel (Jan and Jan 1997). Based on a yeast complementation assay, these studies could show that plant K^+ uptake channels are capable of rescuing growth in K^+ transport-deficient yeast mutants down to the micromolar range of potassium supply (Becker et al. 1996; Bertl et al. 1998; Brüggemann et al. 1999). In line with patch-clamp studies on protoplasts, heterologous expression of, e.g., KAT1 in *Xenopus laevis* oocytes revealed the typical features of a voltage-dependent, K^+-selective ion channel. Animal Shaker potassium channels known at the time were characterized as voltage-dependent K^+ efflux channels (outward rectifiers). As expected from the yeast complementation studies, but in contrast to its animal homologs, KAT1 represents a voltage-dependent K^+ uptake channel (inward rectifier). Patch-clamp studies by Brüggemann et al. (1999) have shown that the inward rectifying K^+ channel KAT1 expressed in Arabidopsis guard cells is capable of mediating potassium uptake from media containing as little as 10 μM of external K^+. Thus, this K^+ uptake channel is working in the concentration range of high-affinity K^+ uptake systems whenever the membrane potential is both negative to the Nernst equilibrium potential of K^+ and to the activation threshold of the channel. Following their identification, plant potassium channels are found in all plant cells and tissues, suggesting a housekeeping role in plant cells of this class of transport proteins. Grass coleoptiles and guard cells have evolved as model systems to study the role of K^+ channels in irreversible plant growth movements on one side and reversible stomatal movement on the other. Since potassium salts represent major osmotica in plants, potassium fluxes through guard cell K^+ channels is pivotal for stomatal opening and closing. Likewise, root hairs and pollen tubes represent ideal systems to investigate the regulation of potassium homeostasis in these polar, tip-growing cell types (see below, and reviews by Chen et al. 2008; Hedrich and Marten 2006; Lebaudy et al. 2007; Roelfsema and Hedrich 2005; Szczerba et al. 2009; Very and Sentenac 2002; and references therein).

Completed plant genome sequencing projects in Arabidopsis, poplar, and rice provided a comprehensive picture of the molecular diversity of plant potassium

channel genes (Ward et al. 2009). In *A. thaliana*, nine genes code for Shaker-like K^+ channels, extended by further six genes belonging to structurally separate potassium channel subgroups (see Fig. 1a and below). Collections of T-DNA insertion mutants provide powerful tools to study the physiology of plant potassium channels and their role in nutrition, growth, or movements. Due to functional redundancy, however, single potassium channel knockout mutants in Arabidopsis hardly display obvious growth phenotypes. One of a few exceptions is represented by the *akt1-1* mutant, which is affected in the root K^+ uptake channel AKT1. This mutant is characterized by impaired growth under limiting K^+ supply, demonstrating the importance of AKT1 for potassium nutrition (Hirsch et al. 1998; Spalding et al. 1999).

In recent studies, increasing evidence is provided for the posttranslational regulation of plant K^+ channels by phosphorylation/dephosphorylation events or through interaction with regulatory proteins. These studies put plant potassium channels into the context of novel, plant-specific signaling networks, paving the way for a forthcoming chapter in plant potassium channel research.

2 Milestones in Plant Potassium Channel Research

Although potassium transport was studied more than 50 years ago using radio tracer flux studies (Epstein 1964; Epstein et al. 1963; Holt and Volk 1945), it was not until the invention of the patch clamp technique when single plant potassium channels were discovered for the first time in protoplasts of guard cells and pulvinus motor cells (Hedrich et al. 1987; Iijima and Hagiwara 1987; Moran et al. 1988; Satter and Moran 1988; Schroeder et al. 1984). Follow-up studies identified potassium channels in plasma membranes and vacuolar membranes from all plant cell types investigated (Hedrich and Becker 1994; Hedrich and Schroeder 1989; Schroeder and Hedrich 1989). Despite the numerous reports on the electrophysiological characterization of plant potassium channels from different species and cell types, it was not until 1992 when the first plant genes encoding voltage-dependent K^+ channels were identified. Using a K^+ uptake-deficient yeast strain in a complementation screen of an Arabidopsis cDNA library, the groups of Rick Gaber and Hervé Sentenac independently succeeded in the isolation of the *A. thaliana* KAT1 and AKT1, respectively (Anderson et al. 1992; Sentenac et al. 1992). KAT1 was shown to exhibit the typical properties of a voltage-dependent K^+ channel following heterologous expression in *Xenopus* oocytes and was the first inward rectifying K^+ channel functionally characterized (Schachtman et al. 1992). Animal Shaker-like K^+ channels known at the time were solely slowly activating, potassium efflux channels (delayed outward rectifiers) opening at depolarized membrane potentials. In contrast, KAT1 opens at very negative, hyperpolarized membrane potentials and thus advanced to a paradigm for structure–function studies on voltage sensing and selectivity.

Following their first molecular identification, genome sequencing projects and bioinformatics revealed the existence of genes encoding potassium channels in all

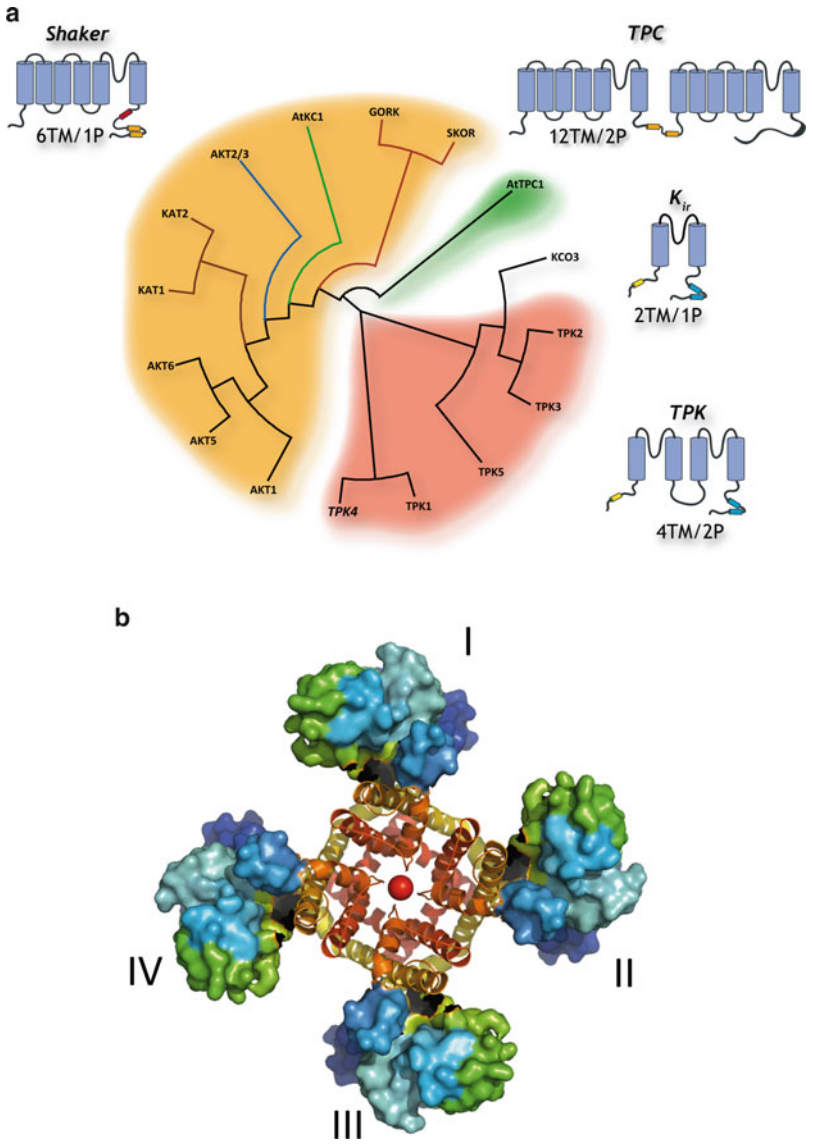

Fig. 1 Phylogeny and Topology of plant K^+ channels. (**a**) Based on sequence comparison plant, K^+ channels can be subdivided into four different families, which also manifest in different topology structures: Shaker-like channels (6TM/1P), TPC-like channels (2 × 6TM/1P), KCO3-like channels (2TM/1P), and TPK-like channels (4TM/2P). *TM*, transmembrane domain, *P* pore domain. (**b**) Top view of a Shaker-like potassium channel model consisting of four homo-subunits. Note that four P-domains are required to build the selectivity filter

living organisms and even in viruses (MacKinnon 2003). A potassium channel "signature" was identified, which encompasses the amino acid sequence TTVTGYGD and is found in the selectivity filter of all potassium selective channels. The simplest architecture of a K^+ channel protein is comprised of only two membrane-spanning helices (TM) connected by a reentry loop, which is only partially crossing the membrane (see Fig. 1a). The *E. coli* KcsA protein resembles the paradigm for this type of potassium channel. The structure of KcsA was the first channel structure solved (Doyle et al. 1998). The KcsA crystal structure showed the symmetrical tetrameric organization of the holochannel protein, with the "signature" containing loop contributing each by one-fourth to the ion permeation pathway (pore region; Fig. 1b). During evolution, gene duplication events seem to have resulted in the emergence of potassium channel exhibiting twice the KcsA structure, i.e., four membrane-spanning regions and two pore regions (P). The Shaker-type K^+ channels, finally, exhibit six transmembrane domains. The last two TM domains, five and six, are connected by the P-loop and resemble the KcsA structure. The first four TM domains harbor a voltage sensor in helix four (S4), a feature that was recently found to be shared with other enzymes such as voltage-dependent phosphatases and proton-permeable channels (Okamura 2007).

The family of plant potassium-selective channels can be grouped into three different structural classes: (1) KcsA-type channels, exhibiting two transmembrane domains (TM) and one pore (P) region, (2) TPK-type channels exhibiting a 4TM/2P structure, and (3) Shaker-type channels characterized by a 6TM/1P topology (Fig. 1). While KcsA-type channels appear absent from poplar or rice genomes, TPK- as well as Shaker-type channels, although varying in number, are conserved in higher plant genomes (for review see Ward et al. 2009; and references therein). A functional K^+ channel is generated by the assembly of four P-loops, connoting that KcsA-type as well Shaker-type potassium channels form tetramers, while TPK channels form dimers. The Shaker-type channels, among them KAT1 and AKT1, are encoded by nine different genes in the Arabidopsis genome and represent the best characterized K^+ channels in plants. Plant Shaker-type channels share a common structure consisting of six α-helices, a reentry loop between TM5 and 6 constituting the selectivity filter (pore = P) and harbor a voltage-sensing domain in TM4 enriched in positively charged residues. However, besides exhibiting discrete expression patterns, plant Shaker-type channels show different biophysical and thus functional properties (Fig. 2). While the Arabidopsis channels KAT1, KAT2, AKT1, and SPIK resemble the typical properties of voltage-dependent, hyperpolarization-activated inward rectifiers (i.e., K^+ uptake channels; K_{in}), *s*telar K^+ *o*utward *r*ectifier (SKOR) and *g*uard cell *o*utward *r*ectifier K^+ (GORK) represent outward rectifying K^+ channels and are activated at depolarized membrane voltages (i.e., K^+ release channels, K_{out}). The phloem-localized AKT2 channel is almost voltage-independent (K_{weak}) and is capable of mediating potassium uptake as well as release. Patch-clamp studies on root hairs revealed the activity of K^+ uptake channels formed by the Shaker subunits AKT1 and AtKC1 (Reintanz et al. 2002). Since in the *akt1* mutant channel activity was completely lost, the remaining AtKC1 channel was considered representing a silent subunit (K_{silent}) regulating

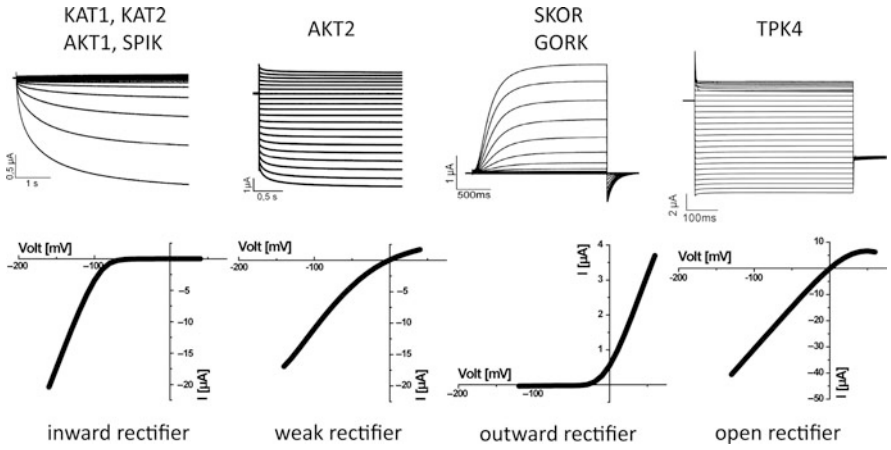

Fig. 2 Functional diversity among plant K^+ channels. (*left* to *right*) Among the structurally related Shaker-like potassium channels, hyperpolarization-activated inward rectifiers (e.g., KAT1), weakly rectifying channels (e.g., AKT2), and depolarization-activated outward rectifiers (e.g., GORK) are found. Members of the TPK family such as TPK4 and TPK1 do not possess an intrinsic voltage sensor and act as the so-called "open rectifiers"

AKT1 activity in a heterotetrameric complex of both channels (see below). Due to its close relationship to SPIK (AKT5), the AKT6 channel is suspected to function as a voltage-dependent inward rectifier.

The Shaker-type channels belong to a species overarching superfamily of voltage-gated K^+ channels (Kv) in which opening and closing transitions (gating) are controlled by the membrane potential. Sensing changes in membrane potential relies on a cluster of positively charged residues on the fourth transmembrane segment (S4), and gating is initiated through conformational changes in the voltage-sensing domains (VSD) of each subunit. Much of our understanding on how movements of the VSD domain translate into channel opening or closing was gained by analyses of potassium channel crystal structures (Doyle et al. 1998; Jiang et al. 2003; Kuo et al. 2003; Long et al. 2005, 2007). When with KvAP, the structure of the first voltage-dependent K^+ channel was solved and the VSD domains appeared as "paddle"-like structures on the channel's outer perimeter surrounding the ion-conduction pore (Jiang et al. 2003). The proposed model suggested these voltage-sensor paddles moving in response to membrane voltage changes, thereby carrying their positive charge across the membrane. Under certain conditions, the patch-clamp technique allows the analysis of these "gating currents."

In outward-rectifying Shaker K^+ channels, an outward displacement of the charges contained in S4 is observed upon channel opening. In plant-inward rectifiers such as KAT1, an inward movement of S4 leading to channel opening is observed, suggesting that the difference between it and outward-rectifying channels resides in the mechanism that couples gating charge displacement with pore opening (Latorre et al. 2003).

Plant K_{in} channels, such as KAT1, KAT2, or AKT1, activate at hyperpolarized membrane potentials (around −80 to −100 mV), and gating of these channels is

almost independent of the prevalent potassium concentrations at both sides of the membrane. In contrast, K_{out} channels, e.g., GORK and SKOR, activate at depolarized membrane potentials, and gating strongly depends on the extracellular K^+ concentration, allowing channel opening positive of the equilibrium potential of potassium (E_K). Thus, even at minute (micromolar) external K^+ concentrations and a steep driving force for K^+ release, channels are kept in the closed state preventing potassium loss from cells under unfavorable conditions of K^+ supply (Ache et al. 2000; Johansson et al. 2006). The weakly rectifying K^+ channels, such as the AKT2 channel, seem to represent a special case of K_{in} channels since it was shown to operate in two gating modes. Depending on its phosphorylation state, the AKT2 channel is shifted from a voltage-independent mode into a K_{in}-like, voltage-dependent mode (Michard et al. 2005). Structure–function studies based on site-directed mutagenesis and domain swapping between K_{in}, K_{out}, and K_{weak} channels have identified key residues involved in gating control and rectification of plant potassium channels (Becker et al. 1996; Gajdanowicz et al. 2009; Geiger et al. 2002; Hoth et al. 2001; Lai et al. 2005; Li et al. 2008; Marten and Hoshi 1998; Poree et al. 2005; Tang et al. 2000). These studies succeeded in the conversion of, e.g., K_{out} in to inward or weakly rectifying channels. The finding that several regions in Shaker-type plant K^+ channels – including the cytosolic N- and C-termini, the S4 domain, the P-region, as well as TM6 – contribute to gating control suggests that distributed residues interact in concert to control the biophysical properties of plant potassium channels (for review see Dreyer and Blatt 2009; Latorre et al. 2003).

Functional diversity of plant Shaker-type K^+ channels additionally is brought about by heteromeric assembly of different channel subunits (see below). Based on yeast-two-hybrid studies, the split-ubiquitin system, as well as electrophysiological studies of coexpressed subunits, it was shown that the C-terminal region is critical for channel assembly (Daram et al. 1997; Dreyer et al. 2004; Naso et al. 2009; Obrdlik et al. 2004). According to these studies, Arabidopsis Shaker-type potassium channels are subdivided into five groups: group I, encompassing the K_{in} channels AKT1, 5, and 6; group II, with the K_{in} channels KAT1 and KAT2; group III, containing the K_{weak} channel AKT2 as the sole member; group IV, resembled by the K_{silent} subunit AtKC1, and group V, represented by the K_{out} channels GORK and SKOR (see Lebaudy et al. 2007 for review). Within a given group, members seem able to form functional heteromers. Assembly of heteromeric channels has been reported between members of group I/III (e.g., AKT1/AKT2), group II/III (e.g., KAT1/AKT2), or group I/IV (e.g., AKT1/AtKC1). Due to structural differences in their C-termini, however, members of group V (i.e., K_{out} channels GORK and SKOR), although assembling into heteromeric K_{out} channel complexes, do not assemble into heterotetramers with any other member of the plant Shaker-type K^+ channels (Ache et al. 2000; Dreyer et al. 2004; Lebaudy et al. 2007). Depending on the localization and coexpression of individual Shaker channels (see Fig. 3), heteromeric assembly of different channel alpha-subunits adds to the functional diversity of plant K^+ channels and resembles a regulatory mean to control K^+ homeostasis in many cell types.

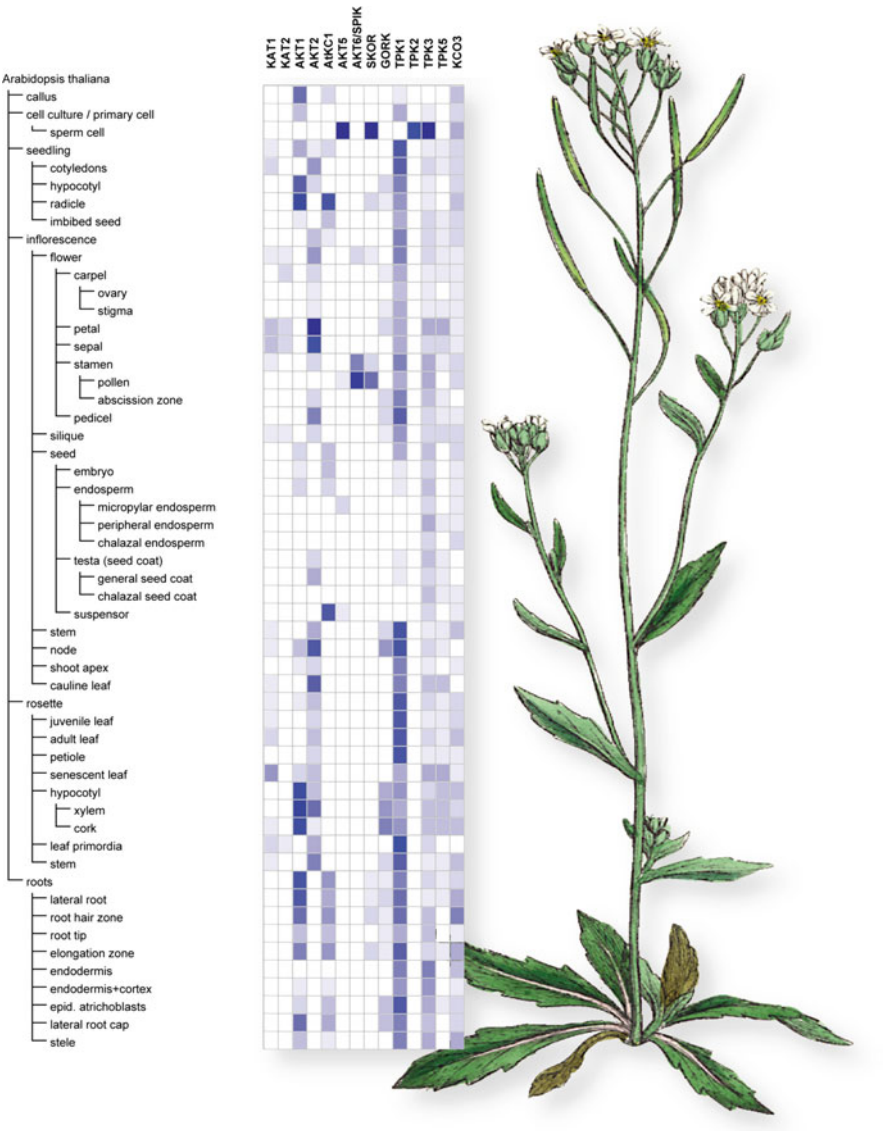

Fig. 3 Expression profile of Arabidopsis K^+ channels. In line with their housekeeping function in potassium homeostasis according to micro array-based transcriptome profiling (Genevestigator) Arabidopsis K^+ channels appear ubiquitously expressed throughout the different organs and life cycle of the Arabidopsis plant

3 Stomatal Movement Is Based on the Reversible Expansion of Guard Cell Pairs

CO_2 uptake and transpiration in plants is accomplished by stomatal pores surrounded by a pair of guard cells in the leaf epidermis. Triggered by various environmental or endogenous signals through reversible volume changes, the two guard cells regulate stomatal aperture. For stomata to open, guard cells take up and accumulate potassium. This process is mediated via K^+-uptake channels. H^+ pumps hyperpolarize the plasma membrane, thereby generating the voltage gradient that drives K^+ inflow. Upon stomatal closure, the membrane is depolarized and efflux of K^+ ions from guard cells is predominantly driven by the chemical gradient (see Fig. 4a). Since K^+ channels allow rapid fluxes of this cation across the plasma and vacuolar membrane, guard cells have been established as a model system to study plant potassium transport (Assmann and Wang 2001; Dietrich et al. 2001; MacRobbie 1998; Roelfsema and Hedrich 2005; Schroeder et al. 2001). The first guard cell K^+ channel, KST1, was cloned from potato by Müller-Rober et al. (1995), while at the same time, the first discovered K^+ channel KAT1 turned out to be expressed in guard cells, too (Nakamura et al. 1995). KAT1, like its counterparts in other plant species, represents a hyperpolarization-activated, pH-sensitive K^+ uptake channel. Extracellular as well as cytosolic protons act on discrete His residues in the channel protein and, by protonation, increase the voltage-sensitivity of KAT1 (Hoth et al. 1997, 2001; Müller-Rober et al. 1995). During stomatal opening when pumping activity of the H^+-ATPase is high, this leads to an increased number of open channels. In addition, the activity of KAT1 is known to be regulated by cytosolic 14-3-3 proteins. The 14-3-3 proteins represent well-known regulatory factors of the nitrate reductase, vacuolar channels, and the plasma membrane H^+ pump (Latz et al. 2007a; van den Wijngaard et al. 2001). Upon interaction with KAT1, 14-3-3 proteins enhance channel activity through an increased channel open probability as well as the total number of channels in the plasma membrane (Sottocornola et al. 2006, 2008).

During stomatal closure, K^+ uptake channels such as KAT1 need to be closed and K^+ efflux channels have to be opened. The phytohormone abscisic acid (ABA) represents a key signal-inducing stomatal closure. Guard cell sensitivity towards ABA is under the control of a pair of negative regulators – the PP2C-like phosphatases *AB*A-*i*nsensitive *1* and *2* – and a positive regulator, the protein kinase open *st*omata *1* (OST1). Patch clamp studies have demonstrated that the activity of guard cell K_{in}^+ channels was reduced in response to ABA. Recent studies by Uozumis group identified the OST1 kinase to exert this effect (Sato et al. 2009). OST1 phosphorylates two threonine residues in the C-terminus of KAT1, thereby reducing the potassium transport activity of the channel. In guard cells, the number of KAT1 channels in the plasma membrane seems further to be regulated by turgor-controlled endo- and exocytosis events (Hurst et al. 2004; Meckel et al. 2005). Upon stomatal opening, when guard cell surface area increases up to 25% (Meckel et al. 2007), a preplasma membrane pool carrying clusters of K_{in}^+ channels is ready

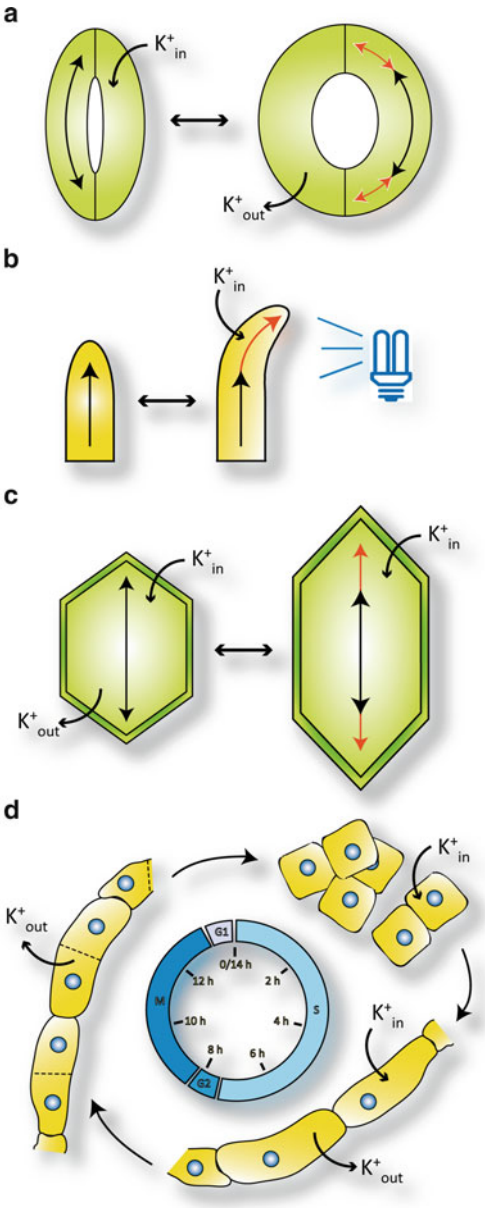

Fig. 4 K^+ channels control reversible and irreversible volume changes in plants. (**a**) Guard cells represent a model system to study the involvement of K^+ channels during reversible, nastic movements in response to a variety of abiotic and biotic signals. (**b**) Grass coleoptiles serve as a model system to investigate the role of potassium channels in irreversible phototropic responses and auxin action. (**c**) Likewise, irreversible plant cell growth is driven by osmotic forces generated by components of the osmotic motor, including proton pumps, ion channels, and transporters. (**d**) Inward and outward rectifying K^+ channels are differentially expressed throughout the cell cycle and their activity feeds back on cell cycle progression

to be incorporated into the plasma membrane. During stomatal closure when the guard cells volume and surface area decreases, endocytic retrieval of membrane vesicles containing K_{in}^+ channels is observed (Sutter et al. 2007).

The initial finding that guard cells exhibit high activity of Shaker α-subunits of K_{in} channels is corroborated by the simultaneous expression of several K^+ channel

genes encoding K_{in} channels, including *KAT1*, *KAT2*, *AKT1*, *AKT2*, and *AtKC1* (Szyroki et al. 2001). The biophysical and pharmacological properties of the "guard cell inward rectifier" resemble a combination of individual α-subunit characteristics such as calcium or pH sensitivity possibly brought about by heteromeric assembly (Ivashikina et al. 2005; Latz et al. 2007b). The body of K_{in} channels far exceeds the physiological requirement of K^+ transport during stomatal opening and might explain why *kat1-1* mutants or transgenic plants expressing a dominant negative *kat1* mutant hardly affect the dynamics of stomatal opening (Kwak et al. 2001). The Arabidopsis *kincless* mutant, however, representing a double mutant expressing a *kat2*-dominant negative construct in the background of the *kat2-1* T-DNA insertion mutant, completely lacks inward potassium currents. Circadian rhythm as well as stomatal opening in response to various stimuli is strongly impaired in *kincless*. Depending on the environmental conditions, this work showed that guard cell K_{in}^+ activity can dramatically feed back on plant biomass production, underlining the general importance of K_{in} channels for stomatal physiology (Lebaudy et al. 2008).

Two outward rectifying K^+ channels, GORK and SKOR, are encoded in the Arabidopsis genome. In contrast to K_{in} channels, GORK represents the only potassium efflux channel expressed in guard cells (Ache et al. 2000; Becker et al. 2003). Expression of a GORK-dominant negative mutant in transgenic Arabidopsis as well as disruption of the GORK gene leads to impaired stomatal closure upon drought stress and enhanced transpirational water loss compared to wild-type plants (Hosy et al. 2003).

4 Growth Results from Irreversible Cell Expansion

Monocot coleoptiles and dicot hypocotyls represent classical as well as modern models for studying auxin-dependent cell expansion. Auxin-induced growth of coleoptiles depends on the presence of potassium, but in contrast to, e.g., stomatal movements represents an irreversible process (Becker and Hedrich 2002; Fuchs et al. 2006). Maize coleoptiles express the Shaker-type channels ZMK1 and ZMK2 in nonvascular cells and the vasculature, respectively (Philippar et al. 1999). Upon gravistimulation, the phytohormone auxin, synthesized in the coleoptile tip, is redistributed towards the lower coleoptile half. This results in enhanced growth of cells facing higher auxin concentrations and upward bending of the coleoptile, away from the gravitropic vector. K^+ accumulation into the lower coleoptile cells plays a fundamental role in this process. In line with a key role of potassium channels in gravitropism, K^+ channel density of the K^+ uptake channel ZMK1, which is under transcriptional control of auxin, is highly increased in these cells. The phototropic response, i.e., bending of the coleoptile towards the light stimulus, represents a differential growth response, and like gravitropism is controlled by auxin gradients across the growing coleoptiles (see Fig. 4b). Light-stimulated coleoptiles growing on a rotating clinostat (at nominally "zero" gravity) exhibited differential ZMK1 transcription in both coleoptile halves. Again, ZMK1 transcripts

were highest in the fast growing, shaded flank of the coleoptile. This suggests that the photo- and gravitropic response of the coleoptile feeds into a common signaling pathway, resulting in auxin redistribution in the coleoptile tip and finally in differential transcription of the K^+ uptake channel ZMK1. In dicotyledonous plants, such as *A. thaliana*, related growth phenomena can be studied on etiolated hypocotyls. In dark-grown Arabidopsis seedlings, the K^+ uptake channel KAT1 is expressed in the cortex and epidermis of etiolated hypocotyls (Philippar et al. 2004). KAT1 transcripts were found to be induced by active auxins as well as in auxin-sensitive tissues characterized by rapid cell elongation, suggesting that KAT1 is involved in elongation growth of Arabidopsis.

5 Polar Growth is Best Studied in Root Hairs and Pollen Tubes

5.1 Root Hairs

Besides guard cells, root hairs represent a versatile cellular model for studying K^+ transport in the context of potassium nutrition. Root hairs represent polar growing epidermal cells exhibiting K^+ influx at the growing tip and K^+ efflux at the base (Ivashikina et al. 2001) (see Fig. 4c). They express the Shaker-type α-subunits AKT1, AtKC1, and GORK (Daram et al. 1997; Ivashikina et al. 2001). In addition to its importance for root hair growth (Desbrosses et al. 2003), AKT1 seems to be essential for overall root K^+ uptake, and the corresponding *akt1-1* mutant shows severe growth retardation under conditions of K^+ starvation (Dennison et al. 2001; Rubio et al. 2008; Spalding et al. 1999). Despite the presence of AtKC1 transcripts, patch-clamp studies on root hair protoplasts revealed a complete absence of K_{in} currents in the *akt1-1* mutant (Reintanz et al. 2002). Based on altered biophysical properties of the root hair K_{in} in wt compared to *atkc1-1* mutants, the AtKC1 channel was designated a modulatory subunit in heteromeric K^+ uptake channel complexes. Verification of this hypothesis was long hampered by the fact that neither AKT1 nor AtKC1 formed functional channels when expressed in heterologous expression systems such as mammalian cell lines or Xenopus oocytes. Recent work, however, has shown that during K^+ starvation, the AKT1 channel protein is subject to activation via a Ca^{2+}-dependent signaling network. According to current models, limiting K^+ supply is suggested to feed back on cytosolic calcium signals sensed via distinct Calcineurin-B-like (CBL) proteins, which contain two calcium-binding EF-hands. Upon assembly with their interacting protein kinase (CIPK), the CBL/CIPK complex is targeted to the plasma membrane to activate the AKT1 channel in a phosphorylation-dependent manner (Hedrich and Kudla 2006; Lee et al. 2007; Li et al. 2006; Xu et al. 2006). While AKT1 phosphorylation leads to channel activation promoting K^+ uptake channel dephosphorylation by the PP2C phosphatase, AIP1 inactivates AKT1 (Lee et al. 2007). Heterologous coexpression of Ca^{2+}-sensors (CBLs), CBL-interacting protein kinases (CIPKs), and AKT1 in

Xenopus oocytes allowed functional characterization of AKT1 channels, and elucidating the role of the root hair expressed AtKC1 channel. In line with previous studies in yeast (Bertl et al. 1997), these studies revealed that AKT1 represents a voltage-dependent inward rectifier and AtKC1 a corresponding regulatory α-subunit of physiological importance (Geiger et al. 2009). Voltage-dependent activation of AKT1 occurs at a more or less fixed voltage (ca. −80 mV) and is independent of the actual K^+ gradient. According to the Nernst equation under conditions of potassium limitation, the equilibrium potential (E_K) would be far negative of the channel's activation threshold (at, e.g., 100 μM K_{soil}^+ and 100 mM $K_{cyt;}^+$ $E_K = -180$ mV), and consequently upon activation of AKT1, a pronounced K^+ efflux is observed (Fig. 3). In plant, however, heteromeric assembly of AKT1/AtKC1 shifts the activation threshold of the root K^+ uptake channel complex towards very negative membrane potentials (negative of E_K) and thus prevents potassium loss under unfavorable conditions of K^+ supply (Geiger et al. 2009; Reintanz et al. 2002). In line with this physiologically important, regulatory role of this channel, AtKC1 alone when transiently expressed in mesophyll protoplasts is retarded in the endoplasmic reticulum and requires, e.g., the AKT1 Shaker-type α-subunit for ER escape (Duby et al. 2008; Geiger et al. 2009).

5.2 Pollen Tubes

Alike root hairs pollen tubes emerging from the germinating pollen grain represent tip growing cells. The Shaker-type potassium channel AKT5 (Shaker Pollen Inward *K*$^+$ channel; SPIK) and the *T*andem *P*ore *K*$^+$ channel TPK4 are located in the plasma membrane of pollen tubes. SPIK was shown to function as a voltage-dependent K^+ channel following functional expression in mammalian COS cells (Mouline et al. 2002) and a T-DNA insertion mutant of SPIK exhibited impaired pollen germination and tube development.

TPK4 is exclusively expressed in pollen and was the first functionally characterized member of the family of tandem pore K^+ channels (Becker et al. 2004). TPK4 represents the only member of this family located in the plasma membrane. Dunkel 2008; Czempinski et al. 2002; Latz et al. 2007a). The TPK4 channel exhibits the hallmarks of a so-called "open rectifier" – i.e., voltage-independent instantaneous gating and saturating K^+ currents at increasing positive membrane voltages. Pollen tubes as well as root hairs achieve polar growth by continuous delivery (exocytosis) of membrane vesicles at their tip regions. The fragile tip region is highly sensitive to mechanical forces and osmotic changes. Turgor control in these cell types thus is believed to take place in a narrow osmotic window through fine tuning of the activities of inward and outward rectifying K^+ channels. In line with this hypothesis, in addition to potassium uptake channels, pollen tubes express the K^+ efflux channels GORK and SKOR, and root hairs GORK only (Fig. 4c).

6 Long Distance K^+ Transport in Plants

Besides cellular K^+ homeostasis plant potassium channels are involved in long distance K^+ transport from root to shoot and *vice versa*. As described above, K^+ uptake from the soil into the root is mediated via the AKT1/AtKC1 potassium channel heteromer. In line with this, both Shaker-type channels are expressed throughout the root tissues with the exception of endodermis cells. The endodermal layer compels a switchover from apoplastic to symplastic nutrient transport. None of the plasma membrane located Shaker-type inward rectifiers seems to be expressed in this cell type, suggesting that K^+ uptake into endodermal cells is mediated by proton-coupled potassium symport. Loading of potassium into xylem vessels and thus root to shoot long distance transport depends on SKOR channel function. This K^+ efflux channel is expressed in pericycle cells and in the parenchyma cells surrounding the xylem vessels (Gaymard et al. 1998). In the *skor-1* mutant, root K^+ content is unaffected but a 50% decrease in shoot K^+ is observed, which – on the basis of root exudate analysis – is likely to result from a decrease in the rate of potassium translocation from roots to shoots via xylem sap flux. The expression of the two Arabidopsis K^+ efflux channels is under the control of the stress hormone ABA. *SKOR* is transcriptionally downregulated by ABA while *GORK* is activated, suggesting that both channels contribute to the whole-plant response to water stress.

Retrograde potassium transport from shoot to root occurs via the phloem. Arabidopsis phloem cells express the AKT2 and the KAT2 channel. While KAT2 represents a voltage-dependent K^+ inward rectifier, AKT2, when expressed in Xenopus oocytes, alike its orthologs from other plants, is only weakly voltage-dependent, capable of exhibiting potassium efflux at depolarized membrane potentials (Ache et al. 2001; Lacombe et al. 2000; Philippar et al. 2003). In a phosphorylation-dependent manner *in planta*, AKT2 activity, however, may be switched from voltage-independent to voltage-dependent inward rectifying (Michard et al. 2005). Studies by Deeken et al. (2002) could show that *AKT2* transcription is under the control of photosynthates and that disruption of the AKT2 channel results in impaired sugar loading into the phloem (Deeken et al. 2002). Active sugar loading into the phloem is coupled to H^+ symport and thus associated with membrane potential depolarization, which negatively feeds back on sugar uptake. In the presence of high AKT2 activity, however, H^+/sugar transport-mediated onset of depolarization is counteracted by concomitant K^+ efflux. This clamps the membrane potential close to the equilibrium potential of potassium (E_K) and provides means for increased sugar accumulation.

7 Potassium Channels Control Cell Cycle Progression

Tobacco bright yellow-2 (BY-2) cells represent suspension cultured cells that advanced to the model system for studying cell cycle regulation in plants (Nagata et al. 2006) (see Fig. 4d). K^+ channel blocker studies with aphidicolin-synchronized

tobacco BY-2 cells revealed that potassium uptake is required for proper cell cycle progression during the transition from G_1 to S phase (Sano et al. 2007). BY-2 cells express four Shaker-like K^+ channel genes. Among them, NKT1 represents an inwardly rectifying K^+ channel that is transcriptionally induced during G_1 phase. NKT1 activity and thus potassium accumulation is highest in elongating S-phase cells. These cells appear hyperpolarized and exhibit elevated osmotic pressure, approximately twice the turgor pressure when compared with depolarized dividing cells. Inhibition of NKT1 activity arrests BY-2 cells in G_1 phase and shows that channel-mediated K^+ uptake in BY-2 cells and turgor formation are fundamental for expansion, growth, and cell cycle transition from G_1 to S phase (Fig. 4d).

In contrast to NKT1, transcripts of the outward rectifier NTORK1 were shown to dominate elongating cells in S phase. Repression of the *NTORK1* gene by means of expression of its antisense construct repressed cell division but accelerated cell elongation even under conditions promoting cell division. In line with this observation, dividing cells (in M-phase) exhibit a decrease in their K^+ content of cells and cellular osmotic pressure. Thus, K^+ uptake is not required for cell division, but cells have to gain a threshold K^+ level to reenter the cell cycle, a process that is regulated through modulation of K^+ channel density and activity (Sano et al. 2009).

8 Vacuolar K^+ Channels

Potassium transport across the vacuolar membrane is accomplished by proton-coupled antiporters and ion channels (MacRobbie and Kurup 2007; Schulz-Lessdorf et al. 1994; Walker et al. 1996). Among the latter, a slow-activating, cation-selective channel (SV channel) is ubiquitously found in plant vacuoles across species (Hedrich and Neher 1987). The SV channel requires elevated cytosolic calcium concentrations for activation and is encoded by *TPC1* gene (Allen and Sanders 1996; Hedrich and Neher 1987; Peiter et al. 2005; Ward and Schroeder 1994). TPC1 resembles the structure of a tandem-pore K^+ channel (see Fig. 1), however, consisting of two interconnected Shaker-type α-subunits. In line with its calcium-dependent activation, TPC1 possesses two EF-hands in the cytosolic interconnection loop. Since TPC1 under some experimental conditions is permeable to potassium as well as sodium and calcium ions, its role in salt stress management and calcium signaling is discussed (Ivashikina and Hedrich 2005; Pottosin et al. 2009; Ranf et al. 2008). Besides TPC1, potassium-selective, tandem pore K^+ channels (KCO; TPKs) mediate K^+ transport across the tonoplast (Fig. 1). TPK channels exhibit four transmembrane domains and two pore regions between TMs 1/2 and TMs 3/4. In their cytosolic N-terminal and C-terminal domains, some TPK channels possess a binding site for 14-3-3 proteins and Ca^{2+}-binding EF-hands, respectively. In Arabidopsis, the TPK family encompasses five members. TPK1, 2, 3, and 5 are located in the vacuolar membrane. According to recent studies by Dunkel et al. (2008), TPK targeting to the vacuolar membrane depends on a structural motif in its EF-hand containing C-terminus together with a diacidic motif accelerating ER-export. Based on mutagenesis studies involving chimeric

channels between the plasma membrane-located TPK4 channel (see above) and other members of the TPK family, it seems very likely that all TPK members represent homomeric, voltage-independent K^+ selective channels (own unpublished results) that, in contrast to Shaker-type channels, do not assemble into heteromeric complexes (Voelker et al. 2006). In fact, the well-known vacuolar K^+ channel (VK) has recently been attributed to be encoded by the *TPK1* gene (Bihler et al. 2005; Gobert et al. 2007). Like TPC1, TPK1 activity requires cytosolic calcium ions. In line with the existence of a binding site in the TPK1 N-terminus for 14-3-3 proteins, these general regulatory factors (GRFs) potentiate potassium currents through TPK1 on top of Ca^{2+}-dependent activation (Latz et al. 2007a). Notably, the same 14-3-3 proteins known to activate TPK1 shut down the activity of the SV channel, TPC1. Interaction of TPK1 with 14-3-3 proteins requires the phosphorylation of conserved Ser/Thr residues in the 14-3-3 binding motif. Own studies provide evidence that phosphorylation of TPK1 involves Ca^{2+}-dependent kinases of the CDPK family and thus suggests that cytosolic K^+ homeostasis through vacuolar potassium channels is regulated via Ca^{2+}-dependent signaling networks, too.

9 Outlook

Shaker-type potassium channels represent the best-characterized transport proteins in plants, so far. With the exception of AKT5, all other α-subunits have been functionally characterized. Given that AKT5 is expressed highest in sperm cells, it is tempting to speculate that this channel exerts an important function in fertilization. Recent studies on the posttranslational regulation of these channels by Calcium-controlled kinase/phosphatase networks have opened the door towards a deeper understanding of plant K^+ channel physiology in the context of plant adaptation towards salt stress or control of cell cycle progression and water status. Currently, little is known about the dynamics or specificity of, e.g., kinase/channel interactions, the calcium dependency of kinase activity, or the respective target sites for phosphorylation within the channel proteins. Likewise, a detailed functional characterization of vacuolar TPK channels – including the vacuolar localized KCO3 channel – is missing. Work in progress addresses the question of how cellular K^+-homeostasis as well as long distance transport/distribution is connected to regulatory signaling networks that control plant movement, growth, and development.

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Mechanism and Evolution of Calcium Transport Across the Plant Plasma Membrane

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Abstract Calcium is an essential plant nutrient, thus the influx of Ca^{2+} into plant cells is a critical process. In addition, the efflux of Ca^{2+} out of a cell is important to prevent toxicity resulting from Ca^{2+} excess, and to modulate levels of cytosolic Ca^{2+} required for signaling functions. Biochemical and genetic analysis of plants has begun to identify the complement of Ca^{2+}-permeable channels and Ca^{2+}-ATPases that function in Ca^{2+} flux across the plasma membrane. In addition to understanding the mechanisms of plasma membrane Ca^{2+} transport, some of the specific functions of these pathways are now emerging. Comparative genomics of higher plant, algal and moss species has identified significant variation in the mechanisms of plasma membrane Ca^{2+} transport between higher and lower plants, and provides insight into the evolution of Ca^{2+} transport processes.

1 Introduction

All organisms exploit the unique properties of Ca^{2+} to carry out essential biological functions (Berridge et al. 2003). Ca^{2+} is an important nutrient with diverse intra- and extracellular functions that include a critical role as a life or death signal (Hofer and Brown 2003). In plants, various external stimuli trigger intracellular Ca^{2+} increases including light, temperature shifts, oxidative stresses, hormones, and pathogen elicitors (McAinsh and Pittman 2009). Furthermore, intracellular Ca^{2+} concentrations in plants have been shown to display circadian oscillations that are

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much more complex than that of animals. In animals, the cell-surface receptors and ion channels responsible for these Ca^{2+} increases have mostly been identified (Berridge et al. 2003); however, homologs of most of these animal counterparts do not exist in the sequenced plant genomes, implying that plants have unique sensory systems and Ca^{2+} channels. Plant biologists continue to search for and characterize the Ca^{2+} transporters on the plasma membrane (PM). Here, we review the present state of the field while acknowledging the dearth of molecularly characterized plant PM Ca^{2+} transporters.

2 PM Ca^{2+} Transport Pathways

Multiple pathways have evolved for the accumulation of Ca^{2+} in plant cells and the regulation of cytosolic Ca^{2+} via PM transport (Bothwell and Ng 2005). These pathways include a variety of Ca^{2+} influx channels and Ca^{2+} efflux transporters (Fig. 1), which, in combination with endomembrane efflux transporters (Pittman and Hirschi 2003), rapidly remove Ca^{2+} from the cytosol, thus preventing cytosolic Ca^{2+} from rising to toxic levels.

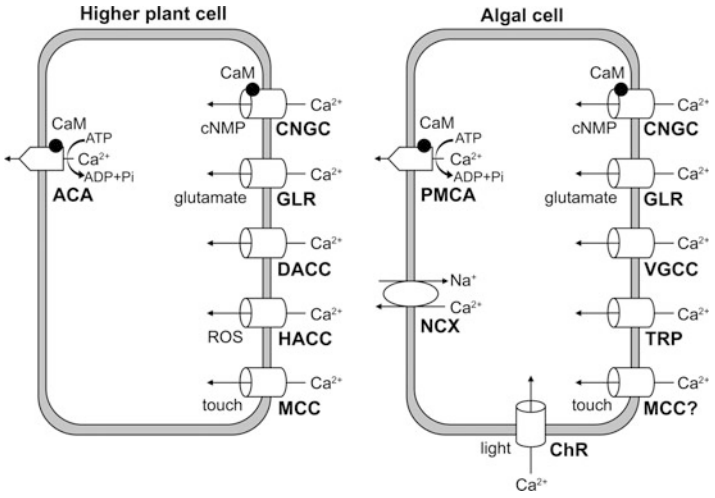

Fig. 1 Summary of PM Ca^{2+} transporters in a typical higher plant and algal cell. Definitions: *ACA* autoinhibited Ca^{2+}-ATPase, *CNGC* cyclic nucleotide-gated channel, *ChR* channelrhodopsin, *DACC* depolarization-activated Ca^{2+} channel, *GLR* glutamate receptor-like channel, *HACC* hyperpolarization-activated Ca^{2+} channel, *MCC* mechanosensitive Ca^{2+} channel, *NCX* Na^+/Ca^{2+} exchanger, *PMCA* PM Ca^{2+}-ATPase, *TRP* transient receptor potential channel, *VGCC* voltage-gated Ca^{2+} channel. Some transporter regulators are indicated, including calmodulin (CaM), cyclic nucleotide monophosphates (cNMP), and reactive oxygen species (ROS)

2.1 Ca^{2+} Influx Channels

Ca^{2+} channels at the PM have the fundamental function of providing Ca^{2+} to the cell, but clearly these transporters are also important for the generation of stimulus-specific Ca^{2+} signals (McAinsh and Pittman 2009). Many PM Ca^{2+} channels have been characterized electrophysiologically from a variety of plant tissues and can be characterized into three main groups: the mechanosensitive Ca^{2+} channel (MCC), the depolarization-activated Ca^{2+} channel (DACC), and the hyperpolarization-activated Ca^{2+} channel (HACC) (Fig. 1) (Demidchik and Maathuis 2007). These can all be regarded as nonselective cation channels as they are permeable to a range of cations in addition to Ca^{2+}, rather than being Ca^{2+}-selective (Demidchik and Maathuis 2007). Unlike in animal cells, there are only a few examples where the molecular identity of the plant PM Ca^{2+}-permeable channel has been established.

2.1.1 Ca^{2+} Influx by Nonselective Cation Channels

The characteristics of MCC and DACC channel types remain largely obscure. MCCs may play a role in the cell's response to mechanical stimuli. MCC activity has been recorded in a variety of cell types and there is good evidence for mechanically induced increases in cytosolic Ca^{2+} (Dutta and Robinson 2004; Monshausen et al. 2007). Recently, two members of the *Arabidopsis* mechanosensitive channel of small conductance-like (MSL) protein family, MSL9 and MSL10, were shown to be localized at the PM and mediate mechanically stimulated Ca^{2+}-permeable channel activity, although they are more permeable to Cl^- than Ca^{2+} (Haswell et al. 2008). In addition, two *Arabidopsis* genes called *MCA1* and *MCA2* are good candidates for encoding MCC activity (Yamanaka et al. 2010). DACCs are thought to play a role in Ca^{2+} influx in response to environmental stress, which often induces PM depolarization, and DACC activity has been identified in a variety of plant species and in different tissues (Demidchik and Maathuis 2007). Although studies suggest that a primary physiological function of DACCs is the efflux of monovalent cations such as K^+, their role in Ca^{2+} influx has been indicated in some tissues, such as maize roots (Roberts and Tester 1997), and they have been proposed to be involved in cytosolic Ca^{2+} signal generation in response to low-temperature stress (White 2009). However, the genes encoding these channels are still unknown.

Our understanding of Ca^{2+} influx into a cell via HACCs is much clearer. HACCs have been reported in many different cell types, but have been most fully studied in the guard cell and root hair systems (e.g., Pei et al. 2000; Foreman et al. 2003). Studies in both of these cell types have clearly demonstrated the role of reactive oxygen species (ROS) in stimulating HACC activity and Ca^{2+} influx (Pei et al. 2000; Foreman et al. 2003; Kwak et al. 2003). For example, in an at*rbohD* at*rbohF* guard cell NADPH oxidase double mutant, HACC activity is significantly impaired, leading to changes in stomatal aperture (Kwak et al. 2003). Similarly,

Ca^{2+} influx and in turn root hair formation are inhibited in the *Arabidopsis rhd2* NADPH oxidase mutant (Foreman et al. 2003). ROS thus appears to be essential for the regulation of HACCs. In addition, there is also evidence for the regulation of guard cell HACC activity by phosphorylation and abscisic acid (ABA) (Köhler and Blatt 2002). The molecular identity of the HACC in both the guard cell and root hair is unclear. In *Arabidopsis* guard cells, a mutation in the gene encoding the ABCC5/MRP5 ABC transporter has inhibited ABA-dependent HACC activation, although it appears that ABCC5 is a regulator of HACC activity rather than the channel itself (Suh et al. 2007).

2.1.2 Gene Candidates for Plant PM Ca^{2+}-Permeable Channels

There are only two gene families to date which are very good candidates for encoding some of the observed PM Ca^{2+}-permeable channel activity. These are the cyclic nucleotide-gated channel (CNGC) genes (Kaplan et al. 2007) and the glutamate receptor-like (GLR) genes (Davenport 2002) (Fig. 1). Cation channels activated by cyclic nucleotides, such as cAMP and cGMP, have been observed in many organisms, including plants. For example, cAMP can stimulate Ca^{2+} influx in carrot cells (Kurosaki et al. 1994) and can stimulate HACC activity in guard cells (Lemtiri-Chlieh and Berkowitz 2004). Twenty CNGC genes have been identified in *Arabidopsis* and functional analysis has begun to confirm a role in Ca^{2+} transport for some of these, although some isoforms may be more important as K^+ transporters (Kaplan et al. 2007). For example, *Arabidopsis* CNGC1 and CNGC2 are permeable to Ca^{2+} with some selectivity for K^+ (Leng et al. 2002; Ali et al. 2006), whereas CNGC3 may be more important in K^+ and Na^+ uptake (Gobert et al. 2006). CNGC1 displays Ca^{2+} permeability when expressed in yeast, but only when a C-terminal calmodulin (CaM) binding domain is removed (Ali et al. 2006). This CaM-binding domain is thought to regulate CNGC activity in a manner similar to animal CNGCs. Some of these channels appear to be important during pathogen-mediated defense responses. *Arabidopsis* mutants lacking *CNGC2* and *CNGC4* exhibit enhanced pathogen resistance but lack the hypersensitive response (Clough et al. 2000; Balagué et al. 2003). Another Ca^{2+}-permeable channel CNGC18 is present at the PM of pollen tubes, preferentially at the growing tip (Frietsch et al. 2007). Cytosolic Ca^{2+} gradients in pollen tube tips are important for regulating pollen tube growth; in a *cngc18* knockout mutant, pollen tube growth is significantly impaired (Frietsch et al. 2007).

Mammalian ionotropic glutamate receptor (iGluR) cation channels are activated by glutamate and glycine and play an important role in Ca^{2+}, K^+, and Na^+ influx, and signal generation in the central nervous system (Davenport 2002). Glutamate and glycine have similarly been shown to stimulate depolarization of the PM and increase Ca^{2+} influx in *Arabidopsis* seedlings (Dennison and Spalding 2000; Dubos et al. 2003). Furthermore, glutamate has been observed to regulate a range of Ca^{2+}-dependent processes including root branching (Walch-Liu et al. 2006). Twenty GLR genes have been identified in *Arabidopsis* (Davenport 2002) and there is

increasingly good evidence from genetic analyses that some of these encode the observed glutamate-responsive membrane depolarization and Ca^{2+} influx. Membrane depolarization and the associated rise in cytosolic Ca^{2+} are significantly impaired in an *Arabidopsis glr3.3* knockout mutant (Qi et al. 2006). Conversely, overexpression of a radish GLR in *Arabidopsis* increases glutamate-activated Ca^{2+} influx (Kang et al. 2006). An interesting variation with the plant GLRs compared to the animal orthologs is the observation that a wide variety of amino acids can mediate membrane depolarization and Ca^{2+} influx (Qi et al. 2006; Stephens et al. 2008). Animal iGluR channels are composed of four subunits. It is likely that plant GLR proteins similarly form a tetrameric complex to give a functional Ca^{2+} channel. It has been suggested that different classes of channels are either homomeric complexes, such as those composed of four GLR3.3 subunits in *Arabidopsis* and only activated by glutamate, or are present as a heteromeric channel complex made of GLR3.3 and GLR3.4 subunits and thus have a broader amino acid binding profile (Stephens et al. 2008).

An increasing number of studies are beginning to implicate annexin genes as candidates for encoding PM Ca^{2+} channel activity. Annexins are small, soluble proteins that can bind Ca^{2+} and associate or insert in membranes in a Ca^{2+}-dependent manner. In a recent study, maize annexin proteins ANN33 and ANN35 were expressed in *Arabidopsis* root protoplasts and shown to mediate a cytosolic Ca^{2+} increase (Laohavisit et al. 2009). Furthermore, incorporation of these annexins into planar lipid bilayers lead to a Ca^{2+}-permeable conductance with characteristics typical of a PM Ca^{2+}-permeable nonselective cation channel. However, annexin-dependent Ca^{2+} influx in vivo has yet to be measured and annexin knockout plants await analysis.

The *Arabidopsis TPC1* (two-pore channel 1) gene has been shown to encode the voltage-dependent slow-activating vacuolar Ca^{2+} channel and is clearly localized to the tonoplast (Peiter et al. 2005). It is interesting to note, however, that *TPC1* gene homologs from rice, tobacco, and wheat appear to encode Ca^{2+}-permeable channels located at the PM, suggesting that TPC1 proteins may be differentially targeted to either the tonoplast or PM in different species (Kurusu et al. 2005; Wang et al. 2005).

2.2 Ca^{2+} Efflux Transporters

2.2.1 Ca^{2+}-ATPases

PM Ca^{2+}-ATPase (PMCA) activity has been described in most eukaryotes including plants. These transporters play a major role in maintaining low "resting" levels of cytosolic Ca^{2+} in the submicromolar range. Ca^{2+}-ATPases usually have a high affinity for Ca^{2+} (~0.1–2 μM) and low capacity (Sze et al. 2000). They are members of the P-type ATPase superfamily and can be subdivided into two phylogenetic groups (Axelsen and Palmgren 1998). The P_{2A} subtype includes the animal

sarcoplasmic/endoplasmic reticulum Ca^{2+}-ATPase (SERCA) and plant endoplasmic reticulum-type Ca^{2+}-ATPase (ECA). The P_{2B} subtype includes the animal PM Ca^{2+}-ATPase (PMCA) and plant autoinhibited Ca^{2+}-ATPase (ACA), both of which are regulated by CaM. The plant Ca^{2+} pumps differ from animal pumps with regard to their membrane localization. Both ECAs and ACAs have been found at the PM, tonoplast, and endoplasmic reticulum (Sze et al. 2000; Boursiac and Harper 2007). While all the *Arabidopsis* ECAs characterized to date are located at internal organelle membranes, the tomato ECA-type Ca^{2+}-ATPase LCA1 is an interesting exception. The *LCA1* gene appears to generate at least two alternative transcripts, one of which is localized to the tonoplast and the other isoform present at the PM (Ferrol and Bennett 1996; Navarro-Avino et al. 1999). As yet, there is no evidence of ECAs from other plant species present at the PM. ACAs have likewise been identified at various cell membranes. The ten ACAs present in *Arabidopsis* can be further distinguished phylogenetically into four clusters (Boursiac and Harper 2007). Membrane localization has been determined for many of the ACAs and it appears that each of these four clusters can be defined by membrane location. Of particular interest is the cluster containing *ACA8*, *ACA9*, and *ACA10*. ACA8 and ACA9 are PM-localized (Bonza et al. 2000; Schiøtt et al. 2004), and ACA10 is likely to share this location, although this awaits experimental confirmation.

Genetic analysis has begun to provide some insight into specific functions of PM Ca^{2+}-ATPases. *Arabidopsis ACA9* is expressed almost exclusively in pollen and knockout of *ACA9* leads to a significant impairment of pollen tube growth and reduced fertility (Schiøtt et al. 2004). In contrast, *ACA8* is expressed throughout the vascular tissue and guard cells, and *ACA10* is expressed ubiquitously (Schiøtt and Palmgren 2005; George et al. 2008). *ACA8* and *ACA10* expression is also regulated by cold stress (Schiøtt and Palmgren 2005) while *ACA8* expression is also stimulated by ABA (Cerana et al. 2006). ACA10 appears to function in adult vegetative development. An *aca10* mutant has altered inflorescences comprising tightly bunched clusters of flowers because of reduced elongation of inflorescence internodes (George et al. 2008). ACA10 Ca^{2+}-ATPase activity therefore appears to regulate *Arabidopsis* reproductive development. Intriguingly, the *aca10* phenotype is completely dependent on the presence of a dominant mutation in *CIF2* which encodes an unknown protein, possibly a novel component of ACA10 Ca^{2+} transport regulation (George et al. 2008).

The mechanisms of posttranslational regulation of the ACAs have been extensively studied and reviewed, and will not be described in detail here. Briefly, the ACAs possess a CaM-binding and autoinhibitory domain at their N-termini, which autoinhibits Ca^{2+} transport when CaM is absent. This mode of regulation is likely to be ubiquitous for all ACAs, including those present at the PM (Chung et al. 2000; Bækgaard et al. 2006). There are also additional mechanisms of regulation. For example, *Arabidopsis* ACA8 is partially activated by heparin binding (Meneghelli et al. 2008), while acidic phospholipids can stimulate PM Ca^{2+}-ATPase activity (Bonza et al. 2001). Some Ca^{2+}-ATPases are regulated via phosphorylation. Phosphorylation sites have been identified on the N-terminal tails of *Arabidopsis* ACA8

and ACA10 (Nühse et al. 2004), although no functional analysis of these sites has yet been performed.

2.2.2 Ca^{2+}/H^{+} Exchangers

In addition to Ca^{2+}-ATPase-mediated Ca^{2+} efflux, low-affinity/high-capacity Ca^{2+} flux out of the cytosol can be driven by H^{+}-coupled transport (Pittman and Hirschi 2003; Shigaki and Hirschi 2006). Ca^{2+}/H^{+} exchange activity has long been observed at the vacuolar membrane in a variety of plant species but it is unclear whether this pathway exists at the PM in higher plants. Biochemical data suggests the presence of Ca^{2+}/H^{+} exchange at the PM in maize (Kasai and Muto 1990); however, the characterization of plant cation exchanger (CAX) genes, which encode Ca^{2+}/H^{+} exchangers, indicates that CAX proteins are almost exclusively tonoplast-localized (Shigaki and Hirschi 2006). Some data does suggest a possible PM location for a CAX, such as GmCAX1 from soybean (Luo et al. 2005). GmCAX1 gives slight tolerance to Na^{+} and Li^{+} stress when overexpressed in *Arabidopsis*, and GmCAX1-GFP was observed at the PM, but no Ca^{2+} transport activity across this membrane by GmCAX1 has yet been demonstrated (Luo et al. 2005). Further work is required to confirm whether PM Ca^{2+}/H^{+} exchange activity is actually present in higher plants, or whether it is restricted to some species, such as maize.

3 Evolution of PM Ca^{2+} Transporters in Plants

The transport of Ca^{2+} across the PM is clearly an ancient trait. The nutritional requirement for Ca^{2+} necessitates a Ca^{2+} influx pathway, but effective Ca^{2+} homeostasis to maintain low cytosolic Ca^{2+} is also critical and long evolved, as evident from the variety of PM Ca^{2+} transporters in bacteria and archaea which are similar to eukaryotic Ca^{2+} pumps and Ca^{2+} exchangers (Axelsen and Palmgren 1998; Shigaki et al. 2006). However, the PM Ca^{2+} transporters in higher plants clearly differ from those in animals (see below). Characteristics of PM Ca^{2+} transport in lower plant species, and surveys of the genomes of green algae and moss, have begun to uncover insights into the evolution of PM Ca^{2+} transport.

3.1 PM Ca^{2+} Transport in Lower Plants

Land plants inhabit a certain range of environments, and their mechanisms of PM Ca^{2+} transport may not necessarily be mirrored in algae or moss that are often found in wide-ranging environments, both aquatic and terrestrial, with very different external ionic conditions. The halotolerant green alga *Dunaliella* is perhaps the

best-studied marine unicellular alga. The saltwater habitat of *Dunaliella* has a high concentration of dissolved minerals and this may have had important consequences for the evolution of ion transporters. Biochemical studies in *D. salina* observed Ca^{2+} extrusion energized by the PM pH gradient (Pick et al. 1986). More recently, however, it has been found that *D. marina* utilize the large Na^+ gradient that exists across the PM to drive Ca^{2+} extrusion by a Na^+/Ca^{2+} exchange mechanism (Karimova et al. 2000). If the Na^+ gradient is artificially reversed, then Ca^{2+} influx is observed. Furthermore, if the Na^+ gradient is abolished, Ca^{2+} is accumulated via channels, which appear to only play a significant role in the absence of functioning Na^+/Ca^{2+} exchange (Karimova et al. 2000). In contrast, in freshwater and soil-living green algae such as *Chlamydomonas reinhardtii*, Ca^{2+} channels play a bigger role in Ca^{2+} absorption, although Na^+/Ca^{2+} exchange can proceed in the opposite direction and also drive Ca^{2+} influx (Karimova et al. 2000). The presence of PM Na^+/Ca^{2+} exchange activity in some unicellular algae is clearly analogous to the animal Na^+/Ca^{2+} exchanger (NCX) proteins, although whether NCX genes do encode this activity is unknown. This also raises interesting questions with regard to the evolution of the Na^+-coupled Ca^{2+} transporters and why they appear to be absent in higher plants (see below). It is clear that unicellular algae have adapted to efficiently use the solutes in their immediate environments to drive biological mechanisms in an entirely different manner than higher plants.

C. reinhardtii exhibits phototaxis by the beating of flagella in response to a light source, and this behavior requires transmembrane Ca^{2+} transport (Nagel et al. 2003). This phototactic response is initiated by light-gated channels, and two of the major effectors in this process, Channelrhodopsin (ChR)-1 and -2 are PM Ca^{2+}-permeable channels (Nagel et al. 2003; Berthold et al. 2008). Homologs of ChR2 have been found in a diverse range algae, including the phototactic chlorophyte *Volvox carteri* (Ernst et al. 2008), but no such channels are known in higher plants. Another *C. reinhardtii* PM Ca^{2+}-permeable channel called CAV2 which is localized at the tips of the flagella is required to mediate another light-mediated swimming behavior, the photophobic response (Fujiu et al. 2009). Ca^{2+} influx is also required for the flagellar excision process in *C. reinhardtii*. This occurs through the Ca^{2+}-catalyzed severing of microtubules in response to a rise in cytosolic Ca^{2+} (Wheeler et al. 2008). A PM Ca^{2+} channel opens following cytosolic acidification, possibly though a mechanism involving inositol 1,4,5-trisphosphate (Quarmby 1992). The molecular identity of this channel awaits confirmation, although one candidate gene, *ADF1* may encode the PM H^+-activated Ca^{2+} channel required for this response (Finst et al. 1998; Wheeler et al. 2008). It is likely that multiple PM Ca^{2+} transporters are involved in flagellar function, such as *C. reinhardtii* CAV2 and PKD2 (see below), although the understanding of the molecular mechanisms involved remains poor.

The marine multicellular phaeophytic alga *Fucus serratus* has been a good model to understand the role of Ca^{2+} influx in polar growth response to osmotic stress. Apical growth of the *F. serratus* rhizoid is driven by turgor pressure and controlled by a Ca^{2+} gradient that develops before germination. A voltage- and mechanosensitive Ca^{2+}-permeable channel activated by hypoosmotic shock has

been identified which has similar characteristics to channels from higher plants (Taylor et al. 1996). It is this channel which appears to generate the apical Ca^{2+} gradient which causes polar growth.

Many mosses appear to use Ca^{2+} influx and a rise in cytosolic Ca^{2+} to induce gametophore bud formation. Voltage-gated PM Ca^{2+}-permeable channels have been identified in *Funaria hygrometrica* which are important for the bud formation (Conrad and Hepler 1988). The influx of Ca^{2+} through these channels appears to be induced by cytokinins (Schumaker and Gizinski 1993). Moreover, the channels can be redistributed throughout the PM in the presence of cytokinins and laterally diffuse to the distal end of target cells, thus leading to localized influx and bud formation at a directed site (Saunders 1986). Ca^{2+} influx is also used in moss for thermoregulation. A recent study with the moss *Physcomitrella patens* identified heat-responsive PM Ca^{2+}-permeable channel activity which appears to regulate a heat shock response (Saidi et al. 2009).

3.2 Analysis of PM Ca^{2+} Transport by Comparative Genomics

It is clear that the physiological and biochemical analyses of PM Ca^{2+} transport in moss or algae are sparse compared to higher plants; however, the recent completion of a number of lower plant genomes (Grossman 2007) has allowed the direct comparison of the genetic complement of putative Ca^{2+} transporters in these species with higher plants, yeast, and human. Initial genomic surveys for predicted PM Ca^{2+} transporters from species including *C. reinhardtii* and *P. patens* have been performed recently (Boursiac and Harper 2007; Wheeler and Brownlee 2008).

A recent comparison of genes encoding Ca^{2+}-permeable channels highlights the variation in Ca^{2+} influx mechanisms between higher plants and green algae (Wheeler and Brownlee 2008) (Fig. 1). Both CNGC and GLR genes which have homologs in animals are present in higher and lower plants alike (Fig. 2), although the numbers of genes in both the CNGC and GLR families have proliferated in *Arabidopsis* and rice compared to *P. patens* and *C. reinhardtii* (one *GLR* and three *CNGC* genes in *C. reinhardtii*; 20 genes in both families in *Arabidopsis*). Interestingly, *Saccharomyces cerevisiae* lack both CNGC and GLR genes. A clear distinction between higher and lower plants is in the presence of the four-domain voltage-gated Ca^{2+} channel (VGCC) and the transient receptor potential (TRP) channel, lacking in higher plants and in moss, but present in chlorophytic algae including *Ostreococcus tauri* and *C. reinhardtii* (Wheeler and Brownlee 2008), and diatom species including *Thalassiosira pseudonana* (Fig. 2). This may suggest that these channels were lost during the evolution of higher plants and moss. The VGCC channels function predominantly as Ca^{2+}-selective channels at the PM in animals and some other species, such as *S. cerevisiae*, and recent evidence indicates that VGCCs also function in PM Ca^{2+} influx in algae. For example, a mutation in the *C. reinhardtii* VGCC gene *CAV2* leads to defective Ca^{2+} influx into the flagella (Fujiu et al. 2009). Many mammalian TRP channels function as ligand-gated Ca^{2+}-permeable

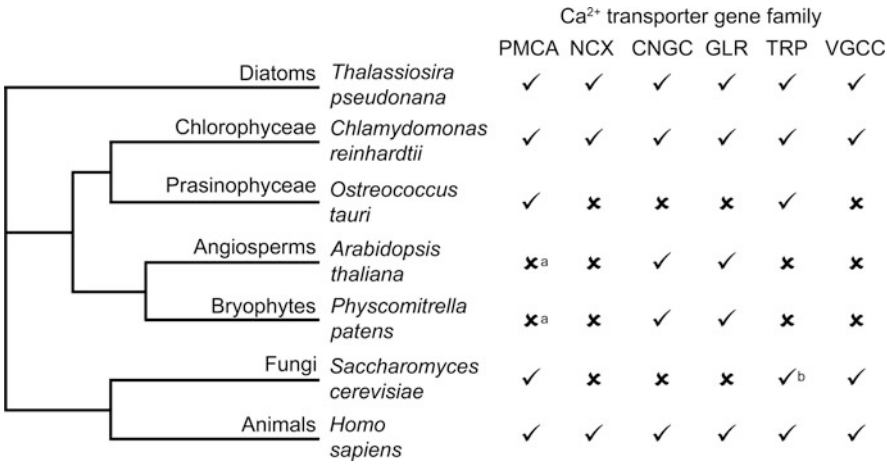

Fig. 2 Comparison of putative PM Ca^{2+} transporter gene families present in the genomes of representative higher plant, moss, diatom, and chlorophytic algal species, with fungi and animal species. Definitions of the gene names are given in the legend of Fig. 1. Gene analysis was taken from Boursiac and Harper (2007) and Wheeler and Brownlee (2008) or was performed using the draft and published genome annotations for *T. pseudonana*, *C. reinhardtii*, *O. tauri*, and *P. patens* available from the JGI (http://genome.jgi-psf.org/). Genes were classified in a gene family as determined by sequence similarity and phylogenetic clustering with human genes from those families. [a]*Arabidopsis* and *Physcomitrella* have ACA-type Ca^{2+}-ATPases rather than PMCAs. [b]The single yeast TRP gene is localized at the tonoplast rather than the PM

channels, which are important in environmental stress perception. *C. reinhardtii* possess 19 TRP channel genes some of which are likely to function at the PM, such as PKD2, a TRP channel at the flagellar membrane (Huang et al. 2007). It is unclear why the VGCC and TRP channels have been lost in higher plants; however, the inability to identify many plant channel genes, such as those encoding HACCs by comparative genomics, indicates that in turn higher plants have evolved their own unique set of Ca^{2+} channel genes. These higher plant PM Ca^{2+} channels may be better suited for the terrestrial environments which plants inhabit.

Ca^{2+}-ATPases are conserved throughout eukaryotes and prokaryotes (Axelsen and Palmgren 1998). ACA genes are present in *P. patens* (Fig. 2) and share significant sequence similarity to those from *Arabidopsis*, although to date no PM-localized Ca^{2+} pumps from *P. patens* have been characterized. In contrast, P_{2B}-type Ca^{2+}-ATPase genes in *C. reinhardtii* and diatoms including *T. pseudonana* are more similar to animal PMCA genes than plant ACAs (Boursiac and Harper 2007). Ion-coupled Ca^{2+} exchangers are similarly conserved in eukaryotes and prokaryotes (Shigaki et al. 2006). Ca^{2+} exchangers are divided into four families including the CAX and NCX gene families. CAX genes in *P. patens* have significant similarity to *Arabidopsis* CAXs. Similarly, *C. reinhardtii* possess CAX genes which have Ca^{2+}/H^{+} exchange activity and are likely to be endomembrane-localized (Pittman et al. 2009). In contrast, *C. reinhardtii* and *T. pseudonana* also possess NCX-like genes, which are absent in higher plants

(Fig. 2). We suggest that the frequent exposure of many aquatic and marine algae to high external Na^+ conditions and thus the steep Na^+ gradients that exist across the PM, has made the presence of a Na^+-coupled Ca^{2+} efflux pathway energetically favorable, while higher plants have adapted to using H^+-coupled mechanisms. It will be of interest for future studies to determine whether these putative NCX transporters are PM localized and whether they do indeed mediate Na^+-coupled Ca^{2+} transport.

4 Conclusions

Plants adapt to conditions by reprogramming physiological and developmental responses. This perception and adaptation is crucial for plant survival. In detecting these signals, intracellular Ca^{2+} changes act as a second messenger, regulating the activation of Ca^{2+}-permeable channels. As we have discussed, several channel candidates in plants such as CNGC, GLR, and TPC have been intensively studied; however, demonstration of their ion channel activities remains a challenge. The plant Ca^{2+} community has invested a large effort to clone Ca^{2+} channels and receptors but several approaches used effectively in animals have failed (e.g., Leyman et al. 1999). Meanwhile, innovative approaches using cell-culture techniques and Ca^{2+} imaging technology have proven successful and portend future advances (Han et al. 2003). Once the ensemble of transporters have been molecularly characterized, the goals will be directed toward functional analysis. The biophysical properties will need to be determined using a plethora of functional assays and Ca^{2+} imagining studies will be used to analyze their mechanisms in controlling basal intracellular Ca^{2+} levels. Finally, we can then begin to ascribe biological functions using phenotypes.

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Sulfate Transport

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Abstract The SulP gene family in plants consists of approximately 14 genes, depending on species, of which around 10 are thought to encode plasma membrane sulfate transporters. On the basis of sequence, five clades or Groups are distinguishable, of which three contain probable plasma membrane sulfate transporters. Isoforms with both high (Group 1) and low affinities (Group 2) for sulfate catalyse cellular uptake in many tissues throughout the plant, particularly at the soil/root interface, and in vascular tissues, respectively. Differential expression of the individual isoforms in response to sulfur nutrition, and in relation to tissue specificity, optimises the uptake and distribution of sulfate depending upon supply and demand. A major contribution to regulation is at the transcriptional level, with transcript abundance and sulfate uptake capacity increasing markedly when sulfur supply is limiting. The sulfate transporters comprise 12 MSDs with long amino and carboxyl regions, the latter containing a STAS domain, which may be involved in functional regulation.

1 Introduction

Plants have an absolute requirement for sulfur for growth, as an essential component of proteins and many secondary metabolites. Sulfur is almost exclusively acquired by the plant as sulfate via the roots from the soil. Sulfate is taken up in an active process usually against a concentration gradient across the plasma membrane of root cells, whereupon it may be directly assimilated into organic forms, temporarily stored in the cell vacuoles or transported to the shoot, the major site of reductive assimilation, via cell to cell transfer and translocation in the xylem.

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In these processes, there is a requirement for transfers of sulfate across multiple membranes: influx and efflux from the cell across the plasma membrane, influx and efflux from the vacuole, and transport into the chloroplast, the major site of activation and reductive assimilation. Catalysing at least some of the steps is a family of transporters, which belong to the SulP major gene family (Saier et al. 1999), for which in plants there exists multiple members fulfilling these roles (Hawkesford 2003).

1.1 Physiology and Energetics of Sulfate Uptake in Plants

Initial uptake measurements into intact barley roots indicated operation of a high affinity, saturable transporter, with a K_m for sulfate of 19 μM (Leggett and Epstein 1956) and a likely second lower affinity component. The high affinity transport system was competitively inhibited by the sulfate analogue, selenate, but not by other major macronutrient anions such as nitrate, phosphate and chloride, clearly indicative of the specificity of this carrier system. A similar K_m, together with a strong pH optimum, was observed for cultured tobacco cells (Smith 1976). The transport of analogous anions such as selenate was exploited subsequently for the first cloning of sulfate transporters in yeast and plants (Smith et al. 1995a, b, 1997), and also is likely to contribute to accumulations of such elements that may have a role in crop quality (Hawkesford and Zhao 2007, Shinmachi et al. 2010).

Sulfate is generally taken up against a concentration gradient and is believed to be coupled to the proton gradient in a co-transport mechanism. Short term uptake studies with the duckweed, *Lemma gibba*, indicated uptake driven by membrane potential, as evidenced by the observed transient depolarisation of the inside negative membrane potential, explainable by co-transport of an excess of positive charge; hence, a $3H^+$/sulfate ratio was suggested. Transport showed a broad peak of activity throughout a wide physiological range (between pH 4 and 8) (Lass and Ullrich-Eberius 1984). Analysis of kinetics at sulfate concentrations between 5 μM and 1 mM indicated for *Lemna* the occurrence of a two-component uptake system with high and low affinity saturable components.

Transport has been characterised in isolated plasma membrane vesicles of *Brassica napus*, a species known for a high sulfur requirement. Transport was shown to be high affinity ($K_m = 10$ μM) with both the flux and the affinity being pH-dependent. Maximal transport was dependent upon a pH gradient, and depolarisation of the inside negative membrane decreased transport. An artificially imposed inside positive membrane potential alone failed to catalyse transport. No low affinity components attributable to active transport could be resolved (Hawkesford et al. 1993).

Studies on intact plant systems have indicated complex influx kinetics and the existence of high and low affinity saturable systems, suggestive of multiple transport systems, which may or may not be co-localised at the cellular level. However, analysis of the transporter kinetics observed in intact plants is not easily separable

from components associated with assimilation or compartmentation into separate pools. Recently, cloning of the gene family and the characterisation of individual isoforms in yeast has conclusively demonstrated contrasting affinities for sulfate for individual sulfate transporters. With such an approach, transporter function of many members of the plant SulP family has been confirmed, with some showing a high affinity for sulfate (K_m generally around 10 μM), consistent with a role in uptake into the cell at low external sulfate concentrations and others having a lower affinity for sulfate, which, coupled with the specificity of tissue expression, indicates a role in cell to cell movements of sulfate, where apoplastic concentrations may be much higher. In both cases, when expressed in yeast, a pH dependency with highest transport activity at lower pH was observed (Smith et al. 1995a, 1997; Takahashi et al. 2000; Yoshimoto et al. 2002).

1.2 Efflux Across the Plasma Membrane

As already indicated, there is a need for efflux from the cell to facilitate transport around the plant, for example, prior to xylem loading in the root but also in cases of cell to cell transport in all tissues, and to facilitate phloem unloading. The molecular identities of these channels have not been determined, and it is possible that influx transporters may act in reverse or that there may be dedicated efflux carriers. A candidate channel for which sulfate is both a substrate and an activator has been identified by electrophysiological studies; this channel anion is not specific for sulfate, catalysing in order of specificity, nitrate, sulfate, chloride and bicarbonate (Frachisse et al. 1999). Critically, the presence of sulfate prevents a run down of activity of the channel, in contrast to the situation with the other anions, which provides a potential for specificity of cellular anion homeostasis (Diatloff et al. 2004; Frachisse et al. 1999) or for the necessary cell to cell transfer of sulfate, as indicated above.

2 The SulP Gene Family

Sulfate transport in plants is catalysed by multiple genes belonging to the SulP family. This gene family occurs in bacteria, yeast (Smith et al. 1995b) and fungi (Ketter et al. 1991) and animals including humans (Schweinfest et al. 1993). In some cases, transport of substrates other than sulfate is the primary function, for example, bicarbonate in a marine cyanobacterium (Price et al. 2004). As already noted, in plants, all members of the family are thought to be able to transport analogous anions such as selenate, chromate and molybdate, in addition to sulfate.

Plants generally possess multiple genes of the SulP family, potentially encoding sulfate transporters. In Arabidopsis, there are 14 related gene members of this family and the phylogenetic relationships and clustering on the basis of sequence

into five clades, or Groups, are shown in Fig. 1. The ten Arabidopsis genes representing the plasma membrane sulfate transporters are found in Groups 1–3. The multiple members have arisen by historical gene duplication, a process that is continuing and a recent example of which has occurred in wheat (Buchner et al. 2004a), and although initially resulting in redundancy of function, often has been followed by specialisation of functional properties or location and conditions of expression (Hawkesford 2003). All plants, for species for which information is available, possess multiple related genes dividable into these Groups, with some species-specific variation (Table 1).

The Group 1 clade represents the high affinity sulfate transporters, which although expressed in many tissues, are particularly strongly expressed in root tips under conditions of sulfur limitation. The transporters are responsible for primary uptake from the soil solution into roots cells, and for uptake into cells throughout the plant. In one instance, expression is restricted to phloem tissues (Yoshimoto et al. 2003), indicating a probable role in phloem loading. In many species, although notably not in wheat, two isoforms are expressed in the same tissue, often showing differential inducibility by sulfur-limitation (Buchner et al. 2004b; Koralewska et al. 2007; Yoshimoto et al. 2002). Heterologous expression in

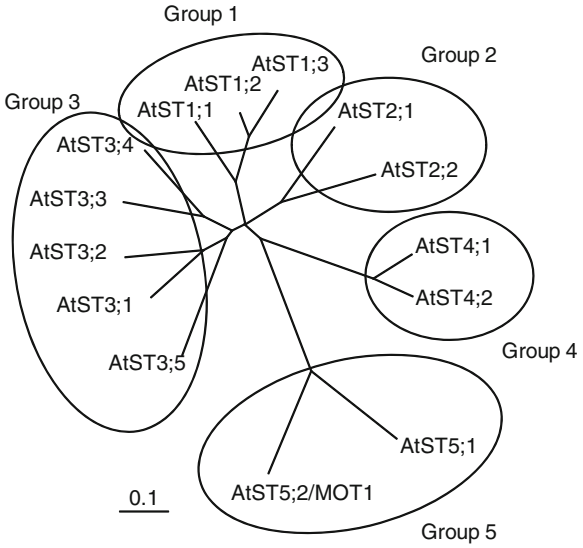

Fig. 1 Phylogenetic representation of the Arabidopsis SulP sulfate transporter amino acid sequences showing subdivision into five groups. Sequences derived from the following genetic loci: AtSultr1;1, At4g08620; AtSultr1;2, At1g78000; AtSultr1;3, At1g22150; AtSultr2;1, At5g10180; AtSultr2;2, At1g77990; AtSultr3;1, At3g51900; AtSultr3;2, At4g02700; AtSultr3;3, At1g23090; AtSultr3;4, At3g15990; AtSultr3;5, At5g19600; AtSultr4;1, At5g13550; AtSultr4;2, At3g12520; AtSultr5;1, At1g80310; AtSultr5;2 or MOT1, At2g25680; Alignments were performed using ClustalX program (Thompson et al. 1997) version 1.83 and the unrooted tree was drawn using the Treeview 1.6.6 program (Page 1996)

Table 1 Occurrence and characteristics of clades of transporters within the SulP family for five plant species

	Numbers of genes								
Group	Arabidopsis	Brassica	Rice	Wheat	Brachypodium	Substrate	Typical K_m	Location	Inducibility
1	3	3	3	2[a]	3	Sulfate	<10 μM	PM	+++
2	2	2	2	1	1	Sulfate	>100 μM	PM	+
3	5	5	6	5	5	Sulfate		PM	No
4	2	2	1	1	1	Sulfate		V	++
5	2	2	2	2	2	Molybdate		M/V	No

PM plasma membrane, *V* vacuole, *M* mitochondrion

[a]Gene numbers were obtained by examination of sequence databases. For wheat, see Buchner et al. 2010

yeast has facilitated the study of function and structure–function relationships for several examples from this group.

Members of the clade representing Group 2 are exclusively expressed in plasma membranes of cells in vascular tissues. Heterologous expression generally indicates a lower affinity (K_m >100 μM) for sulfate (Smith et al. 1995a; Takahashi et al. 1997, 2000), with one exception where a low K_m was reported (Vidmar et al. 2000), consistent with a role in cell to cell transfer at elevated sulfate concentrations as might be found in the vascular tissues. In wheat, expression of only one Group 2 sulfate transporter has been detected (Buchner and Hawkesford, Buchner et al. 2010, see Table 1).

Most plant species contain several members of the clade representing Group 3 sulfate transporters. These have been less well functionally characterised and are generally not well expressed in yeast. One example, the Arabidopsis AtST3;5, whilst showing low transport activity if expressed alone in yeast, when co-expressed with a Group 2 sulfate transporter, AtST2;1, showed sulfate transporter capacity greater than the sum of either expressed alone (Kataoka et al. 2004a). This co-operativity is interpreted, at least in this case, as evidence for heterologous dimers being the *in vivo* functional unit. In contrast, in *Lotus japonicus*, the homologous transporter (SST1) that is specifically involved in sulfate transport into the intracellular symbiosome in root nodule cells, the site of symbiotic nitrogen fixation appears to adequately complement yeast mutants by itself (Krusell et al. 2005). Notably, the transport would be energetically favourable as an anion, as the membrane potential of the symbiosome is thought to be polarised internally positive.

Group 4 also comprises sulfate transporters; however, these are localised in the tonoplast and are involved in efflux of sulfate from the acidic vacuole, possibly in a proton co-transport mechanism analogous to plasma membrane influx (Kataoka et al. 2004b). The distantly related transporters in Group 5, in addition to having a low degree of sequence similarity, also have truncated amino and carboxyl regions. Localisation studies places them in vascular or mitochondrial membranes. Whilst it is likely that all sulfate transporters are able to mediate uptake of molybdate across the plasma membrane, there is a critical role for one Group 5 isoform (Mot1) in molybdenum accumulation, although this may not be in uptake into the cell given the apparent membrane localisation (Baxter et al. 2008; Tomatsu et al. 2007).

2.1 *Structure of the Sulfate Transporters*

Analysis of primary sequences has provided a consensus topology model with potentially 12 membrane spanning domains (MSDs), with both amino and carboxyl regions extending into the cytoplasm (Fig. 2a). Both numbers of MSDs and exact positioning vary depending on prediction method and sequence used, and therefore this consensus should only be considered as a working model. Further sequence analysis indicates possible tertiary structural configuration of some of the MSDs (Shelden et al. 2003) and the importance of specific proline residues in contributing to this structure (Shelden et al. 2001). The relative positions of these MSDs are shown in Fig. 2b. Several individual amino acid residues have been identified as being essential for function or correct assembly in the membrane, including a number of proline residues in the first three MSDs (Shelden et al. 2001) and aspartate and arginine residues (located at the terminal ends of the diagnostic motif, Fig. 2c), which may form a charged couple between MSD 1 and 2 and which may be involved in ion translocation (Shelden et al. 2003). The comparative analysis of sequences from the whole SulP family provides a conserved motif diagnostic for sulfate transporters, which includes the residues specified above, and this is shown in Fig. 2c (Leves et al. 2008).

As already indicated, a quartenary structure involving interactions of heterologous members of the family has been proposed (Kataoka et al. 2004a). Of note is the identified STAS (sulfate transporters and antisigma-factor antagonist) domain (Aravind and Koonin 2000) in the carboxyl region (Fig. 2a), which may also be involved in other protein:protein interactions, possibly involving phosphorylation and hence linked to regulation of activity (Rouached et al. 2005; Shibagaki and Grossman 2004).

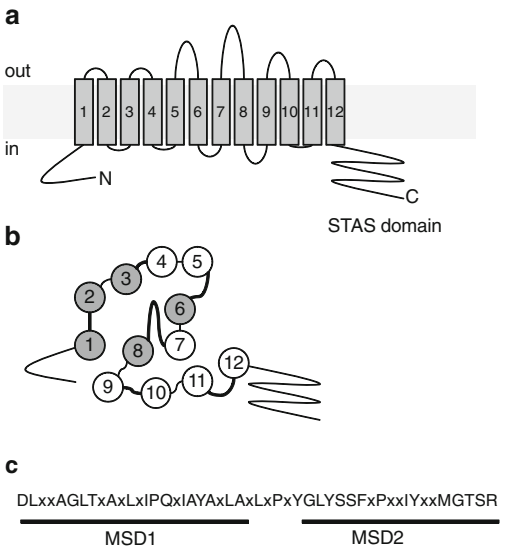

Fig. 2 Possible topology of MSDs and extra-membrane loops for a generalised plant sulfate transporter. (**a**) Positions of 12 *trans*-membrane helices as determined from multiple alignments. Arrangements of extra-membrane loops and STAS domain structure are arbitrary. (**b**) Tertiary arrangement of MSDs. From (Leves et al. 2008; Shelden et al. 2003). (**c**) A diagnostic motif for sulfate transporters, including positions of the first 2 MSDs shown with the bars (Leves et al. 2008)

2.2 Mechanisms of Regulation

Sulfate uptake capacity increases when plants are deficient for sulfur, a condition that can be induced in young seedlings within a few days by removing the sulfur supply (Clarkson et al. 1983; Lee 1982). The observation that plasma membrane vesicles isolated from sulfur-starved plants had a greater capacity (V_{max}) for transport than vesicles isolated from non-starved plants was consistent with the suggestion that plasma membrane-located transporter activity was itself influenced by sulfur nutritional status, either in terms of abundance or conformational state. Furthermore, unless tightly bound, additional non-membrane components were not required to affect allosteric regulation (Hawkesford et al. 1993).

Following the cloning of plant sulfate transporter genes, northern analysis indicated a high degree of regulation of transcript abundance which, coupled with root proliferation, is a major response to enhance scavenging of sulfate by maximising sulfate uptake capability (Hawkesford and De Kok 2006). This regulation of gene expression has been suggested to be mediated by downstream metabolites, either reduced sulfur compounds or intermediates of sulfate assimilation such as the cysteine precursor, *O*-acetylserine (Smith et al. 1997). However, there is not always a strict relationship between abundance of transcripts and the measured sulfate influx capacity, indicating the operation of additional regulatory mechanisms. The abundance of transcripts for the high affinity transporter increases dramatically compared to relatively modest increases in transporter capacity. Moreover, influx capacity usually plateaus or even decreases after an extended period of sulfur deprivation, whilst transcript abundance remains high (Hopkins et al. 2005; Koralewska et al. 2007). When sulfate is re-supplied to sulfur-deprived plants, transporter transcript and protein abundance as well transporter capacity decrease rapidly indicating a rapid turnover of both transcripts and protein (Hawkesford and Wray 2000; Smith et al. 1997). This decrease is often transitory, and a re-induction occurs as the plant re-establishes its sulfur status (Hawkesford and De Kok 2006). The rapidity of the initial response indicates that the signalling system is closely linked to root sulfur pools.

Regulation occurring after transcription is clear from studies of transgene sulfate transporter expression controlled by the constitutive CMV promoter (Yoshimoto et al. 2007), where accumulations of sulfate transporter transcripts and protein were apparent under long-term sulfate starvation. As transcription itself would be unaffected by nutritional status, this could be explained by modification of transcript and/or protein turnover. Transcript abundance for at least some sulfate transporters usually decreases rapidly following sulfate re-supply (Koralewska et al. 2008; Smith et al. 1997), although parallel decreases in uptake capacity are not always seen (Koralewska et al. 2008). An estimated half time for the sulfate transporter protein is 2.5 h, as evidence by protein synthesis inhibition studies or recovery from known repressors (Clarkson et al. 1992; Rennenberg et al. 1989).

Cellular signalling pathways and long distance inter-organ signal molecules remain controversial. It is clear that nutritional status rather than availability results

in transcriptional control. Inhibitor studies indicate that pathways in which sulfur nutritional status determines the level of transcription of the transporters involve phosphorylation/de-phosphorylation steps, and that a phosphatase activity is required for up-regulation of expression (Maruyama-Nakashita et al. 2004a). In addition, cytokinin represses the induction, a phenomenon not seen in cytokinin receptor (cre1/wol/ahk4) mutants (Maruyama-Nakashita et al. 2004b). Finally, a specific 5 bp promoter element, found upstream of many sulfur-regulated genes and involved in the sulfur response, has been identified (Maruyama-Nakashita et al. 2005).

3 Perspective

There is a breadth of knowledge about plant sulfate transporters and their integration with sulfur metabolism and nutrition. Although there has been more focus on regulatory aspects linked to gene expression compared to functional aspects of the transporter itself, recent reports have highlighted the likely importance of the later. As indicated, many paradoxes hinder a full understanding of functional and regulatory aspects of the Sul1 gene family responsible primarily not only for sulfate transport but also for the transport of a number of micronutrient analogues. The area of transcript and protein longevity and turnover has not been systematically researched but these mechanisms may prove to be important for regulation of activity. Similarly the relative importance of monomers, dimers or heterologous dimers as the functional unit requires clarification.

The widespread occurrence of members of the SulP family, the diversity of substrates, the number of functionally evolved isoforms in plants and the importance of sulfur to plant nutrition as well as the existence of several mutations in human homologues, which cause serious disease (Markovich 2001), ensures a continued interest in this gene family. In addition, the transporter is a paradigm for the study of plant nutrient transporters and their integration with plant nutritional status.

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Metal Transport

Aaron Atkinson and Mary Lou Guerinot

Abstract One-third of all proteins require metal cofactors for function, yet metals can be toxic and need to be tightly controlled. Metal homeostasis is basically the equilibrium between metal uptake and metal efflux, making metal transporters key players in controlling cellular metal content. Here, we review the major families of metal transporters that participate in transport of divalent metal cations, focusing on Fe, about which the most is known.

1 Introduction

Metal cations, such as Fe, Cu, Zn, and Mn, play essential structural and catalytic roles throughout the cell in various subcellular compartments. Metal cofactors are critical for such processes as transcription, translation, the production of ATP in the mitochondria, carbon fixation and generation of ATP in chloroplasts, scavenging of toxic free radicals, and transport of oxygen and carbon dioxide. Yet metal cations, like all hydrated ions, cannot passively diffuse into the cell. This is due primarily to the energetically unfavorable cost of dehydration imposed by the hydrophobic environment created by biological membranes (Gouaux and Mackinnon 2005). Therefore, the energized transport of metals across cellular membranes is fundamental for life.

Metals are a highly reactive group of elements. The uptake and distribution of metals within cells is, therefore, highly specific and tightly regulated to avoid hyperaccumulation of unwanted metals within the cell. Furthermore, once metals have been taken up, cells have an enormous capacity to chelate metals (Rae et al. 1999; Outten and O'Halloran 2001; Changela et al. 2003). For instance, under

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standard growing conditions, the "free" concentrations of Cu and Zn have been calculated to be less than one atom per cell, despite total cellular Zn and Cu concentrations that surpass 10^5 and 10^4 atoms per cell, respectively (Rae et al. 1999; Outten and O'Halloran 2001; Changela et al. 2003; Finney and O'Halloran 2003). This furthers the cell's already strong ability to precisely regulate transport, and together, they form part of a system to deliver the reactive metal only to where it is needed while preventing interference from unwanted metals.

Exactly how metalloproteins acquire essential metal cofactors in a cellular environment with both tightly regulated transporters and limited free metal is also just becoming clear now (Waldron and Robinson 2009). In some instances, proteins acting as metallochaperones specifically deliver metal cofactors to cognate proteins and thereby metallate them (Rosenzweig 2002). In other instances, transporters appear to maintain adequate pools of metal and thereby avoid mismetallation of metalloproteins capable of binding various metals through maintaining precise metal availability (Nies 2007).

Despite the cell's best attempts, oxidative damage due to metals does occur. This most often occurs in instances of either chronic disease, or acute exposure to high levels of either essential metals, such as Fe, or nonessential toxic metals, such as Cd (Stohs and Bagchi 1995; Schutzendubel and Polle 2002). This oxidative damage occurs primarily when excess free metal reacts with hydrogen peroxide (or lipid peroxides) to generate OH-, an oxidized metal, and the highly reactive hydroxyl (OH^-) free radical (or lipid radicals). Once generated, these free radicals can damage proteins, nucleic acids, and cellular membranes. An alternative mechanism of oxidative damage occurs when nonreactive metals, such as Cd or Zn, displace reactive metals, such as Fe, Cu, or Mn, triggering the formation of free radicals (Stohs and Bagchi 1995; Schutzendubel and Polle 2002).

In this chapter, we will discuss families of metal transporters involved in the movement of Fe, Mn, Zn, and Cu across the plasma membrane. Family members that localize to other membranes will also be discussed in order to provide as complete a picture as currently possible of these families. Since the most is known about Fe transport, the focus will be on Fe.

2 Iron

Plants fight an uphill battle when it comes to mineral acquisition. Plants must obtain sufficient levels of essential metals, such as Fe, while avoiding toxic metals such as Cd. For example, despite being the fourth most common element in the Earth's crust, Fe is not readily available. Released by the weathering of rock, Fe exists mostly as insoluble Fe^{3+} oxides, which are largely stable and not readily soluble at neutral pH (Guerinot and Yi 1994). In soil, the free Fe^{3+} concentration is estimated at 10^{-17} M, which is well below that required for most soil-based organisms. The low availability of Fe in soil limits plant growth and productivity, making

Fe-limitation third only to phosphate and nitrogen. Furthermore, in response to Fe deficiency, plants are known to accumulate other metals, some of which are toxic to both plants and animals, such as Cd.

For plants, Fe is quantitatively the most important metal cofactor involved in photosynthesis (Raven et al. 1999). In photosynthetic organisms, Fe-deficiency results in chlorosis, which is attributed to the selective reduction and remodeling of photosynthetic components (Spiller and Terry 1980; Terry 1980; Moseley et al. 2002). For example, Photosystem I, which contains 12 atoms of Fe, is the primary target for reduction in photosynthesis due to Fe deficiency (Moseley et al. 2002). However, many other components of photosynthesis, or closely associated processes, also require large amounts of Fe. These include photosystems II, cytochromes b_{563}, f, and c_6, ferrodoxin NAD(P)H/PQ oxidoreductase, catalase, and ascorbate peroxidase (Raven et al. 1999).

The ability of plants to acquire Fe in sufficient levels is crucial to their productivity and survival in competition with other organisms. Under Fe-limited conditions, plants respond with distinct morphological changes, such as increasing the surface area for Fe absorption through generation of secondary roots and root hairs (Romheld 1987; Müller and Schmidt 2004). Plants also respond physiologically to Fe-limited conditions in one of two ways: by using a strategy based on Fe chelators to retrieve Fe^{3+} from the soil, or by using a reduction strategy whereby Fe^{3+} is reduced to Fe^{2+} prior to uptake.

2.1 Chelation Strategy for Iron Uptake

In response to Fe deficiency, grasses increase production and secretion of Fe-specific chelators, termed phytosiderophores (PS), into the soil immediately surrounding the root. PS are small organic molecules, such as mugineic acid, which have a high affinity for Fe^{3+} and are synthesized from L-methionine (Takahashi et al. 2001). In rice and barley, the genes required for sulfur uptake, methionine biosynthesis, and PS synthesis are all upregulated in the first 24 h of Fe deficiency (Kobayashi et al. 2005; Nagasaka et al. 2009). Indeed, a plant's ability to secrete high amounts of PS is correlated with tolerance to low Fe conditions (Marschner 1995; Takahashi et al. 2001; Curie and Briat 2003). Once in the soil, PS chelate Fe^{3+}, thereby enabling subsequent uptake of the Fe^{3+}-PS complex by plant roots.

2.1.1 Yellow Stripe and Yellow Stripe-Like Transporters

Characterization of the chlorotic maize mutant, *yellow stripe 1* (*ys1*), revealed it to be defective in the uptake of Fe^{3+}-PS, suggesting that it lacked the appropriate Fe^{3+}-PS transporter (von Wirén et al. 1994). The *ys1* gene was subsequently cloned and shown to encode a proton-coupled symporter belonging to the oligopeptide transporter (OPT) family (Curie et al. 2001). *YS1* mRNA expression was shown to

increase in response to Fe deficiency in roots and shoots of maize. YS1 was also shown to restore growth to Fe uptake deficient yeast on media containing Fe-deoxymugineic acid as the Fe-phytosiderophore source (Curie et al. 2001). These findings suggest a role for YS1 in both the uptake of Fe from the soil (Fig. 1) and in intercellular Fe transport.

Arabidopsis encodes eight predicted open reading frames that are approximately 80% similar to YS1 and have thus been termed Yellow Stripe-Like (YSL) 1 through 8 (Curie et al. 2009). Arabidopsis does not synthesize phytosiderophores, however, so the role of the YSL family in metal transport in Arabidopsis is proposed to be slightly different than that of YS1. Although Arabidopsis does not synthesize phytosiderophores, it does synthesize a phytosiderophore precursor, nicotianamine (NA), which is known to be abundant in plant sap and to bind metals (Curie et al. 2009). Furthermore, plants lacking functional YSL transporters exhibit mutant phenotypes, which indicate that the role of YSL transporters in Arabidopsis is limited to metal distribution within the plant.

Characterization of Arabidopsis YSL1 suggests that it may have a role in transporting Fe-NA (Le Jean et al. 2005; Waters et al. 2006). Two independent groups have identified and characterized mutant *ysl1* plants. Results from both Waters et al. (2006) and Le Jean et al. (2005) have shown that *YSL1* mRNA

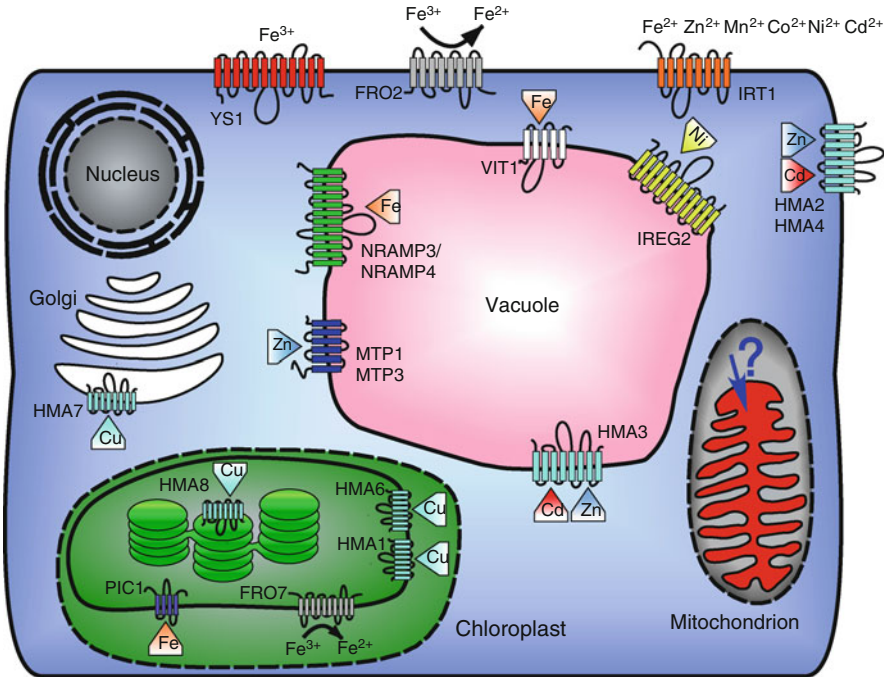

Fig. 1 Metal transporters involved in metal trafficking in Arabidopsis. Only proteins whose localization has been demonstrated experimentally are included on the diagram. Also shown are two ferric chelate reductases that function in Fe transport

accumulates specifically in response to Fe deficiency, indicating that Fe may be a biologically relevant substrate of YSL1. Furthermore, plants with *YSL1* promoter-driven expression of β-glucuronidase (GUS) exhibit staining within the vasculature of roots, shoots, flowers, siliques (seed pods), and within developing seeds (Le Jean et al. 2005). Both groups also note through analysis of publicly available microarray data that *YSL1* message peaks in senescing leaves, and that seeds from *ysl1* mutants contain less Fe. One group also noticed that *ysl1* seeds require more time for germination on Fe-deficient medium, relative to wild-type plants (Le Jean et al. 2005). This is presumably due to lower Fe within seed as Fe supplementation reverses this defect. The delayed germination is the only recorded phenotype for the *ysl1* single mutant. However, a *ysl1 ysl3* double mutant exhibits an additive phenotype, suggesting some redundancy between YSL1 and YSL3 (Waters et al. 2006). The *ysl1 ysl3* double mutant exhibited Fe-deficient symptoms including interveinal chlorosis in the leaves. The double mutant also had lower Fe content in leaves and failed to mobilize Fe from senescing leaves. The fertility of the double mutant was also hampered due to defective anther and embryo development. Together, these data suggest that YSL1 and YSL3 distribute Fe throughout the plant and recover Fe from senescing leaves.

An additional Arabidopsis YSL family member, YSL2, has been implicated in Fe transport into the vasculature. *YSL2* transcript levels respond to the presence of Fe sources (DiDonato et al. 2004; Schaaf et al. 2005). While heterologous expression in yeast is often helpful in determining the substrates of metal transporters, two studies show conflicting results for YSL2, leaving the exact substrate for YSL2 unclear. *In planta*, *YSL2* promoter-driven GUS expression exhibited staining in the vasculature of older leaves and differentiated roots (DiDonato et al. 2004). It was later observed that this pattern was restricted to the endodermis and pericycle that surround the plant vasculature (Schaaf et al. 2005). This tissue is thought to indirectly load the xylem with nutrients for transport to the shoot. In stable transgenic lines expressing GFP-tagged YSL2, the fusion protein localized to lateral membranes within the vasculature. This suggests that YSL2 may transport Fe laterally into the vasculature. Genes with similar expression patterns, including the boron transporter BOR1 (Takano et al. 2002, 2005) and phosphate transporter PHO1 (Hamburger et al. 2002), have been shown to encode proteins that are involved in xylem loading, suggesting that perhaps YSL2 loads the vasculature with a yet undefined substrate, most likely Fe-NA.

Rice also contains YS1 orthologs, with a total of 18 predicted *OsYSL* open reading frames. OsYSL15 is the primary transporter responsible for Fe(III)-PS uptake from the rhizosphere (Inoue et al. 2008; Lee et al. 2009). It is upregulated in response to Fe deficiency and expressed on the plasma membrane in the root epidermis, in addition to the stele, flowers, and developing seeds. Two insertional *osysl15* mutants exhibited chlorotic phenotypes under Fe deficiency and had reduced Fe concentrations in their shoots, roots, and seeds (Lee et al. 2009). This is consistent with the severe germination defects seen when OsYSL15 expression was reduced with RNAi (Inoue et al. 2008). OsYSL18, like OsYSL15, also transports Fe(III)-DMA but does not appear to be involved in the uptake from the

rhizosphere. Rather, based on its expression pattern, it may be involved in DMA-mediated Fe distribution in reproductive organs, lamina joints, and phloem cells at the base of the sheath (Aoyama et al. 2009). Rice *Os*YSL2 has been shown to transport Fe(II)-NA and Mn(II)-NA complexes in *Xenopus* oocytes; however, transport of Fe(III)-DMA was not detected (Koike et al. 2004). *OsYSL2* message is induced in Fe-deficient leaves, and promoter GUS expression reveals *OsYSL2* promoter-driven staining in the phloem (Koike et al. 2004). This finding, together with the functional data, suggests that the role of YSL2 is to load the phloem with NA bound metal.

2.2 Reduction Strategy for Iron Uptake

Arabidopsis is perhaps the best model system for studying the Reduction Strategy of Fe uptake, as many of the essential components were first isolated in Arabidopsis by utilizing the powerful genetics of this model system. Under Fe-deficient conditions, Reduction Strategy plants release protons into the soil through the action of H^+ ATPases at the root surface, chiefly mediated by Arabidopsis H^+ ATPase 2 (AHA2) (Santi and Schmidt 2009). These protons acidify the surrounding soil and thus help solubilize Fe^{3+} as each unit drop in pH (above pH 4.0) results in a 1,000-fold increase in Fe availability. Fe^{3+} is then reduced to Fe^{2+} at the root surface by the ferric chelate reductase FRO2 [Fig. 1(Robinson et al. 1999)]. The Fe^{2+} is then available to be transported by Iron-Regulated Transporter 1 (IRT1) across the plasma membrane (Fig. 1) of the root epidermis (Henriques et al. 2002; Varotto et al. 2002; Vert et al. 2002). IRT1 is critical for Fe uptake as mutant *irt1* seedlings die shortly after germination unless rescued with high (600 mM) amounts of Fe. While Fe is the most critical IRT1 substrate, metal content analysis of *irt1-1* seedlings and functional characterization in yeast indicates that, in addition to Fe, IRT1 does transport significant amounts of Zn, Mn, Co, and the toxic metal Cd (Eide et al. 1996; Korshunova et al. 1999; Rogers et al. 2000; Vert et al. 2002). While IRT1 is a nonspecific metal transporter, the seedling lethality of *irt1* mutants is specific to Fe as supplementing the soil with Zn, Mn, or Co fails to rescue mutant *irt1* seedlings, indicating that Fe uptake is the primary function of IRT1 (Varotto et al. 2002; Vert et al. 2002).

IRT1 orthologs have been identified in other plant species including tomato (Eckhardt et al. 2001), pea (Cohen et al. 2004), rice (Bughio et al. 2002), and barley (Pedas et al. 2008). Actual loss-of-function mutants are not available for most of these orthologs. Nonetheless, the available data on these orthologs agree with data on IRT1 function in Arabidopsis. For instance, the IRT1 orthologs have Fe transport activity demonstrated through expression in yeast, and transcription of the orthologs is induced under Fe-deficient conditions much like IRT1. Interestingly, the barley ortholog is also upregulated by Mn deficiency, and higher expression of *HvIRT1* has been correlated with Mn efficiency (Pedas et al. 2008).

Mounting evidence suggests that the Fe^{2+} uptake of OsIRT1 may supplement the Fe^{3+}-PS uptake activity present in rice (Ishimaru et al. 2006). First, rice secretes inadequate amounts of phytosiderophores to account for the total Fe uptake (Mori et al. 1991). Secondly, the rate of Fe^{3+} uptake is also significantly less than Fe^{2+} uptake when exogenous phytosiderophores are not supplied (Ishimaru et al. 2006). The expression of *Os*IRT1 in Fe-deficient roots with the ability to function as an Fe transporter suggests that rice may utilize an Fe^{2+} uptake strategy, in addition to an Fe^{3+}-PS uptake strategy. It has been suggested that this hybrid Fe^{2+} and Fe^{3+} uptake strategy could be an adaptation to growing in submerged anoxic conditions in rice paddies, where Fe would be in the reduced Fe^{2+} form, but it would be oxidized to Fe^{3+} upon drainage of rice paddies (Ishimaru et al. 2006). Transgenic rice plants overexpressing OsIRT1 showed enhanced tolerance to Fe deficiency as seedlings, demonstrating that Fe^{2+} uptake can be an important source of this essential nutrient (Lee and An 2009). Furthermore, Fe and Zn levels were elevated in the shoots, roots, and mature seeds of overexpressing plants, demonstrating that OsIRT1 can be used for enhancing micronutrient levels in rice grain.

3 ZIP Family of Metal Transporters

IRT1 is the founding member of a large protein family now known to be present in all biological kingdoms. The family has been named the Zrt-, Irt-like protein (ZIP) family for the founding members, Zrt1p and Zrt2p, the primary Zn uptake transporters in *Saccharomyces cerevisiae* (Zhao and Eide 1996a, b) and IRT1 (Eide et al. 1996).

Eukaryotic ZIP family members have been subdivided further into ZIPI/II, LZT (*L*IV1 subfamily of *Z*IP *T*ransporters), and the gufA (gene of unknown function A) subfamilies (Taylor and Nicholson 2003). Four of the five ZIP family members from *S. cerevisiae* and 15 of the 16 ZIP members in Arabidopsis are within the ZIP I subfamily (Taylor and Nicholson 2003). Arabidopsis IAR1 and yeast Yke4p represent the single LZT subfamily member present in these organisms. *iar1* plants partially suppress the phenotype of a mutant that overproduces IAA (Indole-3-acetic acid), the major endogenous form of the plant hormone auxin (Lasswell et al. 2000). This suggests that the *IAR1* participates in auxin metabolism or response.

ZIP transporters predominantly transport metal ion substrates across cellular membranes into the cytoplasm. However, two ZIP family members are suspected of transporting cations in the opposite direction or bidirectionally. *Gm*ZIP1 from soybean is characterized as complementing the Zn uptake defect of *zrt1 zrt2* yeast and localizing to the peribacteroid membrane of symbiosome, which house symbiotic bacteria in legumes (Moreau et al. 2002). Inhibition of Zn uptake in isolated symbiosomes by *Gm*ZIP1 antibodies suggests that *Gm*ZIP1 transports Zn, not into the cytoplasm but out of the cytoplasm into the symbiosome. The direction of substrate transport was called into question further by recent genetic evidence gathered on the *S. cerevisiae* ZIP, Yeast *KE4*, or *YKE4* (Kumanovics et al. 2006).

In Zn adequate conditions, Yke4p transports Zn into the secretory pathway, as evidenced by a Zn suppressible cell-wall defect in yeast lacking Yke4p. However, under conditions of low cytosolic Zn, Yke4p actually removes Zn from the secretory pathway. This was demonstrated genetically where deletion of *YKE4* partially rescues the phenotype of cells lacking an ER Zn importer, Msc2p. While the genetic evidence is compelling, the direct uptake or efflux of Zn was not determined.

3.1 Structure and Function of ZIP Transporters

ZIP family members, also known as the solute carrier family 39A (SLC39A) family in mammals, are predicted to have eight transmembrane domains (TMDs), a histidine-rich loop between TMD III and IV and extracellular amino and carboxy termini. TMD IV and TMD V are the most highly conserved segments among ZIP orthologs and compose a "signature sequence," used to identify additional ZIPs from genomic sequences or EST databases. The signature sequence contains several charged residues that are critical for function and are part of two amphipathic α helices thought to form an intramembranous cation-binding site critical for the passage of substrates across the membrane (Eng et al. 1998).

The predicted intercellular loop between TMD III and IV in Zrt1p has been studied extensively. In the absence of Zn, Zrt1p is a stable plasma membrane protein. However, in response to high levels of Zn, Zrt1p is ubiquitinated at a critical lysine (Lys^{195}) in the predicted intercellular loop between TMD III and IV (Gitan et al. 1998; Gitan and Eide 2000). Substitution of Lys^{195} with Arg blocks the ubiquitination and subsequent endocytic degradation of Zrt1p (Gitan and Eide 2000). Similar Lys residues in the loop between TMD III and IV of IRT1 are also required for Fe-induced turnover of IRT1, suggesting both a role for these lysines in turnover of IRT1 and that the loop containing the residues is in the cytoplasm (Kerkeb et al. 2008).

3.2 ZIP Transporters in Plants

Arabidopsis IRT2 is similar to IRT1, in that it can transport Fe and Zn in yeast, but unlike IRT1, IRT2 does not transport Mn or Cd. *IRT2* mRNA accumulates mainly in the root epidermis in response to Fe deficiency, suggesting that like IRT1, Fe in the soil is a biologically relevant substrate for IRT2. However, expression of IRT2 in mutant *irt1* plants does not rescue the seedling lethal phenotype (Varotto et al. 2002; Vert et al. 2002), and a null mutant of *irt2* does not show chlorosis like *irt1* does when grown with low iron (Vert et al. 2009). Because IRT2 localizes to intracellular vesicles, Vert et al. (2009) have proposed that IRT2 likely prevents toxicity from IRT1-dependent iron fluxes in epidermal cells, via compartmentalization. IRT3 localizes to the plasma membrane and has been shown to transport

Fe and Zn but not Mn when expressed in yeast (Lin et al. 2009). Overexpression of IRT3 in Arabidopsis leads to increased accumulation of Fe in the roots and Zn in the shoots of transgenic plants, with no differences seen in the accumulation of Mn, Cu, Mo, Mg, or Ca. Because the expression of IRT3 is increased in response to Zn deficiency, its main role may be in Zn transport, but whether it functions in uptake from the soil or transport to the shoot remains to be determined; its expression pattern is rather ubiquitous, with strong expression seen in the stele.

ZIP1, ZIP2, and ZIP3 from Arabidopsis were isolated through their ability to suppress the growth defect of a Zn transport mutant of yeast; a subsequent family member, ZIP4, was then identified by its similarity to ZIP1, ZIP2, and ZIP3 (Grotz et al. 1998). ^{65}Zn uptake competition assays demonstrated that in addition to Zn, ZIP1 and ZIP2 transport Cd and Cu. The Cu transport of ZIP2 and ZIP4 was confirmed through their ability to suppress the growth defect of Cu transport yeast mutants (*ctr1*) on respiration selective media (Wintz et al. 2003). However, direct transport of Cd by either ZIP2 or ZIP4 has yet to be demonstrated, so whether Cd inactivates ZIP2 or ZIP4 or whether it is transported remains unresolved. The substrate specificity of ZIP3 is potentially broader. ^{65}Zn uptake of ZIP3 is inhibited not only by Cd and Cu but also by Mn, Fe, and Co. The differential cation selectivity of Arabidopsis ZIP transporters is a valuable source of information as to potential residues involved in cation specificity.

The role of ZIP1, ZIP3, ZIP4, ZIP5, and ZIP9 in Zn transport is supported by the observation that expression of these transporters is increased in response to Zn deficiency; *ZIP2* and *ZIP4* also respond to Cu-deficiency (Grotz et al. 1998; Hall and Williams 2003; Wintz et al. 2003). *ZIP4* and *ZIP9* are expressed in roots and shoots, whereas *ZIP1*, *ZIP3*, and *ZIP5* are mainly expressed in the roots. Until plant mutant phenotypes have been resolved and the specific localization for each transporter determined, the exact role of these transporters in plants is unclear.

In addition to *Os*IRT1 and *Os*IRT2, there are at least ten additional known ZIP family members in rice (Ishimaru et al. 2005). *Os*IRT2 is similar to *Os*IRT1 and is a functional Fe and Cd transporter expressed in Fe-deficient roots of rice, where it localizes to the plasma membrane (Ishimaru et al. 2006; Nakanishi et al. 2006). Of the ten remaining ZIP family members in rice, only four, ZIP1, ZIP2, ZIP3, and ZIP4, have been characterized thus far. The expression of these transporters is stimulated by Zn-deficiency. *OsZIP2* mRNAs accumulates solely in Zn-deficient roots. In contrast, *OsZIP1*, *OsZIP3*, and *OsZIP4* are expressed in both Zn-deficient roots and shoots (Ramesh et al. 2003; Ishimaru et al. 2005). Not surprisingly, the metal specificities vary among *Os*ZIP1, *Os*ZIP2, *Os*ZIP3, and *Os*ZIP4. All four transporters are able to suppress the growth defect of yeast lacking high affinity Zn uptake. However, where the expression of *Os*ZIP1 confers Cd sensitivity to yeast, *Os*ZIP2 and *Os*ZIP3 do not (Ramesh et al. 2003). Mineral content analysis by ICP-MS (Inductively Coupled Plasma – Mass Spectrometry) shows that yeast expressing *Os*ZIP1 contain three to four times more Cd than to cells expressing *Os*ZIP3, or with vector only. Competition assays also show that ^{65}Zn uptake of *Os*ZIP1 is strongly inhibited by Cd, where the ^{65}Zn uptake of yeast expressing *Os*ZIP3 is not. The prospect of using this and similar information on ZIP family members to

improve the nutrient quality of rice grain would be of great value. Rice grain is a staple for over three billion people (Khush 2005), yet the mineral content of polished rice is extremely low (Simmons et al. 2003; Reeves and Chaney 2004). Rice also represents the primary source of Cd intake for several populations, including industrialized nations like Japan (Simmons et al. 2003; Tsukahara et al. 2003; Reeves and Chaney 2004; Liu et al. 2005).

In addition to *Arabidopsis thaliana* and agricultural crops such as rice, tomato, and soybean, ZIP family members have also been characterized in plants that hyperaccumulate metals. Such metal hyperaccumulating plants are able to tolerate levels of metal that are toxic to their nonhyperaccumulating cousins (i.e., *A. thaliana*). For example, the hyperaccumulator, *Arabidopsis halleri*, is known to accumulate Zn and Cd. In *A. halleri* shoots, Zn represents up to 1.5% of the dry weight, which surpasses the critical Zn toxicity of most species (0.01–0.03% Zn in dry biomass) including *A. thaliana*, which accumulates up to 0.015% Zn in dry shoots (Marschner 1995; Becher et al. 2004). While Cd does not accumulate to levels as high as Zn, Cd hyperaccumulating plants are defined as those that have 0.01% or more Cd on a dry weight basis when grown in their natural environment (Baker and Brooks 1989). Exploiting the fact that *A. halleri* is 94% identical to *A. thaliana* at the nucleotide level within coding regions, an *A. thaliana* genome microarray has been used with *A. halleri* cDNA to show that *ZIP6*, *ZIP9, ZIP10*, and *IRT3* expression levels are higher in *A. halleri* relative to the expression levels in *A. thaliana* (Becher et al. 2004; Weber et al. 2004; Talke et al. 2006). Gene duplications may account for some of the increase in expression, because 10 copies of *ZIP6* and 13 copies *ZIP9* are thought to be present in *A. halleri* (Talke et al. 2006). This data suggests that overexpression of *ZIP6*, *ZIP9*, *ZIP10*, and *IRT3* in *A. halleri* leads to hyperaccumulation of Zn and Cd. Similarly, increasing the expression of ZIP transporters in crop plants could enhance the mineral content; however, the risk of Cd accumulation warrants attention.

4 NRAMP Family of Metal Transporters

Members of the natural resistance-associated macrophage protein (NRAMP; SLC11A family in mammals) compose a large super family of metal transporters that are conserved from bacteria to mammals. While the major physiological role of NRAMP transporters is in Mn and Fe transport, some members have the capacity to transport other cations, including Ni, Zn, Cu, Co, and Cd (Gunshin et al. 1997; Liu and Culotta 1999; Portnoy et al. 2000). Prominent NRAMP family members include mammalian NRAMP1 and NRAMP2 (also known as DMT1 or DCT1; for *d*ivalent *m*etal *t*ransporter 1, or *d*ivalent *c*ation *t*ransporter 1 respectively), yeast *SMF1* (*S*uppressor of *M*itochrondrial import *F*unction 1), and Arabidopsis *At*NRAMP1, *At*NRAMP3, and *At*NRAMP4. Mammalian NRAMP1 confers resistance to bacterial infection and functions at the lysosomal membrane of macrophages and neutrophils (Atkinson et al. 1997; Gruenheid et al. 1997; Govoni and

Gros 1998). Opposing characterizations of NRAMP1 in CHO (Chinese hamster ovary cells) and *Xenopus laevis* oocytes offer conflicting models as to how NRAMP1 is involved in bacterial resistance (Mackenzie and Hediger 2004). Evidence for one model is based on data gained through ectopic expression of NRAMP1 on the plasma membrane of CHO cells. In this case, NRAMP1 mediates the uptake of Fe^{2+}, Mn^{2+}, and Co^{2+} with the proton gradient (Forbes and Gros 2003). This data suggests that NRAMP1 on the lysosomal membrane can extract nutrients from the lysosome into the cytoplasm thus depriving bacteria of essential minerals. A second group found that expression of NRAMP1 in *X. laevis* transported metal against the H^+ gradient (Goswami et al. 2001). This led them to hypothesize that NRAMP1 on the lysosome membrane would not deprive the bacteria of nutrients but rather would concentrate toxic amounts of metals within the lumen of the lysosome.

Mammalian DMT1 (NRAMP2) is essential for Fe^{2+} uptake from food in the duodenum (Gunshin et al. 1997; Fleming et al. 1998), and for transporting Fe across endosomal membranes (Fleming et al. 1998; Gruenheid et al. 1999). DMT1 expression is stimulated in direct response to dietary Fe starvation (Canonne-Hergaux et al. 1999). Transport studies in *X. laevis* oocytes have demonstrated that DMT1 is capable of transporting Fe^{2+}, Mn^{2+}, Co^{2+}, Cd^{2+}, Cu^{2+}, Ni^{2+}, Pb^{2+}, and possibly Zn^{2+} in a pH-dependent manner (Gunshin et al. 1997). Two naturally occurring but equivalent substitutions in DMT1 (G185R) are responsible for the microcytic anemia of *Belgrade* rat (Fleming et al. 1998) and microcytic anemic (*mk*) mice (Fleming et al. 1997). Mammalian DMT1, but not NRAMP1, is able to functionally complement the Mn^{2+} transport defect of mutant yeast lacking the NRAMP orthologs *SMF1* and *SMF2* (Pinner et al. 1997).

Yeast have three NRAMP family members, Smf1p, Smf2p, and Smf3p (Portnoy et al. 2000). The triple *smf1 smf2 smf3* mutant is hypersensitive to the metal chelator, EGTA (ethylene glycol bis(2-aminoethyl ether)-*N*,*N*,*N*′*N*′-tetraacetic acid), whereas the *smf1* single mutant is more sensitive to EGTA relative to the other single mutants (Cohen et al. 2000). Smf1p has a high affinity for Mn^{2+}, but a low specificity as it also transports Mg^{2+}, Co^{2+}, Zn^{2+}, Cu^{2+}, Fe^{2+}, Ni^{2+}, and Cd^{2+} (Gadd and Laurence 1996; Liu et al. 1997; Cohen et al. 2000). In fact, overexpression of Smf1p, but not Smf2p or Smf3p, can rescue the Fe uptake mutant *fet3 fet4* (Cohen et al. 2000).

A. thaliana has six NRAMP genes, four of which have been characterized. *At*NRAMP1, *At*NRAMP3, and *At*NRAMP4 are all able to functionally complement the Fe uptake defect of *fet3 fet4* yeast in a pH-dependent manner (Curie et al. 2000; Thomine et al. 2000), and to rescue the Mn uptake defect of *smf1* mutant yeast (Thomine et al. 2000). Furthermore, expression of *At*NRAMP1, *At*NRAMP3, and *At*NRAMP4 leads to cellular Cd accumulation, rendering yeast sensitive to 10 mM Cd (Thomine et al. 2000). In Arabidopsis, steady state mRNA levels of *NRAMP1*, *NRAMP3*, and *NRAMP4* increase in response to Fe-deficiency, with mRNAs for *NRAMP1* and *NRAMP3* primarily accumulating in roots, and *NRAMP4* mRNA accumulating in both roots and shoots (Curie et al. 2000; Thomine et al. 2000). *In planta*, overexpression of NRAMP1 increases the resistance of transgenic plants to high levels of Fe (Curie et al. 2000). This suggests a possible role for NRAMP1 in

sequestering Fe, rather than in Fe uptake. This hypothesized role for NRAMP1 is supported by the fact that it has a plastid targeting sequence, and plastids are a known site for Fe storage in plants (Terry and Abadia 1986).

A role for NRAMP3 and NRAMP4 in Arabidopsis has recently been documented. *NRAMP3* and *NRAMP4* have overlapping expression patterns and localize to the vacuolar membrane [Fig. 1 (Thomine et al. 2003; Lanquar et al. 2005)]. Neither single mutant has a dramatic phenotype, yet the double mutant *nramp3 nramp4* seedlings exhibit a transient Fe-dependent delay of cotyledon greening and root growth (Lanquar et al. 2005). Furthermore, when double mutant plants are germinated on Fe-deficient calcareous soil, 90% of the double mutant seedlings die. Analysis of *nramp3 nramp4* mutant seeds found that there is no decrease in the amount of stored Fe. However, a time course of germinating seeds examined by electron microscopy revealed that Fe stored in vacuoles, as electron dense globoids, did not diminish overtime in *nramp3 nramp4* seeds as observed in wild-type seeds. Furthermore, overexpression of *NRAMP3* downregulates *IRT1* and *FRO2* mRNA levels, both of which have been shown to accumulate in response to Fe-deficiency (Thomine et al. 2003). These results favor the hypothesis that NRAMP3 and NRAMP4 function to mobilize Fe stored in the vacuole into the cytoplasm (Lanquar et al. 2005). Interestingly, AtNRAMP3 and, to a lesser extent, AtNRAMP4 also play a role in resistance against the bacterial pathogen *Erwinia chrysanthemi* (Segond et al. 2009). The susceptibility of the double *nramp3 nramp4* mutant is associated with the reduced accumulation of reactive oxygen species and ferritin, an iron storage protein known to participate in *A. thaliana* defense. This finding suggests that the function of NRAMP proteins in innate immunity has been conserved among plants and animals.

NRAMP6 has recently been suggested to serve as an intracellular transporter and to contribute to Cd toxicity (Cailliatte et al. 2009).

4.1 P_{1B}-ATPase Family of Metal Transporters

The P-type superfamily of ATPases are characterized by the formation of a phosphorylated (P-type) intermediate and by inhibition by vanadate. With a total of 46 P-type ATPases, Arabidopsis encodes for more P-type ATPases than any other organism, including rice (Baxter et al. 2003). P-type ATPases can be subdivided into distinct subfamilies including the heavy-metal-transporting P_{1B}-ATPases, originally named CPx-ATPases. Arabidopsis has eight P_{1B}-type ATPases designated Heavy Metal ATPase (HMA) 1–8 (Baxter et al. 2003; Cobbett et al. 2003; Williams and Mills 2005). As their name indicates, HMA proteins use the energy of ATP hydrolysis to catalyze the transport of heavy metals, such as Cu, Zn, Cd, Pb, and Co across membranes. Through sequence analysis of the P-type ATPase superfamily, the P_{1B}-ATPases were further subdivided, based on covariance of conserved amino acids and transport specificity (Axelsen and Palmgren 1998;

Argüello 2003). HMA1 through HMA4 group with the $Zn^{2+}/Cd^{2+}/Pb^{2+}$ transporting class, also known as group IB-2 (Argüello 2003; Cobbett et al. 2003; Williams and Mills 2005). HMA5 through HMA8 group with the Cu^{+}/Ag^{+} transport class, also known as group IB-1 (Argüello 2003; Cobbett et al. 2003; Williams and Mills 2005).

The structure of P_{1B}-ATPases is characterized by eight TMDs that presumably form an ionic pore; a CPx/SPC motif in TMD VI is thought to bind the metal for transport; and predicted metal-binding domains (MBD) at the amino and/or carboxy termini (Williams et al. 2000; Banci et al. 2006). The ionic specificity of P_{1B}-type ATPases is attributed to N and C terminal MBD and membrane transport sites (Banci et al. 2006).

P_{1B}-type ATPases serve essential functions in plants. For instance, HMA6 (PAA1) and HMA8 (PAA2) are two closely related P-type ATPases crucial for delivering Cu to and within the chloroplasts (Shikanai et al. 2003; Abdel-Ghany et al. 2005). In this instance, the Cu content of chloroplast fractions and subcellular localization support a role for HMA6 in transporting Cu across the plastid envelope and HMA8 in transporting Cu across the thylakoid membrane (Fig. 1). Another P_{1B}-type ATPase, HMA7 (RAN1, responsive to antagonist 1) was identified in a genetic screen for mutations that alter sensitivity to the ethylene antagonist, trans-cyclo-octene or TCO (Hirayama et al. 1999). Mutant *hma7* seedlings exhibit a TCO-dependent ethylene phenotype that can be reversed through the application of Cu (Hirayama et al. 1999). Cloning of *HMA7* revealed that it was similar to the P-type ATPases ATP7A, ATP7B (Menkes and Wilson's disease proteins respectively), and CCC2 (Ca^{2+} cross-complementing 2) from *S. cerevisiae*. In fact, HMA7, ATP7A, or ATP7B expression rescues the Cu-dependent phenotype of mutant *ccc2* cells (Hung et al. 1997; Payne and Gitlin 1998; Borjigin et al. 1999; Hirayama et al. 1999; Forbes and Cox 2000; Mercer et al. 2003). Ccc2p localizes to a late Golgi compartment or post-Golgi compartment where Ccc2p is required to deliver Cu to the metallo-oxidase Fet3p (Yuan et al. 1995, 1997). Using this evidence, the proposed model for RAN1/HMA7 is that it is required to deliver Cu into the Golgi (Fig.1) for metallation of the ethylene receptor apoprotein, ETR1 (Hirayama et al. 1999; Woeste and Kieber 2000), which requires a Cu cofactor for high affinity ethylene binding (Rodriguez et al. 1999).

Other HMA family members function in metal detoxification. For instance, HMA5 functions in Cu compartmentalization and detoxification (Andres-Colas et al. 2006). As evidence for this, elevated Cu levels primarily in roots specifically stimulate *HMA5* expression. Two T-DNA insertion mutants, *hma5-1* and *hma5-2*, are both hypersensitive to Cu but not other heavy metals such as Cd. These T-DNA mutants also accumulate greater amounts of Cu in their roots relative to wild-type plants. Furthermore, a strong interaction was documented between HMA5 and the Arabidopsis Cu chaperone, ATX1, in a yeast two-hybrid assay. This interaction is analogous to that seen with Atx1p and Ccc2p in *S. cerevisiae* and further suggests that HMA5 is a Cu transporter. In addition, a study of natural variation in Cu tolerance among Arabidopsis accessions identified HMA5 as a major QTL associated with Cu translocation capacity (Kobayashi et al. 2008).

HMA4 and HMA2 appear to have partially redundant functions in Arabidopsis. No growth phenotypes were observed with either *hma2* or *hma4* single mutant plants (Hussain et al. 2004). However, *hma2 hma4* double mutant plants require high levels of exogenous Zn to avoid severe chlorosis, growth inhibition, and failed seed set. Characterization through expression in bacteria and yeast suggests that HMA4 functions in Zn, Cd, and Pb efflux at the plasma membrane [Fig. 1 (Mills et al. 2003; Mills et al. 2005; Verret et al. 2005)]. Similarly, the ATPase activity of HMA2 is activated by Zn and Cd (Eren and Argüello 2004). Consistent with the characterization in yeast, *hma2 hma4* plants had two- to fourfold lower Zn content in their shoots relative to wild-type plants (Hussain et al. 2004). In contrast, the Zn accumulation in roots was twofold higher in *hma2 hma4* plants, suggesting that HMA2 and HMA4 are not involved in Zn uptake in the roots but rather in Zn translocation to the shoot. Root to shoot Cd translocation is also decreased to about 2% that of wild type in the *hma2 hma4* mutant (Wong and Cobbett 2008), suggesting that these two transporters are also the major mechanism for Cd translocation in Arabidopsis. Plants that overexpress *HMA4* have higher shoot Zn and Cd content, further supporting a role for HMA4 in root to shoot translocation of Zn and Cd (Verret et al. 2004). *HMA2* and *HMA4* promoter GUS fusions revealed heavy staining in the plant vasculature (Eren and Argüello 2004; Hussain et al. 2004; Verret et al. 2004), consistent with a role for HMA2 and HMA4 in transport of Zn and Cd into the vasculature. Interestingly, Zn hyperaccumulation and full hypertolerance to Zn and Cd in *A. halleri* depend on HMA4 (Hanikenne et al. 2008). The enhanced expression of HMA4 in *A. halleri* relative to *A. thaliana* turns out to be due to a combination of modified *cis* regulatory sequences and copy number expansion (Hanikenne et al. 2008).

Prior to any functional characterization, HMA1 was predicted to transport Co^{2+} based on its similarity to the P_{1B}-type ATPase, CoaT, from *Synechocystis* (Rutherford et al. 1999; Argüello 2003). However, yeast expressing HMA1 targeted to the plasma membrane contained 6.4-fold more Zn and 2.8-fold more Cu compared to vector only controls as determined by elemental analysis using inductively coupled plasma – atomic emission spectrometry or ICP-AES (Seigneurin-Berny et al. 2006). *In planta*, HMA1 is primarily expressed in green photosynthetic tissues, where it localizes to the chloroplast envelope (Fig. 1). This was substantiated upon analysis of chloroplasts from mutant *hma1* seedlings, which contained approximately 30% less Cu than wild-type chloroplasts. Furthermore, mutant *hma1* plants were photosensitive when grown under high light conditions. Thus, the role for HMA1 appears to be similar to that of HMA6 and HMA8, although the phenotype of the single *hma1* mutant demonstrates it has a function distinct from that of HMA6 and HMA8 in chloroplast Cu homeostasis. This data also demonstrates there are additional specificity determinants beyond highly conserved amino acids among P_{1B}-type ATPases.

The Arabidopsis HMA3 protein had been previously shown to localize to the vacuolar membrane in yeast and to rescue the Cd sensitivity of yeast lacking the glutathione conjugate pump, Ycf1p (Gravot et al. 2004); such yeast are sensitive to Cd as they are not able to sequester glutathione-Cd conjugates within the

vacuole (Li et al. 1997). A role for HMA3 in vacuolar sequestration of Cd and other heavy metals has now been directly demonstrated in plants. A *hma3* mutant is more sensitive to Cd and Zn whereas overexpression of *HMA3* improved plant tolerance to Cd and Zn as well as Co and Pb (Morel et al. 2009). As expected from the studies in yeast, HMA3 localizes to the vacuole and is highly expressed in vascular tissues.

5 COPT Family of Metal Transporters

Another group of transporters that primarily move Cu as a substrate in plants is the COPT (COPper Transporter) family (Burkhead et al. 2009). COPT1 was the first member identified through functional complementation of yeast defective in high-affinity copper uptake (Kampfenkel et al. 1995). Isolation of COPT1 led to a family of similar membrane proteins through database searches (Sancenon et al. 2003) that are related not only to COPT1 but also to the Ctr1 and Ctr3 Cu transporting ATPases of *S. cerevisiae*. Collectively, these transporters form the SLC31 copper transporting family. SLC31 members described to date are composed of three TMDs with an extracellular amino terminus and intracellular carboxy terminus. Early cross-linking and molecular weight analysis suggested that human CTR1 (hCTR1), together with yeast Ctr1 and Ctr3, have a mass equivalent to the expected size of a homotrimeric complex (Dancis et al. 1994; Pena et al. 2000). Electron cyrstallographic analysis of hCTR1 revealed a trimer with a central channel large enough to accommodate a Cu cation (De Feo et al. 2009).

Subsequent work on additional Arabidopsis SLC31 members also demonstrated that expression of COPT2 could also functionally complement yeast defective in Cu uptake. However, COPT3 and COPT5 were unable to restore growth of uptake deficient yeast. While the expression of those transporters unable to complement *ctr1 ctr3* yeast (COPT3, COPT4, and COPT5) was unaffected by exogenous copper, the expression of those ORFs able to complement *ctr1 ctr3* yeast (COPT1 and COPT2) was downregulated in response to copper. The Cu-stimulated regulation of COPT1 and COPT2 implies that Cu is the biologically relevant substrate of these transporters. In addition, short-term radiological uptake experiments of *COPT1* antisense lines show a 40–60% reduction in ^{64}Cu uptake in the suppressed *COPT1* lines relative to wild-type plants (Sancenon et al. 2004). Long-term accumulation of Cu was also reduced in the *COPT1* antisense lines by 40–60% as determined by atomic absorption spectroscopy of rosette leaves. In addition, the *COPT1* antisense lines also exhibit increased sensitivity to Cu-limitation. GUS staining in *COPT1-GUS* lines localizes to several cell types, but most heavily in the elongation zone of both primary and secondary roots. Together, these results suggest that COPT1 functions in Cu uptake from the medium at the elongation zone of roots. While exact subcellular localization is pending, the proposed function for COPT1 is similar to that demonstrated for the hCtr1 ortholog in intestinal absorption of Cu (Nose et al. 2006)

and the plasma membrane Cu uptake performed by the Ctr1 and Ctr3 orthologs of *S. cerevisiae* (Dancis et al. 1994; Pena et al. 2000).

6 CDF Family of Metal Transporters

The Cation Diffusion Facilitator (CDF), or SLC30 family of metal transporters, is another large and ubiquitous family of membrane proteins. CDFs are primarily known as metal/H^+ antiporters that either transport metals out of the cell or into the lumenal space of intracellular compartments. However, recent characterization of a murine ZnT5 (Zinc Transporter 5) splice variant, Znt5B, showed that it was capable of bidirectional Zn transport across the plasma membrane (Valentine et al. 2007). Structurally, the CDF family members are very similar, with six TMDs and extracellular amino and carboxy termini. The substrates of characterized CDFs include Co, Cd, Zn, Mn, and possibly Fe. In yeast, several CDF members have been characterized including: Zrc1 (Zinc Resistance Conferring 1), Cot1 (Cobalt Toxicity 1), Msc2 (Meiotic-sister chromatid recombination 2), and Zrg17 (Zn Regulated Gene 17). Both Zrc1 and Cot1 localize to the vacuolar membrane and are thought to sequester Zn in the vacuole (MacDiarmid et al. 2000; MacDiarmid et al. 2003) and both have also been implicated in ER function (Ellis et al. 2004). Msc2 has been shown to localize to the ER, where it is required for ER function (Li and Kaplan 2001). Similarly, Zrg17 localizes to the ER and has been shown to interact with Msc2 to form a heteromeric Zn transporting complex (Ellis et al. 2005).

In Arabidopsis, there are 12 predicted CDF family members that are referred to as MTPs (metal tolerance proteins). Both AtMTP1 and AtMTP3 localize to the vacuolar membrane (Kobae et al. 2004; Desbrosses-Fonrouge et al. 2005; Arrivault et al. 2006). Overexpression of either MTP1 or MTP3 confers resistance to high levels of Zn while loss of function confers Zn hypersensitivity (Desbrosses-Fonrouge et al. 2005; Arrivault et al. 2006). These phenotypes support a role for MTP1 and MTP3 transporters in loading Zn into the vacuole (Fig. 1). MTP11 has been implicated in Mn homeostasis. Arabidopsis *mtp11* mutants are hypersensitive to elevated levels of Mn, whereas plants overexpressing *MTP11* are hypertolerant (Delhaize et al. 2007; Peiter et al. 2007).

Evidence for Zn transport was established via suppression of the Zn-hypersensitive phenotype of yeast lacking both Zrc1 and Cot1, and metal accumulation in Xenopus oocytes expressing AtMTP1. Suppression of AtMTP1 using RNA interference was effective in lowering the Zn content of several organs and in sensitizing plants to exogenous Zn (Desbrosses-Fonrouge et al. 2005). One of a set of three AtMTP1 orthologs present in *A. halleri*, AhMTP1-3, has also been shown to localize to the vacuole and complement mutant *zrc1 cot1* yeast. Furthermore, a closely related homolog from a hybrid poplar, PtdMTP1, has also been shown to both localize to the vacuoles of yeast and plants and functionally complement the *zrc1 cot1* mutant yeast strain (Blaudez et al. 2003). These combined results further

suggest that MTP1 in these species functions to transport Zn into the vacuole (Fig. 1).

CDF family members have been isolated from other species that are either metal tolerant or that hyperaccumulate metals, such as MTP1 from *A. halleri*. For instance, *Stylosanthes hamata*, is a tropical legume tolerant to both low pH and high Mn^{2+} concentrations (Delhaize et al. 2003). Screening of a Stylosanthes yeast expression library identified four CDF family members denoted ShMTP1 through 4 that conferred tolerance to toxic levels of Mn^{2+} to wild-type *S. cerevisiae* (Delhaize et al. 2003). Phylogenetic comparison to AtMTP family members shows that ShMTP1 through 4 form a distinct subgroup together with AtMTP8 through 11, perhaps suggesting that these MTPs in Arabidopsis might also transport Mn^{2+}. Expression of ShMTP1 in Arabidopsis confers increased resistance to Mn^{2+}, as it did in yeast. Localization of an ShMTP1 GFP fusion to yeast, Tobacco, and Arabidopsis vacuolar membranes indicates that ShMTP1 confers resistance to Mn^{2+} through vacuolar sequestration (Delhaize et al. 2003).

7 SLC40 Family of Metal Transporters

Thus far, the only iron exporter identified is ferroportin (Fpn), which is also known as Ireg1 or Mtp1 (Abboud and Haile 2000; Donovan et al. 2000; McKie et al. 2000). While all are orthologous genes, ferroportin was isolated through positional cloning in an anemic zebra fish mutant, whereas IREG1 (Iron-Regulated mRNA 1) and MTP1 (Metal Transporter Protein 1) were isolated using either subtractive cloning among anemic mouse litermates or in a library enriched for IRP1 (Iron-Regulated Protein 1) binding sites, respectively. Fpn is highly expressed in iron-deficient macrophages and intestine. Fpn localizes to the basolateral membrane in duodenal enterocytes where it mediates export of Fe into the bloodstream. The hormone hepcidin binds to ferroportin and induces its internalization and turnover. Certain mutations of Fpn cause an autosomal-dominant disease (type IV hemachromatosis) leading to iron overload, apparently due to the resistance of mutant Fpn to hepcidin-mediated internalization.

Arabidopsis has three SLC40 transporters. *AtIREG2* mRNA expression is induced under Fe-limited conditions and downregulated upon exposure to Fe in a manner similar to IRT1. Given that AtIREG2 shows 34.8% similarity to Fpn, it was thought that it might function as an Fe exporter as well. However, expression of AtIREG2 in yeast could not suppress strains that were either sensitive to exogenous iron or defective in Fe-uptake (Schaaf et al. 2006). Complementation screening in other yeast strains demonstrated that AtIREG2 expression could suppress the growth defect of yeast lacking the vacuolar transporter, Cot1 (Cobalt Toxicity 1), under conditions of low pH and high exogenous Ni^{2+} concentrations. This result was substantiated with radiological uptake assays showing ^{63}Ni accumulation over other metals. Consistent with suppression of Cot1, an AtIREG2-GFP fusion protein localizes to plant vacuoles when expressed in either Arabidopsis protoplasts or root

cells. *In planta*, overexpression of AtIREG2 led to both increased tolerance to high Ni and increased Ni accumulation in roots. In addition, two independent insertion alleles of *ATIREG2* not only contained less Ni in roots but were also more sensitive to Ni in the medium. Interestingly, the Ni sensitivity of the mutant plants was only observed under Fe limitation. Taken together, the data suggests that AtIREG2 detoxifies Ni through vacuolar import perhaps due to increased expression of broad substrate transporters, such as IRT1, under Fe-deficient conditions (Fig. 1).

8 Cation Selectivity

The majority of transport proteins need to get specific ions across the membrane while simultaneously excluding other ions (Gouaux and Mackinnon 2005). To attain this critical specificity, transporters must "feel" the ion to guarantee that only certain ions cross the membrane. In order for a transporter to "feel" an ion, energetically favorable binding sites in the protein must compensate for the energetic cost of dehydrating the ion. The specificity of a transporter results when the binding sites are more energetically favorable for one type of ion than they are for others (Gouaux and Mackinnon 2005). The major factors that contribute to ion selectivity are the atomic composition of the binding site by polypeptide main-chain and side-chain groups and the size of the binding site. These factors provide coordination spheres particular to types of ions. However, these may not be the only selectivity determinants. For instance, metal transporters with conserved MBD are known to transport different substrates (Argüello 2003). Nonetheless, successful ion transport can be viewed as a dynamic process where ions are moved between successive coordination spheres, which perhaps involve conformational changes in the protein that shift coordination spheres among separate TMDs and external loops. The determinants of cation specificity are only just becoming apparent in many disparate emerging systems.

Cation selectivity in ZIP family members

Although members of the ZIP family are capable of transporting a variety of metals including Fe, Mn, Zn, Ni, Cd, and Co, the most common substrate among ZIP family members identified to date is Zn. Extensive radiological uptake and competition assays identified several ZIP transporters that are highly specific for Zn transport, including Zrt1p (Zhao and Eide 1996a), Zrt2p (Zhao and Eide 1996b), human ZIP1 (Gaither and Eide 2001), and mouse ZIP1, ZIP2, ZIP3, and ZIP4 (Zhao and Eide 1996a, b; Gaither and Eide 2000; Gaither and Eide 2001; Dufner-Beattie et al. 2003a, b; Kim et al. 2004; Wang et al. 2004a, b, c). However, other ZIP transporters exhibit broader substrate specificity when tested, including the Arabidopsis ZIP family members IRT1, ZIP2, and ZIP3 (Grotz et al. 1998; Korshunova et al. 1999), *Le*IRT1 and *Le*IRT2 from tomato (Eckhardt et al. 2001), *Os*ZIP1 from rice (Ramesh et al. 2003), human ZIP2 (Gaither and Eide 2000, 2001), and ZupT from *E. coli* (Grass et al. 2002, 2005). Despite this biochemical evidence, our

knowledge of the residues governing substrate specificity in ZIP family members is limited.

IRT1 is capable of transporting Fe, Zn, Mn, Co, and the toxic metal Cd (Eide et al. 1996; Korshunova et al. 1999; Rogers et al. 2000; Henriques et al. 2002; Varotto et al. 2002; Vert et al. 2002), as discussed previously. The substitution of conserved residues capable of binding metal to alanine (A) results in either altered substrate specificity or lack of transport activity in IRT1 (Rogers et al. 2000). Two of the three substitutions that affect IRT1 specificity reside in the first extracellular loop, indicating that this loop is important for substrate specificity. The two substitutions that alter IRT1 selectivity in the first extracellular loop are D100A and E103A.

The D100A allele of IRT1 was unable to support growth of yeast with defects in either Fe or Mn uptake (Fig. 1), indicating a loss of Fe and Mn transport (Rogers et al. 2000). As with IRT1, expression of the D100A allele also rendered wild-type yeast sensitive to Cd in the medium, suggesting that the D100A variant retained that ability to transport Cd. D100A was also capable of complementing Zn uptake in mutant yeast. However, in ^{65}Zn uptake assays, yeast expressing D100A contained 70% less cell-associated ^{65}Zn than yeast expressing wild-type IRT1. This indicates that Zn uptake by D100A was greatly diminished. Despite the reduced overall ^{65}Zn transport, mutant D100A transporters exhibited a higher specificity for Zn. This enhanced specificity was seen in competition studies in which Fe had less of an effect on ^{65}Zn uptake by D100A relative to wild-type IRT1.

Zn transport in the E103A variant was abolished as evidenced by both ^{65}Zn uptake activity and failure of E103A to functionally complement Zn uptake deficient yeast (Rogers et al. 2000). E103A was, however, able to transport Fe as demonstrated through yeast complementation and direct radiological measurement ^{59}Fe uptake in yeast expressing E103A. E103A was also able to rescue yeast deficient in Mn transport and render yeast sensitive to Cd. This indicates that Mn and Cd transport through E103A are maintained. Radiological competition assays show no difference between E103A and IRT1 in ^{59}Fe uptake upon addition of either Zn, or Mn, or Cd. The similar decrease in ^{59}Fe uptake in E103A and IRT1 due to Zn indicates that E103A may bind Zn yet cannot transport Zn successfully.

Given the altered selectivity of D100A, and E103A transporters, the double mutant D100A E103A was also characterized in yeast. It was predicted that the D100A E103A double mutant would not be able to transport Fe, Mn, and Zn (Rogers et al. 2000). The only common substrate that is transported by both the D100A and E103A single mutants is Cd. Therefore, the D100A E103A double mutant was predicted to transport Cd. However, complementation studies in yeast demonstrate that the double D100A E103A mutant transports both Zn and Cd (Rogers et al. 2000).

The carboxyl groups on D^{100} and E^{103} are both likely binding sites for the hard Lewis acids Fe, Mn, and Zn. As such, the loss of transport activity in IRT1 mutants lacking these conserved residues is highly conceivable. However, the Zn transport of the D100A E103A double mutant suggests that there is an alternative route for

Zn uptake via IRT1 in the D100A E103A double mutant. A possible explanation could be that Fe, Mn, and Zn have different ionic radii. The ionic radius of Zn^{2+} is 0.74 Å, while the atomic radii for Fe^{2+} and Mn^{2+} are 0.645 Å and 0.46 Å respectively. In the absence of both D^{100} and E^{103}, the larger ionic radius of Zn^{2+} could allow for binding to more distant ligands that would not be available for binding by Fe^{2+} and Mn^{2+}.

9 Conclusion

The controlled uptake and distribution of metals within plants and other organisms is now just becoming clear. Precisely controlling the distribution of metals is affected not only by regulation of transporters but also by the specificity of the transporters as well. As we have seen, controlling metal homeostasis involves numerous transporters among other factors not discussed in detail here, including metal responsive transcription factors, metallochaperones, and chelators such as phytochelatin and metallothionein. Understanding these systems in detail will allow us not only to add a molecular diagnosis to disease but also to address long-standing plant mineral deficiencies that have downstream effects on agricultural productivity and human health. For instance, both mineral deficiencies and concentration of unwanted toxic metals in staple foods could be resolved through an understanding of plant metal uptake, transporter specificity, and metal distribution.

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Organic Carbon and Nitrogen Transporters

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Abstract During growth and storage phases, plant cells import large amounts of carbon (C) and nitrogen (N) assimilates to drive their metabolism and to facilitate synthesis of storage products. Following assimilation, organic C and N metabolites are transported via the phloem (C) or via the xylem followed by phloem (N) to sink tissues. These translocation processes involve participation of metabolite transporters located in source and sink cells. While information on transporters mediating cellular efflux of C and N metabolites is scarce, cell import systems have been identified and characterized. An overview is provided on transporters importing amino acids, peptides, and sugars into plant cells with particular emphasis on their substrate selectivity, expression, and function.

1 Introduction

Carbon (C) and nitrogen (N) assimilates are the major organic nutrients for plant growth and reproduction. N is generally acquired from the soil as nitrate or ammonium and reduced for inclusion in amino acids in roots or leaves (Miller and Cramer 2005). In addition, roots may take up amino acids, peptides, or proteins (Hirner et al. 2006; Lee et al. 2007; Svennerstam et al. 2007; Komarova et al. 2008;

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Paungfoo-Lonhienne et al. 2008; for review, see Rentsch et al. 2007) as well as amides, amino acids, or ureides derived from atmospheric N_2 reduced in a plant–bacteria symbiosis (Smith and Atkins 2002; Vessey et al. 2005). Following uptake or assimilation in roots, inorganic or organic N is transported in the xylem to shoots, especially to photosynthesizing leaves (Pate 1980). Xylem-derived or leaf-assimilated organic N is loaded into leaf phloem and transported to developing sink organs (Lalonde et al. 2003). The dominant phloem-transported N forms are amino acids and ureides, but other N-containing compounds such as peptides, urea, nucleotides, and RNA are also translocated. Movement of N metabolites from source to sink, from cell to cell, and within cells requires proteins to mediate membrane transport. Most N metabolite transporters characterized to date, are localized to the plasma membrane (PM) and function as proton-coupled importers of amino acids or peptides when analyzed using heterologous expression systems (i.e., *Saccharomyces cerevisiae* mutants and/or *Xenopus* oocytes) or in transgenic plants with increased or decreased transcript levels. This review focuses on these transporters and their function (Table 1). For a more inclusive and detailed description of organic N transporters, see recent work and reviews on amino acid (Delrot et al. 2000; Wipf et al. 2002; Liu and Bush 2006; Tegeder and Weber 2006; Rentsch et al. 2007; Sekito et al. 2008), ureide (Desimone et al. 2002; Pélissier et al. 2004; Rentsch et al. 2007), peptide (Lubkowitz 2006; Waterworth and Bray 2006; Rentsch et al. 2007; Tsay et al. 2007), urea (Liu et al. 2003; Kojima et al. 2006; Wang et al. 2008a), and other N transporters (de Koning and Diallinas 2000; Klein et al. 2006; Haferkamp 2007; Gournas et al. 2008; Maurel et al. 2008).

Sucrose is the dominant form in which photosynthetically reduced C is transported from leaves to heterotrophic sink organs (Lalonde et al. 2003). However, in some plant groups, the principal transported sugars are polyols (e.g., sorbitol, mannitol) or members of the raffinose family of oligosaccharides (RFOs) such as raffinose, stachyose, or verbascose (Lalonde et al. 2003). With the exception of symplasmically loaded RFOs, phloem loading and unloading of sugars includes membrane transport to and from source or sink apoplasms. Apoplasmically unloaded RFOs are hydrolyzed by cell-wall-bound α-galactosidases to sucrose and galactose (Chrost et al. 2007). Sucrose released to, or produced in, the sink apoplasm can be hydrolyzed to glucose and fructose by extracellular invertases (Lalonde et al. 2003). Thus, PM transporters for sucrose and monosaccharides (polyols; hexoses) may operate to load phloem and sink cells. Similarly, sugar transport into and from vacuoles depends upon tonoplast-localized transporters. In addition, amylolytic turnover of starch in chloroplasts releases glucose (Weber et al. 2005) and/or maltose (Nittyla et al. 2004) across the chloroplast envelope. Functional information about the sugar transporters responsible for PM efflux is scant (Carpaneto et al. 2005). The monosaccharide and sucrose transporters (SUTs) localized to PMs and tonoplasts that have been identified and/or characterized belong to the major facilitator superfamily (Lalonde et al. 2004). For greater depth than that summarized here, the interested reader is referred to recent reviews on monosaccharide (Büttner 2007) and sucrose (Sauer 2007) transporters.

Table 1 Overview on functional analyses of amino acid and peptide transporters in plants

Accession	Approach	Species	Function in/ effect on	References
Amino acid transporters				
At1g58360	*ataap1*	*Arabidopsis*	Root uptake	Lee et al. (2007)
AJ318809	*Legumin* (cotyledon-specific) promoter – *VfAAP1*	Pea; *Vicia narbonensis*	Seed size, seed protein, vegetative biomass increase	Rolletschek et al. (2005), Weigelt et al. (2008)
At1g44100	*ataap5*	*Arabidopsis*	Root uptake	Svennerstam et al. (2008)
At1g10010	*ataap8*	*Arabidopsis*	Seed development	Schmidt et al. (2007)
At5g40780	*atlht1*	*Arabidopsis*	Root uptake, mesophyll cell uptake	Hirner et al. (2006), Svennerstam et al. (2007)
At5g04770	*atcat6*	*Arabidopsis*	N supply of sink cells and giant cells	Hammes et al. (2006)
At3g11900	*atant1*	*Arabidopsis*	Proportions of phloem amino acids	Hunt et al. (2006)
AB073084	*35S-HvProT*	*Arabidopsis*	Biomass, leaf proline levels	Ueda et al. (2008)
	RC (root cap)-*HvProT*	*Arabidopsis*	Root elongation, root tip proline levels	Ueda et al. (2008)
Peptide transporters				
At3g54140	*atptr1*	*Arabidopsis*	Root uptake, biomass, N content	Komarova et al. (2008)
At2g02040	*35S-AtPTR2* antisense	*Arabidopsis*	Flowering, seed development	Song et al. (1997)
At5g46050	*atptr3*	*Arabidopsis*	Germination under salt stress, pathogen defense	Karim et al. (2005, 2007)
At5g01180	*atptr5*	*Arabidopsis*	Pollen germination	Komarova et al. (2008)
	35S-AtPTR5	*Arabidopsis*	Pollen germination, root uptake, biomass, N content	Komarova et al. (2008)

2 Organic Nitrogen Transporters

2.1 Amino Acid Transporters

Plant amino acid transporters are grouped into the amino acid–polyamine–choline (APC) and amino acid transporter (ATF) families (Wipf et al. 2002). While some

putative amino acid transporters have been analyzed in potato (Koch et al. 2003), *Nicotiana sylvestris* (Lalanne et al. 1997), *Nepenthes* (Schulze et al. 1999), castor bean (Bick et al. 1998; Neelam et al. 1999), and legumes (Montamat et al. 1999; Tegeder et al. 2000, 2007; Miranda et al. 2001, 2003; Tan et al. 2008), characterization of the substrate selectivity and functional analysis of these transporters *in planta* has mainly focused on *Arabidopsis* proteins (Table 1).

2.1.1 Amino Acid Transporter Family

Most amino acid transporters (ATFs) analyzed so far belong to the ATF family consisting of AAP, lysine/histidine transporter (LHT), ProT, ANT, γ-aminobutyric acid transporter (GAT), and AUX/LAX subfamilies (Rentsch et al. 2007). The *Arabidopsis* AAPs (amino acid permeases), with eight members, mediate proton-coupled transport of neutral and acidic amino acids, and with the exception of the high-affinity AAP6 transporter, exhibit moderate affinity for their substrates (Fischer et al. 1995, 2002; Boorer et al. 1996). Basic amino acids appear to be transported only by AtAAP3 and AtAAP5 (Boorer and Fischer 1997; Fischer et al. 2002). *Arabidopsis* AAP1 has been localized to the PM (Lee et al. 2007), whereas AAP3 was additionally associated with endomembrane structures (Okumoto et al. 2004). RNA expression and promoter:*GUS* studies have demonstrated that *AAP*s display isoform-specific expression patterns and that these are developmentally and environmentally regulated (Fischer et al. 1995; Rentsch et al. 1996; Hirner et al. 1998; Schulze et al. 1999; Okumoto et al. 2002, 2004; Koch et al. 2003; Lee et al. 2007; Tegeder et al. 2000, 2007; Miranda et al. 2001, 2003; Tan et al. 2008; for review, see Liu and Bush 2006). *AAP1* is expressed in root hairs, epidermal cells, and root tips, and *aap1* seedlings showed reduced uptake of glutamate and neutral amino acids, consistent with a role in amino acid acquisition from the rhizosphere (Lee et al. 2007). T-DNA insertion mutations of the constitutively expressed *AAP5* (Fischer et al. 1995) exhibited reduced uptake of basic amino acids (Svennerstam et al. 2008). Antisense repression of the potato-source-leaf-specific amino acid transporter *AAP1* reduced free amino acid levels in tubers, thus suggesting a role of AAP1 in phloem loading (Koch et al. 2003). With respect to sink tissue, only *Arabidopsis AAP8*, which is expressed in the silique vasculature and seeds up to 4 days after pollination has been analyzed so far (Schmidt et al. 2007). Seed number in *aap8* siliques was strongly reduced, indicating a role of AAP8 in delivering amino acids to young embryos. While a direct function of a transporter in import of amino acids by embryos has yet to be shown, embryo-specific overexpression of *AAP1* from *Vicia faba* in pea increased protein content, demonstrating that amino acid transporters can influence seed protein accumulation (Rolletschek et al. 2005; Weigelt et al. 2008).

Naming of the ten *Arabidopsis* "lysine/histidine" transporters (LHTs) was based on the initial biochemical analysis of LHT1 in yeast mutants (Chen and Bush 1997). However, recent suggest that LHT1 and LHT2 preferentially transport neutral and

acidic amino acids with high affinity (Lee and Tegeder 2004; Hirner et al. 2006). All LHTs analyzed so far are targeted to the PM (Hirner et al. 2006; Foster et al. 2008), and RNA localization and promoter:*GUS* studies of four *LHT*s showed distinct and highly regulated expression in reproductive organs, indicating a reproductive function of these transporters. Mutant analyses of *LHT1*, which is expressed in the root epidermis and leaves, showed that the transporter contributes to amino acid uptake from the rhizosphere and N import into mesophyll cells (Hirner et al. 2006; Svennerstam et al. 2007).

The three *Arabidopsis* ProT proteins as well as the tomato ProT1 protein transport proline, glycine betaine, and related quaternary ammonium compounds including γ-aminobutyric acid (GABA) with moderate or low affinity (Rentsch et al. 1996; Breitkreuz et al. 1999; Schwacke et al. 1999; Grallath et al. 2005). Mangrove homologs transport proline and glycine betaine (Waditee et al. 2002), whereas barley ProT is reported to be a specific proline transporter (Ueda et al. 2001). All *Arabidopsis* ProTs are targeted to the PM, and their gene expression patterns are both complementary and cell-specific (Grallath et al. 2005). Salt stress impacts expression of *Arabidopsis ProT2* and *ProT*s of other plant species (Rentsch et al. 1996; Waditee et al. 2002). While data on *proT* mutant analysis is lacking, overexpression of barley *HvProT* in the *Arabidopsis* root cap led to proline accumulation in root tips and enhanced root growth, thus supporting a role in proline transport (Ueda et al. 2008).

Arabidopsis ANT1 is the only one of 13 transporters characterized within the aromatic and neutral amino acid transporter (ANT) subfamily, and it displays moderate affinity for aromatic and neutral amino acids (Chen et al. 2001). While cellular and subcellular localization of ANT1 has not been resolved, mutant plants show differences in amino acids composition of phloem sap compared to wild type (Hunt et al. 2006).

The γ-aminobutyric acid transporter (GAT) subfamily consists of two members in *Arabidopsis*. GAT1, a high-affinity PM transporter of GABA and GABA-related compounds (Meyer et al. 2006), is expressed most strongly in flowers and can be induced by wounding and senescence.

The four Arabidopsis auxin-resistant (AUX/LAX) transporters form the sixth subfamily. AUX1 and LAX3 transport indole-3-acetic acid (IAA), which is structurally similar to the aromatic amino acid tryptophan (Yang et al. 2006; Swarup et al. 2008). The *aux1* mutant exhibits an agravitropic phenotype caused by reduced auxin uptake within *Arabidopsis* roots (Bennett et al. 1996) and the *lax3* mutation results in altered auxin-dependent lateral root formation (Swarup et al. 2008).

2.1.2 Amino Acid–Polyamine–Choline Transporter Family

The APC family is divided into the cationic amino acid transporter (CAT) and L-type amino acid transporter (LAT) subfamilies. There are six predicted LATs (also called glycoprotein-associated amino acid transporters) in *Arabidopsis* that

have not been characterized, but show homology to mammalian proteins containing the light chain of hetero(di)meric amino acid transporters (HATs, Verrey et al. 2004). There are nine predicted CATs in *Arabidopsis* based on their similarity with mammalian systems. *Arabidopsis* CAT1 and CAT5 are high-affinity transporters for basic amino acids (Frommer et al. 1995; Su et al. 2004; Verrey et al. 2004), while CAT6 preferentially transports large, neutral and cationic amino acids with moderate affinity (Hammes et al. 2006). All CATs analyzed so far are localized to the PM, but CAT6 is also targeted to the ER and Golgi. *CAT6* is expressed in root tips as well as in other sink tissues, and its knockout led to growth inhibition of seedlings when exposed to glutamine as sole N source, suggesting a function in supplying sink cells with amino acids (Hammes et al. 2006).

2.2 Peptide Transporters

Plant peptide transporters belong to three gene families, each recognizing peptides of defined length. Di- and tripeptides are transported by members of the peptide transporter/nitrate transporter 1 (PTR/NRT1) family, whereas transport of peptides of four to five amino acids and glutathione is mediated by members of the oligopeptide transporter (OPT) family (Tsay et al. 2007; Rentsch et al. 2007). The PTR/NRT1 and OPT families also comprise transporters for other substrates (for review, see Lubkowitz 2006; Waterworth and Bray 2006; Tsay et al. 2007). In animals, transporters for larger peptides have been identified among ABC transporters (Herget and Tampé 2007). Three homologs are present in the *Arabidopsis* genome, but their function in peptide transport is yet to be demonstrated (Rentsch et al. 2007; Verrier et al. 2008).

2.2.1 Peptide Transporter/Nitrate Transporter 1 Family

Functionally characterized di/tripeptide transporters comprise *Arabidopsis* PTR1 (Dietrich et al. 2004), PTR2 (formerly NTR1 or PTR2B; Frommer et al. 1994; Rentsch et al. 1995; Song et al. 1996; Chiang et al. 2004), and PTR5 (Komarova et al. 2008), as well as barley PTR1 (West et al. 1998). Furthermore, complementation of a yeast peptide transport mutant has been shown for *Arabidopsis* PTR3 (Karim et al. 2007) and *V. faba* PTR1 (Miranda et al. 2003). A more detailed characterization of *Arabidopsis* PTR1 and PTR2 demonstrated that multiple di- and tripeptides are transported with different affinities and that naturally occurring modified peptides like phaseolotoxin and peptide-like substrates lacking a peptide bond, i.e., 4-aminophenylacetic acid are recognized (Chiang et al. 2004; Dietrich et al. 2004). Electrophysiological analyses revealed that PTR2 transports peptides and protons simultaneously by a random binding mechanism (Chiang et al. 2004). PM localization of *Arabidopsis* PTR1 (Dietrich et al. 2004), PTR5 (Komarova et al. 2008), and barley PTR1 (Waterworth et al. 2000) as well as tonoplast localization

of *Arabidopsis* PTR2 (Carter et al. 2004; Dunkley et al. 2006; Shimaoka et al. 2004) suggests involvement in inter- and intracellular transport, respectively. Transcript analyses showed that *PTR* expression is transporter-specific, pointing to different functions in plants. Expression of *V. faba PTR1* during cotyledon development and *PTR1* in the scutellum of germinating barley grains indicates a role in seed and seedling development (West et al. 1998; Miranda et al. 2003). *Arabidopsis PTR1* is expressed during germination and in vascular tissues (Dietrich et al. 2004). Germination and growth studies with *ptr1* mutants showed that PTR1 contributes to dipeptide uptake into roots (Komarova et al. 2008; Table 1). *PTR2* mRNA was detected throughout the plant (Frommer et al. 1994; Song et al. 1996), and *PTR2* antisense lines displayed a late flowering phenotype and arrested seed development (Song et al. 1997; Table 1). However, this phenotype could not be detected in *PTR2* T-DNA insertion lines (Dietrich and Rentsch, unpublished), indicating that additional transcripts are downregulated in the *antisense* lines. *PTR5* promoter activity was localized in *Arabidopsis* pollen and ovules (Komarova et al. 2008) and analysis of *ptr5* mutants and 35S:*PTR5* overexpression lines demonstrated that PTR5 functions in dipeptide transport during pollen germination and tube growth (Komarova et al. 2008; Table 1). *PTR3* expression is regulated by amino acids, salt, salicylic acid, methyl jasmonate, and abscisic acid, and *ptr3* mutants showed impaired germination under salt stress (Karim et al. 2005) as well as reduced defense against virulent bacterial pathogens (Karim et al. 2007). Consistent with a role in transporting protein degradation products, *PTR* mRNA from the carnivorous plant *Nepenthes* was detected in phloem cells of the pitcher organs of the plant (Schulze et al. 1999).

2.2.2 Oligopeptide Transporter Family

Transport of selected tetra- and pentapeptides by *Arabidopsis* OPT1, 4, 5, 6, and 7 has been shown by yeast growth assays (Koh et al. 2002), but OPT2 and 3 function in peptide transport could not be demonstrated. However, OPT3 has been shown to function in metal transport (Stacey et al. 2008). A more detailed electrophysiological analysis of *Arabidopsis* OPT4 using *Xenopus* oocytes revealed a high affinity for the tetrapeptide KLGL (Osawa et al. 2006). *Arabidopsis* OPT6 (Cagnac et al. 2004), a rice homolog (OsGT1, Zhang et al. 2004) and Brassica juncea homolog (GT1, Bogs et al. 2003) expressed in yeast also mediate transport of glutathione and glutathione conjugates. In addition, *Arabidopsis* OPT6 appears to transport cadmium and cadmium/glutathione conjugates (Cagnac et al. 2004). In contrast, no transport of glutathione could be shown when *Arabidopsis* OPT4 was expressed in *Xenopus* oocytes (Osawa et al. 2006). Functionally characterized *Arabidopsis OPT*s show the highest expression in vascular tissues (Stacey et al. 2006). However, differential tissue-specific expression is also seen, as *Arabidopsis OPT1* is expressed in pollen and growing pollen tubes and only *OPT6* transcripts are detected in ovules (Stacey et al. 2006). With the exception of mutations in the

gene encoding the metal transporter OPT3 (Stacey et al. 2002), no phenotypes of *opt* mutants have been described to date.

3 Sugar Transporters

3.1 Monosaccharide Transporters

Phylogenetic analyses have identified 53 putative members of the monosaccharide transporter (*MST*) gene family in *Arabidopsis* (Büttner 2007) and 65 putative members in rice (Johnson and Thomas 2007). These can be grouped into seven subfamilies (Büttner 2007; Johnson and Thomas 2007) comprising sugar transport proteins (STPs), vacuolar glucose transporter-like (VGT), tonoplast monosaccharide transporter (TMT), plastidic glucose transporter (GlcT), polyol transporter (PLT), inositol transporter (INT), and early responsive to dehydration-like (ERD) genes. To date, no ERD isoforms have been functionally characterized (Büttner 2007; Antony et al. 2008) and hence will not be discussed further.

3.1.1 Sugar Transport Protein Subfamily

Arabidopsis contains 14 predicted STP members. The eight biochemically characterized members function as PM-localized proton symporters with apparent K_m values of 10–100 μM glucose (Büttner 2007) except STP3 with an apparent K_m value of 2 mM glucose (Büttner et al. 2000). STPs exhibit a broad specificity for D-hexoses and D-pentoses (Büttner 2007), with exception of STP6 that shows a preference for hexoses (Scholz-Starke et al. 2003). *STP3* expression is localized to green leaves whereas the remaining *STP*s are preferentially expressed in sink tissues; *STP6* (Scholz-Starke et al. 2003) and *STP11* are expressed exclusively in pollen (Büttner 2007). High levels of redundancy amongst STPs represent a major impediment to tests of physiological function (Table 2). Putative STP homologs identified in other species include rice (29 members; Johnson and Thomas 2007), grape (Fillion et al. 1999; Vignault et al. 2005; Hayes et al. 2007), tomato (Gear et al. 2000), barley (Weschke et al. 2003), and walnut (Decourteix et al. 2008). Functionally characterized STP homologs reflect transport properties found for *Arabidopsis* STPs, and the genes encoding these proteins are expressed predominantly in sink tissues with several being anther-specific (*OsMST8*, Mamun et al. 2006; *pmt1*, Yistra et al. 1998). Notable differences from *Arabidopsis* STPs are higher apparent K_m values recorded for rice STPs (200–500 μM glucose; Wang et al. 2008b) and a capability of rice MST4 to transport both glucose and fructose equally (Wang et al. 2007). Downregulation of tobacco *MST1* expression caused a growth phenotype under in vitro conditions when transformants were deprived of sugar supply (Leterrier et al. 2003).

Table 2 Overview on functional analyses of monosaccharide and sucrose transporters in plants

Accession	Approach	Species	Function in/effect on	References
Monosaccharide transporters				
At1g11260	*atstp1*	*Arabidopsis*	Seed germination, growth inhibition protection	Sherson et al. (2000)
AJ001061	Cosuppression of *NtMST*s by *VvHT1*	Tobacco	Sink uptake under low C	Leterrier et al. (2003)
At2g43330	*atint1*	*Arabidopsis*	Vacuole myo-inositol release	Schneider et al. (2008)
At1g20840	*attmt1* or *attmt1-3*	*Arabidopsis*	Vacuole glucose transport	Wormit et al. (2006)
At3g03090	*atvgt1*	*Arabidopsis*	Vacuole glucose transport	Aluri and Büttner (2007)
Sucrose transporters				
At1g71880	*atsuc1*	*Arabidopsis*	Pollen germination, anthocyanin biosynthesis	Sivitz et al. (2008)
At1g22710	*atsuc2*	*Arabidopsis*	Phloem loading	Gottwald et al. (2000)
Q40583	*35S-NtSUT1* antisense	Tobacco	Phloem loading	Bürkle et al. (1998)
AF176638	*rolC* (phloem specific)-*LeSUT1* antisense	Tomato	Phloem loading	Hackel et al. (2006)
Q43653	*35S-StSUT1* antisense	Potato	Phloem loading	Riesmeier et al. (1994)
	rolC-StSUT1 antisense	Potato	Specifically phloem loading	Kühn et al. (1996)
	B33 patatin (tuber specific)-*StSUT1* antisense	Potato	Phloem unloading/sink cell uptake in young tubers	Kühn et al. (2003)
	Vicilin (cotyledon specific)-*StSUT1*	Pea	Seed sucrose levels, cotyledons biomass gain	Rosche et al. (2002)
A36574	*35S-SoSUT1*	Potato	C partitioning in leaves and tubers	Leggewie et al. (2003)
At1g09960	*ppyk20* (nematode syncitia specific)-*AtSUC4* RNAi	*Arabidopsis*	Development of female syncitia	Hofmann et al. (2007)
AF237780	*35S-StSUT4 RNAi*	Potato	Slower phloem loading	Chincinska et al. (2008)
At1g71890	*atsuc5*	*Arabidopsis*	Endosperm fatty acid levels, embryo development	Baud et al. (2005)
At5g06170	*atsuc9*	*Arabidopsis*	Prevention of premature flowering	Sivitz et al. (2007)
Os03g07480	*Ubiquitin-OsSUT1* antisense	Rice	Seed sucrose loading, seed germination	Scofield et al. (2002)
Q9FVL6	*35S-LeSUT2* antisense	Tomato	Pollen tube growth, possibly phloem unloading	Hackel et al. (2006)

3.1.2 Polyol Transporter Subfamily

PLTs have been cloned from celery (*MaT1*, Noiraud et al. 2001; *MaT2*, Juchaux-Cachua et al. 2007), sour cherry (*SOT1*, *SOT2*, Gao et al. 2003), apple (*SOT3*, *SOT5*, Watari et al. 2004), common plantain (*PLT1*, *PLT2*, Ramsperger-Gleixner et al. 2004), olive (*MT1*, Conde et al. 2007), and *Arabidopsis* (*PLT5*, Klepek et al. 2005). These transporters function as proton symporters, with apparent K_m values ranging from 0.3 to 2 mM, except for plantain PLT1 and PLT2 (12 and 20 mM, respectively; Ramsperger-Gleixner et al. 2004). PLTs transport a broad spectrum of polyols, although the apple PLTs, SOT3 and SOT5 exhibit high sorbitol selectivity (Watari et al. 2004). Most *PLT*s are expressed in leaf vasculature (but see Gao et al. 2003), particularly in collection phloem where they are localized to companion cells (Ramsperger-Gleixner et al. 2004) and/or sieve elements and phloem parenchyma cells (Juchaux-Cachua et al. 2007) consistent with a phloem loading function.

3.1.3 Inositol Transporter Subfamily

*INT*s have been cloned from common ice plant (*Mitr1*, *Mitr2*, Chauhan et al. 2000), *Arabidopsis* (*INT1-4*, Schneider et al. 2006, 2007, 2008), and pineapple (*INT1*, Antony et al. 2008). *Arabidopsis* INT 1, 2, and 4 are proton symporters (apparent K_m values of 0.2–1.0 mM) whereas *INT3* is a pseudo gene. In contrast, Mitr1 and 2 function as sodium symporters (Chauhan et al. 2000). *Arabidopsis* INT2 and 4 are PM transporters whereas *Arabidopsis* INT1, pineapple INT1, and *Mesembryanthum* Mitr1 and 2 are tonoplast localized. In *Arabidopsis*, an *int1* knockout produced a reduced root length phenotype with myo-inositol accumulated in vacuoles. The phenotype could be rescued by high exogenous levels of myo-inositol consistent with a function in supplying vacuolar-synthesized myo-inositol to the cytoplasm (Schneider et al. 2008; Table 2).

3.1.4 Vacuolar Glucose Transporter-Like and Tonoplast Monosaccharide Transporter Subfamilies

To date, five hexose transporters, *Arabidopsis* TMT1-3 (Carter et al. 2004; Wormit et al. 2006), *Arabidopsis* VGT1 (Aluri and Büttner 2007), and pineapple MST1 (Antony et al. 2008) have been localized to the tonoplast. *Arabidopsis tmt1* and *tmt1-3* knockouts do not exhibit a morphological phenotype, but vacuolar glucose uptake and accumulation was reduced. Comparison of wild type and *tmt* mutants demonstrated that glucose transport across the tonoplast was coupled to the proton motive force (Wormit et al. 2006). TMT homologs have been detected in sugarcane (Casu et al. 2003) and barley (Endler et al. 2006). Functional characterization of *Arabidopsis* VGT1 demonstrated a probable proton antiporter energy coupling, hexose specificity, and an apparent K_m value of 3.7 mM for glucose (Aluri and

Büttner 2007). *vgt1* mutants exhibit late flowering, reduced germination, and retarded shoot growth suggestive of a central role in cell turgor maintenance for cell expansion (Aluri and Büttner 2007; Table 2).

3.2 Sucrose Transporters (SUTs or SUCs)

In contrast to MSTs, SUTs are encoded by smaller gene families. For example, *Arabidopsis* contains nine (Sauer 2007) and rice five (Aoki et al. 2003) *SUT* genes. Phylogenetic analyses place SUTs into three (Aoki et al. 2003; Lalonde et al. 2004) or four (Sauer 2007) clades. The fourth clade (group) arises from dividing clade III between monocot and dicot SUTs. Using the nomenclature put forward by Lalonde et al. (2004), clade I contains high-affinity transporters (apparent K_m values 0.5–2.0 mM sucrose, except for *Arabidopsis* SUC9 with an apparent K_m of 0.07 mM, Sivitz et al. 2007) in contrast to lower-affinity transporters (apparent K_m values of 4–20 mM sucrose) of clades II and III (Lalonde et al. 2004; Zhou et al. 2007; Reinders et al. 2008). Dicot clade III transporters are structurally distinguished from clade II members by an elongated N-terminus (~20 amino acids) and an enlarged cytoplasmic loop of ~60 amino acids that is not present in monocot members of the clade (Lalonde et al. 2004). Except for several legume members of clades I and II, that function as sucrose facilitators (*Phaseolus vulgaris* SUF1 and pea SUF1, SUF4; Zhou et al. 2007), all remaining characterized SUTs function as proton symporters (Sauer 2007).

3.2.1 Clade I (SUT1/SUC2)

Clade I SUTs are peculiar to dicots (Aoki et al. 2003; Lalonde et al. 2004) and a plant species may contain several isoforms. For example, *Arabidopsis* has seven genes encoding clade I members, of which *SUC6* and *SUC7* are pseudogenes, whereas common plantain contains two SUT1 isoforms (Sauer 2007). *Arabidopsis* SUC2 (Chandran et al. 2003) and SUC9 (Sivitz et al. 2007) exhibit a weak selectivity for α-linked gluocosides and will transport certain β-glucosides. Interestingly, *Arabidopsis* SUC5 also transports biotin (Ludwig et al. 2000). *In planta* PM localization has been confirmed for *Arabidopsis* SUC2 (Endler et al. 2006), SUC1 (Endler et al. 2006; Sivitz et al. 2008), and SUC9 (Sivitz et al. 2007). SUT1s are expressed along the phloem from source to sink in the PM of sieve elements in the Solanaceae, in companion cells of *Arabidopsis* and common plantain (Sauer 2007), or in both cell types (e.g., *AmSUT1*, Knop et al. 2004 but see Schmitt et al. 2008). Reverse genetics demonstrated that SUT1 isoforms play key roles in phloem loading and possibly phloem unloading (Table 2). Phloem-localized SUT1s also are expressed in sink tissues such as developing seeds that load nutrients from apoplasmic spaces (Zhang et al. 2007; Sauer 2007), nematode syncitia (Hofmann et al. 2007), and pollen grains (Sauer 2007). Some SUTs are sink-cell-specific including

tobacco *SUT3,* which is expressed in pollen (Lemoine et al. 1999), *Arabidopsis SUC5*, which is expressed in endosperm (Baud et al. 2005), and *Arabidopsis SUC1*, which is expressed in pollen, trichomes, and roots (Sivitz et al. 2007). Significant roles for SUT1s in various sink cells have been verified for *Arabidopsis* SUC1, SUC5, and SUC9 as well as potato SUT1 (Table 2).

3.2.2 Clade II (SUT4)

Single SUT4 isoforms have been discovered in a number of dicot species including *Arabidopsis*, tomato, potato (Weise et al. 2000), *Lotus japonicus* (Flemetakis et al. 2003; Reinders et al. 2008), and pea (Zhou et al. 2007). SUT4 isoforms have also been found in monocot species such as barley (*HSUT2*, Weschke et al. 2000) and rice (*SUT2*, Aoki et al. 2003). *L. japonicus* SUT4 transports both α- and β-glycosides but with a greater substrate specificity than that of SUT1s (Reinders et al. 2008). Potato SUT4 and tomato SUT4 have been immunolocalized to the PM of sieve elements (Weise et al. 2000); a PM localization was confirmed using a GFP fusion (Chincinska et al. 2008). In contrast, *Arabidopsis* SUT4, barley SUT2 (Endler et al. 2006), and *L. japonicus* SUT4 (Reinders et al. 2008) are localized to tonoplasts of mesophyll cells (Endler et al. 2006) as well as various sink cell types (also see Weschke et al. 2000; Flemetakis et al. 2003). Since SUT4s, except PsSUF4 (Zhou et al. 2007), are proton symporters, tonoplast-localized SUT4 is likely function in the release of vacuolar sucrose to the cytoplasm. RNA interference with *SUT4* expression slows early development of female nematode syncitia in *Arabidopsis* and enhances phloem loading in potato, possibly via posttranscriptional regulation of heterodimerization with SUT1 (Chincinska et al. 2008; Table 2).

3.2.3 Clade III (SUT2)

In contrast to dicots, some monocot species contain more than one clade III isoform (e.g., four isoforms in rice, Aoki et al. 2003). Relative to SUT1s, SUT2s exhibit a greater specificity for sucrose (Meyer et al 2000; Schulze et al. 2000; Sivitz et al. 2005; Reinders et al. 2006). In addition, gene chimeras demonstrate that the extended central loop exerts no influence on transporter kinetics (Meyer et al. 2000; Schulze et al. 2000) whereas the N-terminus determines transporter affinity for sucrose (Schulze et al. 2000). SUT2s are localized to the PM of sieve elements in tomato (Barker et al. 2000), common plantain (SUC3, Barth et al. 2003), *Arabidopsis* (SUC3, Meyer et al. 2004), and wheat (SUT1A, 1B, and 1D, Aoki et al. 2004). In contrast, rice SUT1 localizes to sieve elements and companion cells (Scofield et al. 2007). SUT2s are also present in mesophyll cells (Barth et al. 2003), cells at the periphery of sugarcane vascular bundles (Rae et al. 2005), a range of vegetative sinks (Barth et al. 2003; Meyer et al. 2004), and

developing seeds (Zhang et al. 2007). Repressed expression of genes encoding SUT2s results in impaired pollen development, possible slowing of phloem unloading, and reduced seed fill (Table 2). Maize SUT1 is capable of reversed transport under conditions where the free energy of the transmembrane sucrose gradient exceeds that of the opposing proton motive force (Carpaneto et al. 2005). Such findings imply a possible role for SUT2s localized to nonvascular leaf cells in apoplasmic phloem loading (Meyer et al. 2000) and in apoplasmic phloem unloading in vegetative (Carpaneto et al. 2005) and seed (Zhang et al. 2007) sinks containing an active extracellular invertase.

4 Conclusions

Intra- and intercellular membrane transport processes are critical for sustaining the complexity of plant metabolism and growth. While knowledge of the location and function of sugar transporters has greatly increased over the last decade, relatively little is known about the substrate selectivity and physiological role of plant organic N transporters or their importance in phloem and seed loading. Further, research has only focused on a small number of amino acid and peptide transporters. As a result, the functions of whole subfamilies remain largely unknown. Similarly, most N and C metabolite transporters characterized to date are proton symporters and are targeted to the PM to mediate metabolite import into plant cells. Future studies will need to address intracellular membrane transporters and identify alternative transport mechanisms such as transporters involved in cellular assimilate efflux. Efforts must be made to dissect the role of these transporters in metabolism and partitioning of metabolites within cells, between cells, and over long distances.

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ABC Transporters and Their Function at the Plasma Membrane

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Abstract The plasma membrane (PM) constitutes the outer boundary of the plant cell. As such, it functions in communication with the neighboring cells and with the environment. Moreover, the PM serves as the principal barrier for the small molecules that enter or exit the cell. To function in this regulatory capacity, the PM harbors a large number of transporters, channels, and carriers that provide selectivity and responsiveness to internal and external stimuli. As opposed to carriers and channels where transport is driven by chemiosmotic gradients, transporters directly utilize energy stored in adenosine triphosphate (ATP) to mobilize substrates, often against the chemiosmotic membrane gradient. One group of ATP-hydrolyzing transport proteins functioning at the PM, the ATP-binding cassette (ABC) transporters, will be reviewed in this chapter.

1 ABC Transporters and Their Function

All transporters that are members of the ATP-binding cassette (ABC) protein superfamily have the ability to bind and hydrolyze ATP as an energy source to drive transport across a membrane. Individually, these proteins can function as exporters, importers, or ATP-regulated channels. They perform essential tasks in almost all phyla, including nutrient uptake, detoxification, and hormone translocation (Nikaido et al. 1998; Singh and Rohm 2008). In animals, some of their functions include the maintenance of the blood–brain, blood–placenta, and blood–gut barrier (Hagenbuch et al. 2002; Deeken and Löscher 2007) and the transport of sterols (Wang et al. 2008). The biological importance of ABC transporters in humans is underscored by the observation that genetic defects in these proteins can lead to severe pathologies.

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Cystic fibrosis in humans results from mutations in the *CFTR* gene, which encodes an ATP-regulated chloride channel (Zhang et al. 2009). Misregulation of ABC transporter abundance can also affect disease therapies. For example, overexpression of the mammalian drug exporter MDR1/ABCB1 contributes to multidrug resistance in tumor cells (Fuchs et al. 1991; Stallard et al. 1989; Vollrath et al. 1991).

In plants, ABC transporters function in the accumulation of plant secondary metabolites in specialized organs such as the rhizome (Shitan et al. 2003) and glandular trichomes (Stukkens et al. 2005), deposition of lipids that form the cuticula (Pighin et al. 2004; Panikashvili et al. 2007; Luo et al. 2007), malate scavenging and regulation of stomatal movement (Lee et al. 2008), detoxification of xenobiotics (Frelet-Barrand et al. 2008; Martinoia et al. 2002; Lee et al. 2005), and transport of the hormone auxin (reviewed in Titapiwatanakun and Murphy 2009). A consensus nomenclature that is consistent with systematic nomenclature applied to other phyla has been developed for plant ABC transporters (Verrier et al. 2008). This nomenclature identifies nine plant ABC subgroups (ABCA through ABCI). Of these, the best-characterized subfamilies are the ABCB subgroup, which includes both the P-glycoprotein transporters and the transporter-associated with antigen presentation/heavy metal tolerance (TAP/HMT) transporters; the ABCC multiple drug resistance-related (MRP) transporters; and the ABCG subgroup containing both the white brown complex (WBC) and pleiotropic drug resistance (PDR) transporters. Members of the ABCB and ABCG subgroups have been shown to act at the plasma membrane, whereas, to date, all plant ABCC proteins have been localized at the tonoplast where they function primarily in detoxification.

Plant plasma membrane ABC transporters were originally presumed to function in detoxification and defense, and deletion of some *ABCG*/*PDR* transporters can lead to an increase in heavy metal sensitivity and greater susceptibility toward pathogens (Stein et al. 2006). However, plant plasma membrane ABC transporters have now been shown to also play important roles in growth and development. For example, *Arabidopsis* ABCB1 and ABCB19 translocate the essential growth hormone auxin, and deletion of these proteins leads to dwarf phenotypes. A similar dwarf phenotype is also observed in the maize mutant defective in the *ABCB1* ortholog *brachytic2* (*BR2*) (Multani et al. 2003).

2 Structure and Evolution of Plant Plasma Membrane Transporters

Members of the large and diverse ABC protein superfamily share common structural and functional attributes. All members have a highly conserved ATP-binding site [hereafter called nucleotide-binding fold (NBF)] and transmembrane domains (TMDs). The so-called full-length transporters have two sets of transmembrane domains and nucleotide-binding folds, which are arranged either (TMD–NBF)–(TMD–NBF) or (NBF–TMD)–(NBF–TMD) in the primary structure. The ABCB

transporters share a (TMD–NBF)–(TMD–NBF) organization, whereas both subgroups of ABCGs (WBCs and PDRs) displayed a reverse order of (NBF–TMD)–(NBF–TMD). The effects of this reverse orientation on protein function are unknown.

Each TMD consists of six transmembrane α-helices (TMHs), although deviations from this number occur. "Half-transporters" in both the ABCB and ABCG groups have only one TMD and NBF, and they require hetero- or homodimerization with another half-transporter for function. Dimerization is thought to perform an important regulatory role for these transporters.

The two TMDs form a membrane-spanning barrel structure through which the substrate moves across the PM. This structure can have two different conformations: open (toward the cytosol) or closed (Fig. 1a, b). The transition between these states is mediated by ATP binding and hydrolysis at the cytosolic NBFs.

Plant ABC transporter structures have been computationally modeled using partial and complete crystal structures that have been determined for bacterial

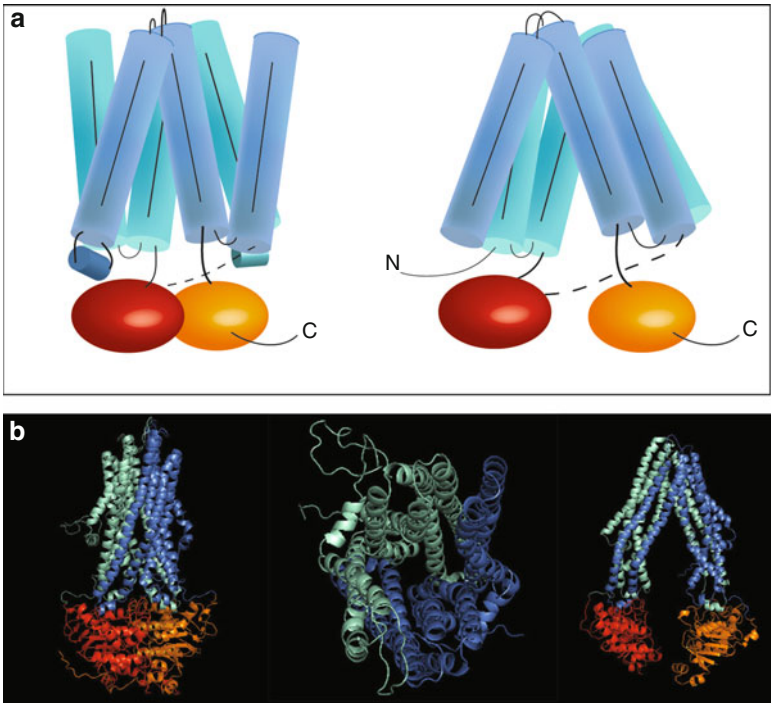

Fig. 1 Basic structure of an ABC transporter. ABCB1 is used as the model, kindly provided by Haibing Yang. (**a**) Schematic model of ABCB1 with closed (*left*) and open (*right*) conformations. TMD1 (*light blue*), TMD2 (*dark blue*), NBFs (*red* and *orange*), helix-joining loops (*solid lines*), linker region connecting the two halves of the transporter (*dashed lines*). The coupling helices that connect one TMD with the opposite NBF are shown. Each TMD rod-shaped structure represents two α-helices that are located close together. Thus, each TMD consists of six α-helices. (**b**) Computational model of ABCB1. Closed conformation (*left*), closed conformation, top view (*center*), open conformation (*right*). Colors as in (**a**)

and animal transporters, including the vitamin B importer BtuCD from *Escherichia coli* (Locher et al. 2002), sav1866 from *Staphylococcus aureus* (Velamakanni et al. 2008; Dawson and Locher 2007), the metal chelate importer from *Haemophilus influenzae* (Pinkett et al. 2007), and a murine P-glycoprotein (Aller et al. 2009). Mutational analyses of nonplant ABC proteins have also provided insights into structure–function relationships, including their positioning and localization in the membrane. In particular, ATP turnover at the NBFs has been extensively studied (Lewis et al. 2004). Arginine scanning and screening for point mutations in the transmembrane barrel of the proteins has also shown that the TMDs contribute to substrate specificity and transport efficiency (Loo et al. 2009a). These studies indicate that the TMDs and NBFs work closely together and are coupled via a so-called transmission interface. Application of this information to plant ABC transporter analyses has aided elucidation of their function.

3 The Nucleotide-Binding Fold: The Motor That Drives ABC Transport

The ABC nucleotide binding fold contains the catalytic center that binds and hydrolyzes ATP. The catalytic center is composed of a Walker A (GxxGxGKST) motif, followed by a Walker B motif (ϕϕϕϕDE, with ϕ being a hydrophobic amino acid). Furthermore, four other motifs, the Q-loop (xxQxx), C-loop (LSGGQ, also called the signature motif), D-loop (SALD), and H motif (xxHxx), are highly conserved (reviewed in Procko et al. 2009).

Crystallization of the NBF from *Salmonella typhimurium* showed that the NBF has an L-shaped form (Hung et al. 1998). A long arm that contains the ATP-binding site is formed by a six-stranded β-sheet and a unit composed of five β-sheets and five α-helices. A second arm is composed of only α-helices that face the transmembrane portion of the protein. In this structure, the two β-sheets form hydrogen bonds between the long arms and oppose the ATP-binding site. Thus, the ATP molecules bind to the outside of the protein on each side of the NBF dimer. However, a different structure of the NBF dimer has been proposed for some eukaryotic ABC transporters. The crystal structure of the NBF monomer from the cystic fibrosis transmembrane conductance regulator (CFTR) appears to form a dimer wherein two ATP molecules are sandwiched between the two NBFs (Lewis et al. 2004). Dimerization of the two NBFs is believed to be initiated by ATP binding, and the two ATP molecules appear to hold the two units together. In either model, ATP hydrolysis causes a separation of the two NBFs. This movement is translated into a conformational change in the transmembrane helices, which opens the channel toward the cytosol. This transmission is most likely mediated by two transmission interfaces, consisting of two small coupling helices that are arranged parallel to the membrane that are in direct contact with the NBF.

Studies with the bacterial maltose transporter MsbA showed that without ATP, the two NBFs are separated from each other, and the transporter is open toward the cytosol. Upon ATP binding, the two halves undergo a closing motion of about 20–30 Å at the cytosolic side, which causes an opening on the extracellular side of about 7–10 Å. (Zou and McHaourab 2009; Zou et al. 2009). The authors further investigated the structure of MsbA in the ATP-bound state in comparison with the vanadate-trapped state (designated ADP-vi), which allows for ATP hydrolysis but prevents dissociation of ADTP-vi. Since the ADT-vi-bound state is very similar to the ATP-bound state, the authors concluded that ATP binding causes the conformational change that triggers substrate translocation.

4 The Two Halves of Full-Length ABC Transporters Are Similar But Not Identical

There is still a question as to how many molecules of ATP are required for an ABC transporter to translocate a substrate molecule. A fully assembled transporter has two ATP-binding sites, which suggests that two ATP molecules are bound and hydrolyzed during one cycle of opening and closing. However, in some eukaryotic systems, only one of the ATP-binding sites is conserved, while the other one is degenerated and appears to exhibit lower ATP affinity, suggesting that only one hydrolysis event might be required for functionality (Procko et al. 2009). The plant ABC transporter COMATOSE (CTS) exhibits this type of functional asymmetry in the two NBFs, as specific amino acid substitutions in NBF1 abolish CTS function and the same substitutions in NBF2 have no effect (Dietrich et al. 2009).

The structural asymmetry of the two ABC transporter halves is not restricted to the NBFs. Mammalian TAP1 and TAP2 proteins are half-transporters that form a heterodimer to become fully active. Domain swapping experiments showed that the position of the two coupling helices did not alter activity, as long as the assembled protein contains each of the distinct coupling helices. The combination of two identical coupling helices was shown to abolish transport activity (Oancea et al. 2009). Further, different combinations of half-transporter pairs may alter the functionality of the entire protein.

The WHITE BROWN and SCARLET system in *Drosophila* is a heterodimeric ABC transporter that is composed of a WHITE subunit with either a BROWN or SCARLET subunit. Depending on whether BROWN or SCARLET comprises the second half, either tryptophan or guanine is accepted for transport. The WHITE/BROWN transporter transports guanine, and a disruption of this transporter leads to the brown eye phenotype, whereas the WHITE/SCARLET complex transports tryptophan (Mackenzie et al. 1999). Mutations in helix five of the WHITE subunit causes a significant reduction in guanine transport, yet has only a small effect on tryptophan transport, suggesting that this section of the protein is important for

substrate determination. Conversely, a mutation in the intracellular loop between helix 2 and 3 of the WHITE complex reduces transport of both compounds, suggesting that this region is necessary for general transport and does not impart substrate selectivity. Similar interactions may regulate WBC-like ABCG transporter function in plants.

5 The Transmembrane Domain Forms the Pore and Functions as the Substrate Acceptor Site

The TMD region shows greater sequence variation among ABC transporters than the NBF domains. In most cases, the transmembrane domains consist of six helices each, although deviations from this number are possible. In eukaryotes, the four units (two NBFs and two TMDs) are present in either one single polypeptide or in two halves that are transcribed separately. In some bacterial systems such as the maltose transport system from *E. coli*, all four units are transcribed independently (Orelle et al. 2008; van der Heide and Poolman 2002; Böhm et al. 2002). The maltose transport system, as well as many other importer systems in prokaryotes, requires an additional substrate acceptor subunit, e.g., the maltose-binding protein (Gould et al. 2009; Grote et al. 2009). These substrate-binding proteins are thought to enhance substrate loading of the transporter by providing binding sites near the entrance of the pore (van der Heide and Poolman 2002).

In eukaryotic systems that lack these additional substrate-binding proteins, substrate acceptance is believed to take place at the TMDs. For instance, the mutations clustered in two predominant "hotspots" localized at the leaflet–leaflet interface with extension into the inner or outer leaflet of the mammalian ABCB1 protein lead to altered substrate affinities, and helices 1 and 11/12 appear to be particularly important for substrate binding (Shilling et al. 2006). It is not clear whether these two putative binding sites represent a pathway for a single molecule, or whether the sites are utilized simultaneously to transport two molecules. It is further unclear whether import transporters have accession sites directly open to the extracellular space/apoplast or also utilize binding sites within the membrane, especially when hydrophobic or amphipathic molecules are transported.

6 The Transmembrane Helices Determine the Shape of the Pore

The helices that form the two TMDs can be of different length and shape, which has a significant impact on how the TMDs assemble and integrate in the PM. It has been reported for bacterial systems that exporters tend to have shorter helices, while importers have longer helices that extend into the extracellular

space and are believed to aid substrate recognition (Procko et al. 2009). For example, the bacterial vitamin B12 transporter BtuCD from *E. coli* has comparatively short transmembrane helices that span the entire width of the plasma membrane. Therefore, the helices are upright in the membrane, and this parallel arrangement forms the pore. In contrast, the twisted structure seen in the sav1866 transporter results in a more complex pore interior than the straight-barrel pore of the BtuCD vitamin B12 transporter or the funnel-shaped MsbA daunorubicin transporter from Gram-negative bacteria (Locher et al. 2002). The coupling helices of sav1866 are domain-switched, which means that the transmission interface from one half is connected to the NBF of the other half. This twisted organization accommodates the fitting longer helices to the lipid bilayer and allows for concerted movement of both TMDs. This conformation is not unusual and has reported for the bacterial molybdate transporter pdb2onk from *Methanosarcina acetivorans* and is therefore likely common for many prokaryotic ABC transporters.

However, eukaryotic and prokaryotic membranes differ in their membrane composition. Whereas eukaryotic membranes contain sterols that influence the fluidity and width of the lipid bilayer (Pencer et al. 2005), prokaryotic membranes generally do not contain sterols, but instead, contain hopanoids that appear to perform a similar function (Welander et al. 2009). As a number of eukaryotic ABC transporters have been shown to be localized in ordered lipid domains of the PM thought to be equivalent to detergent-resistant membrane fractions (DRMs), the organization of eukaryotic TMDs in the membrane may differ.

7 The Inner Lumen of the Transmembrane Barrel Determines Substrate Translocation

The α-helices of the transmembrane barrel provide structural strength, determine the shape of the barrel, enable proper membrane insertion, and contribute to substrate specificity. Mutational analysis and arginine scanning analysis have identified important amino acid residues on the inside of the barrel and clarified which residues comprise the inner lumen of the transporter (Loo et al. 2009a). In the case of sav1866 (and all models based on it), helices 1–6 (TMD1) form one half of the barrel, whereas helices 7–12 (TMD2) form the other half. When viewed from the top, human ABCB1 (modeled based on sav1866) shows a slightly distorted interior of the barrel due to the tilted arrangement of the helices and the positioning of helices 6 and 9 that are positioned slightly inward from the barrel circumference, thus roughly dividing the lumen in two "chambers" (Loo et al. 2008). This suggests that these two helices may have a special function in the translocation mechanism. Indeed, arginine replacement in helices 5 and 6 (A302R, L339R) reduced the affinity of the transporter for verapamil, but had no effect on

colchicines or rhodamine B. Further, the mutation F770R in helix 8 increased activity for colchicines but not for vincristine (Loo et al. 2009a). Thus, the mammalian multidrug resistance transporter appears to have several binding pockets for different substrates, which allows for transport of a wide range of structurally unrelated compounds.

Another indication of the importance of helix 6 is derived from domain swapping experiments (Zhou et al. 1999). This study took advantage of the fact that human ABCB1 and its homolog human ABCB4 share a 78% sequence similarity, yet ABCB4 does not transport most of the known ABCB1 substrates. Instead, ABCB4 has been shown to translocate the membrane phospholipid phosphatidylcholine. However, the introduction of ABCB1 helix 6 into the ABCB4 backbone partially conferred ABAB1-like substrate affinity. More specifically, the authors were able to demonstrate by site-directed mutagenesis that introducing residues Q330, V331, and L332 in helix 6 were sufficient to confer ABCB1 activity. Other studies have shown that the replacement of a cysteine residue in helix 6 in the mammalian multidrug resistance transporter LmrA drastically reduces transport of the chemical Hoechst 33342 (Poelarends and Konings 2002). Studies in the *Drosophila* WBC system also suggest a role for helix 5 and 6 in substrate binding and translocation. Mutations previously shown to affect substrate selectivity are localized in these helices (Ewart et al. 1994). One of these mutations involved a glycine to serine substitution at position 588, whereas another mutation resulted from a deletion of an isoleucine residue at 581, which caused reduced red pigmentation.

Although these two helices appear to be important for substrate recognition, several studies with yeast and mammalian ABC transporters have shown that the opposite side of the barrel, formed by helices 9, 10, 11, and 12, also influences substrate translocation. The mammalian ABCB1 transporter consists of 17 TM helices, with the last 12 being homologous to the core TMDs. Mutating the threonine residue T550 in helix 10 (homologous to helix 5) to alanine caused increased vincristine resistance, but decreased doxorubicin resistance.

Zhang et al. (2009) extended previous studies to implicate helix 16 (homologous to helix 11) in determination of substrate specificity (Daoud et al. 2001; Qian et al. 2001). Taken together, these findings suggest that two regions on opposite sides of the barrel form binding sites through which the transported substrate interacts with as it traverses the structure. These two binding pockets are formed by helices 5 and 6 on the one side and helices 9, 10, and 11 on the other.

Amino acid substitutions in other regions of mammalian ABCB1 have also been shown to disturb transport activity (Loo et al. 2009a). Considering the broad substrate specificity of ABCB1, these findings are not surprising. ABCB1 can accommodate molecules of various sizes and different polarities, which most likely requires more than a single binding pocket. However, the presence of multiple binding pockets with restricted binding properties cannot necessarily be extrapolated to plant ABC transporters.

8 Substrate Translocation Mechanism Within the Transmembrane Domain

Substrate translocation is largely believed to be mediated by the TMDs, although the transmission interface is involved in mediating ATP hydrolysis when a substrate is bound, and ATP affinity is increased upon the binding of a substrate molecule (Sauna et al. 2008). However, recent progress in resolving the structural properties of ABC transporters has not fully elucidated the mechanism of substrate translocation. A model has been proposed in which two binding sites of different affinities switch their affinities from high to low after ATP hydrolysis (McDevitt et al. 2008; Procko et al. 2009). This switch in affinities would result in an alternating binding and release of the substrate between the sites, thus leading to substrate translocation through the inner pore. However, although ABC transporters may share a common translocation mechanism, the differences observed in substrate specificity and the properties of transported molecules suggest a considerable degree of variation on a common theme.

Sequence homology between ABC transporters from plants and other phyla is not a good predictor of substrate affinity. For example, mammalian ABCB1 isoforms transport a broad range of cancer drugs such as verapamil, taxol, and vinblastine (reviewed in Ambudkar et al. 2003), but the closely related plant ortholog ABCB1 transports the plant hormone auxin [indole acetic acid (IAA)], but not the aforementioned cancer drugs (Geisler et al. 2005). Comparison of computational models based on existing crystal structures may be more informative in this regard than simple sequence alignments. For instance, the modeled electrostatic surfaces of several nonplant ABCB transporters in the closed conformation (with the barrel open toward the extracellular space) exhibit a polarity of the inner pore surface that is often the opposite of the substrate charge (Flanagan and Huber 2007). However, models of the *Arabidopsis* ABCB1 transporter display discrete polar and charged regions within a generally neutral surface. This may contribute to the increased substrate specificity of the plant ABCB1 transporter. More generally, it is likely that the inner surface charge indicates the types of molecules that are transported. If so, then amino acid substitutions that change residue polarity are more likely to alter transport activity, especially if those changes also alter helix bending and positioning.

9 Membrane Insertion

When comparing the human CFTR transporter with human ABCB1, Enquist et al. (2009) found that 10 out of 12 helices of CFTR insert efficiently in the membrane, whereas only two helices in ABCB1 self-insert and the other helices require the flanking residues or even neighboring helices on both sides for proper insertion.

Of these, the weakly hydrophobic helices 3, 6, 7, and 9 would be expected to self-insert very poorly. These helices are thought to create a microenvironment within the TMD that may facilitate the membrane translocation of polar and amphipathic molecules across the membrane, many of which are believed to enter the transporter channel from within one membrane leaflet.

Defects in folding/maturation of ABC proteins are linked to pathologies in mammals. Both CFTR and ABCB1 exhibit maturation defects linked to mutation, leading to amino acid substitutions in cytosolic loops of the proteins (Loo et al. 2009b). These defects can be corrected by a class of "corrector" drugs that restore normal maturation and insertion of the proteins. A similar misfolding defect is seen in the *Arabidopsis* auxin transporter ABCB19 when plants are treated with the compound gravicin or when a glutamate to lysine amino acid substitution mutation is induced in the cytosolic C-terminal portion of the protein (Rojas-Pierce et al. 2007). This suggests that the maturation and membrane insertion of at least some plant ABC transporters are similar to what is seen in mammals. Further, evidence that the FKBP immunophilin-like protein FKBP42/TWISTED DWARF1 functions in activation/insertion of a subset of plant ABC proteins (Geisler et al. 2003; Rojas-Pierce et al. 2007) suggests that this may be a more general function of this type of protein.

However, water-filled cavities have been detected in the lumen of LmrA (Poelarends and Konings 2002), suggesting that the inside channel of these transporters forms an aqueous rather than lipid environment. Replacement of amino acids with the bulky polar amino acid arginine was without effect on either substrate specificity or protein folding, suggesting that the inner lumen of the protein can tolerate the insertion of polar residues. Therefore, it is likely that the interior of the channel is hydrophilic. An analysis of the mammalian multidrug resistance transporter ABCB11 concluded that most helices display hydrophobic and hydrophilic sides that align with either the membrane bilayer or the lumen. An exception is helix 6 of the protein, which is completely hydrophilic and is positioned more inwardly in the TM barrel structure (Loo et al. 2009a). A natural conclusion of this analysis is that the first energy-consuming step in translocation is the ATP-facilitated movement of a substrate molecule from the lipid membrane phase to the inner channel aqueous phase, and that a second step involves release of the substrate to the extracellular aqueous environment.

10 Membrane Microdomains

Many ABC transporters are localized in lipid-ordered, sterol-rich regions in the membrane, and many ABC transporters function in translocation of lipids and sterols (Wang et al. 2008). The mammalian ABCG1 transporter accepts sphingomyelin bound to cholesterol and releases it into the extracellular space. Similarly, mammalian ABCB4 recognizes phosphatidylcholine bound to cholesterol and exports both as a complex (Kimura et al. 2007). The authors propose a model in

which sterols can fill in the empty space in the lumen when binding a small molecule, which helps accommodate a wide range of molecules of different sizes. Some plant WBC-like ABCG transporters appear to transport lipids (Luo et al. 2007; Pighin et al. 2004), but the contribution of those lipids to the transport of other substrates is yet to be determined. However, although the *Arabidopsis* auxin transporters ABCB1and ABCB19 do not appear to export or cotransport sterols, addition of cholesterol to mammalian cell cultures expressing plant ABCB auxin transporters increase their transport activity (Titapiwatanakun et al. 2009). Other ABC transporters may contribute to sterol transport, as sterol transport appears to take place in the endosomal trafficking system (Grebe et al. 2003).

In plants, raft-like membrane domains have been observed in *Arabidopsis* and tobacco (Borner et al. 2005; Mongrand et al. 2004; Titapiwatanakun et al. 2009). Similar to mammalian lipid rafts, these microdomains are rich in sphingolipids and sterols (Martin et al. 2005; Grennan 2007). A number of plant ABCB transporters, including ABCB1, 4, 10, and 19, have been localized to DRMs associated with membrane microdomains (Borner et al. 2005; Mongrand et al. 2004; Titapiwatanakun et al. 2009). Of these, ABCB19 appears to characterize and even stabilize a unique membrane domain to which the PIN1 auxin transporter is recruited (Blakeslee et al. 2007; Titapiwatanakun et al. 2009).

11 Plant Plasma Membrane ABC Transporters: Two Subfamilies and Their Functions

11.1 ABCBs: Transport of Hormones, Organic Acids, and Alkaloids

The full-length plasma membrane ABCB transporters ABCB1 and ABCB19 are the closest orthologs to the mammalian ABCB1 protein that contributes to multidrug resistance in tumor cells. However, unlike mammalian ABCB1, plant orthologs examined to date exhibit much greater substrate specificity and accept only a small number of related compounds. The ABCB subfamily contains both exporters (e.g., *Arabidopsis* ABCB1 and 19) as well as apparent importers (e.g., *Arabidopsis* ABCB4 and 14, *Coptis japonica* ABCB1). This observation is somewhat striking, as all mammalian ABCBs described to date function in substrate export, and the characterized bacterial importer systems all rely on an additional substrate-binding protein to facilitate import and possess a straight-barrel structure not seen in eukaryotes.

The mechanism that allows for reverse transport in some plant ABCB transporters is unknown, although apparent structural differences and an additional substrate-binding site in models of the proteins suggest a mechanistic logic for this activity (Yang and Murphy 2009). The structure and extension of the N- and C-terminus as well as the loop between the first NBF and TMD vary among

members of this group, and it has been proposed that, in addition to documented protein–protein interactions at these sites (Geisler et al. 2003; Bouchard et al. 2006), these sites could also constitute regulatory sites for substrate specificity and transport directionality (Yang and Murphy 2009).

Probably the best-characterized members of the ABCB family in plants are the principle auxin exporters *Arabidopsis* ABCB1 and ABCB19. One additional auxin transporter, ABCB4, has been characterized, although it differs from ABCB1 and 19, in that it can function as a concentration-dependent reversible transporter when expressed heterologously in *Schizosaccharomyzes pombe* (Yang and Murphy 2009) and mammalian cells (Terasaka et al. 2005).

ABCB1 and ABCB19 (and perhaps additional ABCB transporters) comprise an independent auxin transport system that functions independently but coordinately with polarized full-length PIN efflux carriers to establish and maintain polar auxin transport (Blakeslee et al. 2007; Mravec et al. 2008). The predominant endogenous auxin is IAA, an amphipatic molecule that can cross the plasma membrane from the acidic apoplast in its protonated state but that is believed to become trapped in the more neutral cytoplasm once dissociated (Goldsmith and Goldsmith 1977). Since the dissociated molecule cannot leave the cell by diffusion, it requires protein-mediated export.

The polar localization of auxin export proteins can generate a polar auxin flux that plays an important role in establishing the apical–basal axis as well as organogenesis. PIN proteins provide the primary postzygotic polar auxin stream that coordinates polar development and organogenesis (Friml et al. 2003; Reinhardt et al. 2003; Benkova et al. 2003). ABCB auxin transporters are localized both polarly and nonpolarly according to developmental stage and tissue type (Blakeslee et al. 2007). Unlike PIN proteins that are directed to and from polar subcellular localizations by GNOM-dependent dynamic trafficking mechanisms (Geldner et al. 2003), ABCB proteins are trafficked by GNOM-independent pathways and exhibit greater membrane stability (Blakeslee et al. 2007; Titapiwatanakun et al. 2009). However, ABCB19 does appear to enhance the stability and activity of PIN1 in tissues when the two proteins colocalize (Blakeslee et al. 2007; Titapiwatanakun et al. 2009).

ABCB auxin transporters appear to function primarily in regulation of localized auxin accumulations in apical tissues and restriction of auxin to long-distance polar streams in mature tissues (Zazimalova et al. 2010). The cells of the apical meristem are a site of auxin biosynthesis and have higher levels of auxin. However, meristematic cells are unelongated, and auxin that leaves these cells can enter from any side of these small cells, preventing auxin loading into the long-distance auxin transport stream. Thus, energy-dependent export from this region is necessary to overcome the effects of auxin reflux and to allow loading of the polar auxin transport stream (Fig. 2).

In *Arabidopsis* seedlings, *ABCB1*/*PGP1* is predominantly expressed in the shoot and root apical meristem as well as in lateral root tips (Sidler et al. 1998; Geisler et al. 2005; Mravec et al. 2008). In root and shoot apices, ABCB1 can be detected apolarly on all sides of the PM. However, in tissues above the distal elongation

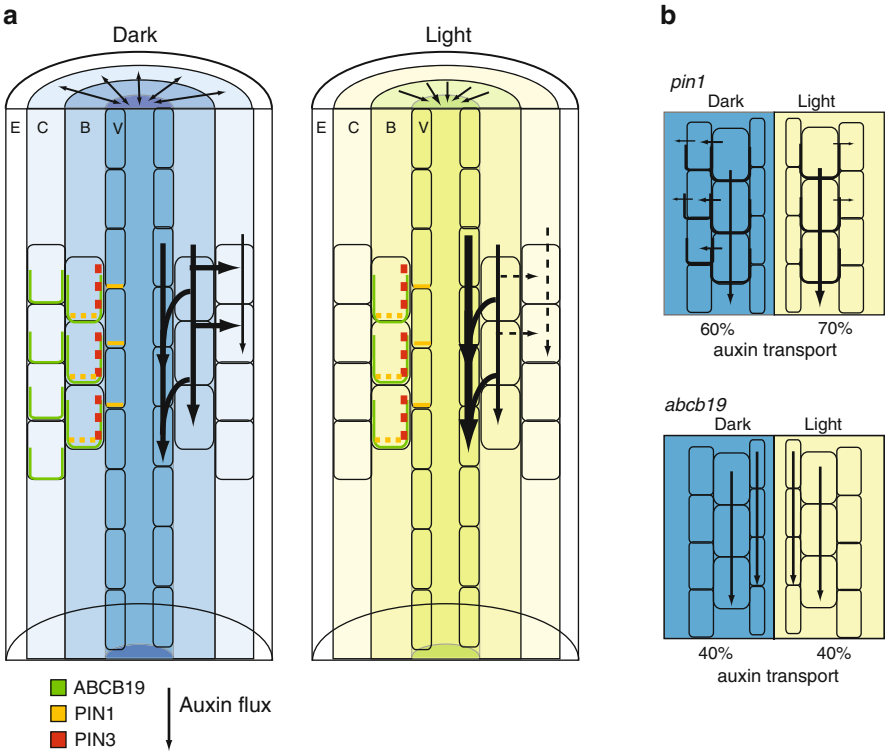

Fig. 2 Auxin transport in the *Arabidopsis* shoot apex. (**a**) Auxin efflux from unelongated meristematic cells is mediated by ABCB1, preventing auxin (IAA) from diffusing back into the cell. ABCB19 and PIN1 create a sink for IAA in existing or newly forming vasculature, where polar auxin transport takes place. (**b**) Magnification of (**a**)

zone, a clear polar signal was observed on the basal side of cortical and endodermal cells when a PGP1pro:PGP1-cmyc protein was visualized by immunolocalization (Geisler et al. 2005).

ABCB19 is predominantly localized in the bundle sheath cells in light-grown hypocotyls and the in tissues near and above the cotyledonary node where ABCB19 mediates auxin transport, and ABCB19 and PIN1 localization strongly overlap in vascular tissue (Blakeslee et al. 2007) (Fig. 3a). ABCB19 is also localized in cotyledons (Lewis et al. 2009) and anther filaments (Titapiwatanakun et al. 2009). In roots, ABCB19 is localized to stelar and cortical cells (Blakeslee et al. 2007; Wu et al. 2007) as well as epidermal cells adjacent to the root apex (Titapiwatanakun et al. 2009). In etiolated seedlings, ABCB19 distribution expands to cortical cells. In the bundle sheath cells of dark-grown seedlings, ABCB19 colocalizes with the auxin efflux facilitator PIN1, and interactions of these two proteins have been shown to enhance auxin transport (Blakeslee et al. 2007) (Fig. 3a). This interaction appears to be specific, that is, only PIN and ABCB transporters that colocalize *in planta* have an additive effect when expressed together in heterologous systems.

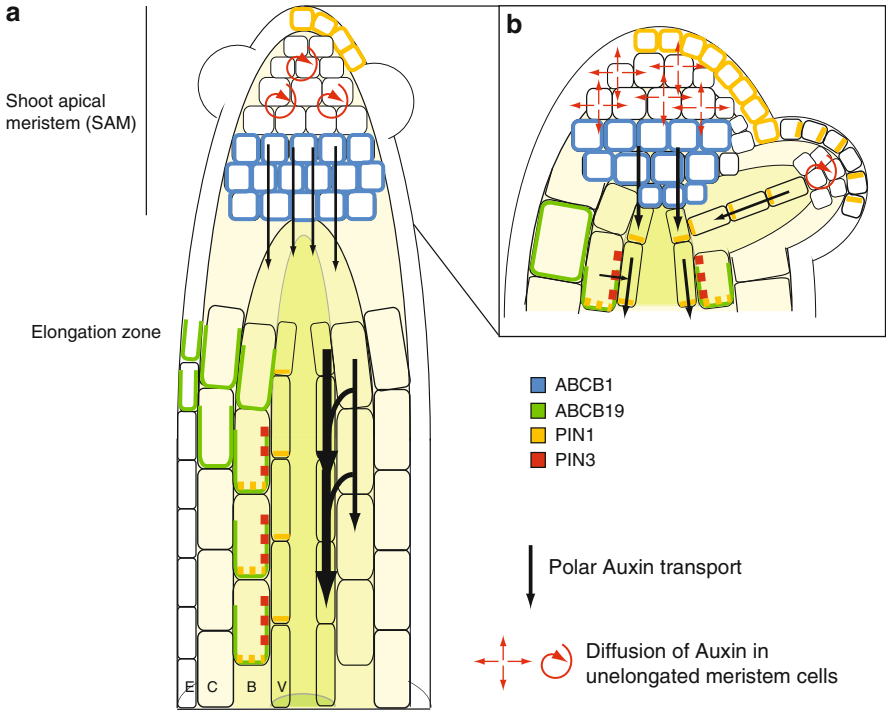

Fig. 3 Auxin transport in light- and dark-grown *Arabidopsis* seedlings. (**a**) Auxin transport in light- and dark-grown *Arabidopsis* seedlings, mediated by ABCB19, PIN1, and PIN3. In etiolated seedlings, ABCB19 is localized in cortical and bundle sheath cells (*left*). In light-grown seedlings, ABCB19 is no longer localized on the plasma membrane in cortical cells, restricting auxin transport activity to the bundle sheath cells (*right*). Thereby, IAA is funneled in the polar auxin transport mediated by PIN1. PIN3 facilitates loading PAT from the bundle sheath cells. (**b**) Consistent with this model, auxin transport in *pin1* is to 60% or 70% reduced in dark- or light-grown seedlings, respectively (*top*) (Blakeslee et al. 2007). In *abcb19*, auxin transport is reduced to 20% in hypocotyls and 40% in inflorescence stems (*bottom*) (Noh et al. 2001)

It has been proposed that the major role of *Arabidopsis* ABCB1 is to function in concert with ABCB19, PIN1, and PIN3 to export IAA from meristematic cells at the shoot and root apices to load auxin into the long-distance auxin transport stream (Fig. 2). ABCB19, on the other hand, is thought to function coordinately with PIN1 to direct auxin transport streams from the shoot to root apex. In dark-grown seedlings, ABCB19 is also localized in the neighboring cortical cells, which would result in a less-efficient auxin transport mediated by PIN1, as less IAA reaches the vascular bundle (Blakeslee et al. 2007). In agreement with this, dark-grown *pin1* mutants display 60% of wild-type auxin transport but show 70% when grown in the light. The restriction of ABCB19 to the bundle sheath cells appears to promote polar auxin transport even in the absence of PIN1, although the transport is still less efficient (Fig. 3b). Conversely, the *Arabidopsis abcb19* mutant displays

only 35–40% of wild-type auxin transport in the hypocotyl (Blakeslee et al. 2007) and inflorescence stems (Noh et al. 2001), suggesting that PIN1 plays an important role in determining the polarity of auxin transport, but a large portion of auxin transport motive force is provided by ABCB19 (Fig. 3b).

ABCB4 is an *Arabidopsis* auxin transporter that has been shown to function primarily in the root (Terasaka et al. 2005; Cho et al. 2007; Lewis et al. 2007). ABCB4 is localized to the lateral root cap, cortical, and epidermal cells of the root. ABCB4 is apolarly localized in young root tissues, exhibits a basal (top) polar localization in three tiers of epidermal cells in the central elongation zone, and largely apical (bottom) subcellular localization on the PM in mature root tissues. In *abcb4* mutants, the number of lateral roots is increased (Santelia et al. 2005), and the length of the primary root is decreased (Terasaka et al. 2005), suggesting a role for ABCB4 in regulating root growth. ABCB4 also regulates auxin levels in *Arabidopsis* root hairs (Cho et al. 2007) where it appears to mediate auxin uptake until threshold levels of auxin are reached and the direction of auxin transport is reversed (Yang and Murphy 2009).

Both *Arabidopsis* ABCB1 and ABCB19 have been shown to be involved in light responses. When grown under red, far-red, and blue light, *abcb19* displays shorter hypocotyls and epinastic cotyledons (Noh et al. 2003). Dark-grown seedlings exhibit wavy hypocotyls (Lin and Wang 2005). Dark-grown *abcb1* also has a shorter hypocotyl, although the epinastic leaf phenotype is milder than in *abcb19* (Lin and Wang 2005). The blue light receptors PHOT1 and CRY1 appear to regulate *ABCB19* expression in *Arabidopsis*. Upon illumination, ABCB19 slowly disappears from the cortical PM, whereas it remains stable in the bundle sheath cells (Titapiwatanakun et al. 2009; Nagashima et al. 2007). When grown under directional red light conditions, *abcb19* displays greater hypocotyl bending, and both phytochromes and cryptochromes enhance hypocotyl bending by suppressing *ABCB19* expression. Hypocotyl bending under red light and the dark-grown wavy hypocotyl phenotype are enhanced in the double mutant *abcb1abcb19* compared to *abcb19* (Nagashima et al. 2008). ABCB auxin transporters also function in mobilizing auxin streams required for tropic bending. In comparison to the wild type, etiolated *abcb19* seedlings display greater hypocotyl bending after 90° reorientation (Noh et al. 2003). Further, *abcb19* is also insensitive to the gravitropism inhibitor gravacin (Rojas-Pierce et al. 2007).

Two more plant PM ABCB transporters have been characterized to date: *C. japonica* ABCB1 (CjMDR1), an alkaloid transporter localized in the root (Shitan et al. 2003), and *Arabidopsis* ABCB14, which is a stomatal malate transporter (Lee et al. 2008). The *C. japonica* ABCB1 appears to mediate berberine uptake and appears to exhibit relative specificity for related alkaloid compounds. Little else is known of its function. Protoplast heterologous expression transport assays suggest that *Arabidopsis* ABCB14 functions primarily as a malate importer in guard cells. This import can be competitively inhibited by the organic acids fumarate and, to a lesser extent, succinate and citrate (Lee et al. 2008). The knockout line *abcb14-1* flowers earlier than the wild type, and when grown under high CO_2 and drought stress conditions, it produces fewer rosette leaves at the time of bolting. Inversely,

overexpression of *ABCB14* results in later flowering and more rosette leaves at time of bolting when grown under high CO_2 or drought.

11.2 Full-Length ABCG/PDR Proteins: At the Line of Plant Defense

Members of the full-length ABCG/PDR transporter subfamily are structurally similar to full-length ABCBs, but differ in that the orientation of the TMD and NBF domains is reversed (NBF–TMD). So far, no member of this transporter family in animals has been described. However, in yeast, PDRs are involved in exporting toxins and organic acids (Sipos et al. 2006; Crouzet et al. 2006; Mamnun et al. 2004). In *Arabidopsis*, 3 of the 15 members of this subfamily, ABCG36, ABCG37, and ABCG40, have been characterized to date.

Earlier findings in *Nicotiana plumbaginifolia* implicated PDR-like proteins function in defense processes. *Nicotiana plumbaginifolia ABCG1*/*PDR1* is upregulated by sclareol, an antifungal diterpene, and by the plant defense hormone jasmonic acid; it is also constitutively expressed in roots, glandular trichomes, and flower petals (Stukkens et al. 2005). One *Arabidopsis* ortholog, *PDR12*/*ABCG40*, is upregulated by methyl jasmonate as well as ethylene, and an *abcg40* knockout mutant is more sensitive to sclareol (Campell et al. 2003). ABCG40 may also mediate ABA influx into guard cells. This suggests that a discrete subcluster of plant ABCG proteins share similar functions in defense.

Arabidopsis ABCG36/PDR8 groups to a different subcluster (Verrier et al. 2008) and is localized to the PM (Kobae et al. 2006; Strader and Bartel 2009). Expression of *ABCG36* has been detected in the leaf palisade cells and around the hydathodes. T-DNA knockout insertion lines for *ABCG36* show no apparent phenotype when grown under axenic conditions but manifest severe leaf chlorosis and lesions when grown under nonaxenic conditions. Further, when infected with *Phytophtora infestans*, *abcg36* mutants exhibited macroscopic cell death and lesions. Little penetration of the fungal hyphae was observed in wild-type plants, whereas the pathogen was able to spread under the epidermal surface in *abcg36* loss-of-function mutants (Kobae et al. 2006). This suggests a role for ABCG36 in preventing pathogen entry into the leaf by secretion of an antifungal compound. Loss of *ABCG36* function also results in reduced resistance toward nonhost pathogens (Stein et al. 2006).

In addition, ABCG36 appears to function in transport of the auxin indole butyric acid (IBA) as well (Strader and Bartel 2009). Although IBA is an active auxin per definition, IBA function in plants is still controversial (Ludwig-Müller 2007; Bartel et al. 2001). However, auxins are produced by numerous rhizobacteria strains (Howden et al. 2009; Spaepen et al. 2008), and ABCG36 may regulate the uptake of bacterially produced auxins. ABCG36 also appears to confer resistance toward heavy metals such as cadmium and lead, and *ABCG36* expression is upregulated

after Cd and Pb exposure (Kim et al. 2007). It is likely that ABCBCG36 transports chelating organic acids under Cd and Pb stress to alleviate metal toxicity (Kim et al. 2007) rather than functioning as a metal exporter. Taken together, these results suggest that PDR-like ABCGs are likely to have very broad substrate specificities and perform a wider range of tasks than ABCB transporters.

The related *Arabidopsis AtABCG37/PDR9* is expressed in roots and also localizes to the PM. Knockout studies have shown that ABCG37 confers resistance to auxinic herbicides such as 2,4-dichlorophenoxyacetic acid, but it does not alter the sensitivity to the endogenous auxin IAA (Ito and Gray 2006). Consistent with this, ABCG37 does not have IAA transport activity (Titapiwatanakun et al. 2009) but can transport indole butyric acid and other organic acids (Ruzicka et al. 2010). Multifunctionality of ABCB37 is suggested by evidence that an ortholog in tobacco, *NtPDR3*, is inducible by iron deficiency as well as after treatment with methyl jasmonate, NAA, and the diterpene cembrene (Ducos et al. 2005).

Only a few ABCGs have been isolated and partially characterized from other plant species. For example, expression of *ABCG36*/*PDR9* from rice is also induced by heavy metals and other abiotic stress (Moons 2003), and a subsequent study showed that half of the *ABCG* genes in rice are induced by abiotic stress and jasmonic acid treatment (Moons 2008). Salicylic acid was found to induce expression of *PDR20*, while ABA markedly increased the transcript levels of *ABCG48*/*PDR3* and *ABCG31*/*PDR6*. *ABCG48*/*PDR3, ABCG37*/*PDR8*, and *ABCG36*/*PDR9* were also upregulated after application of organic acids such as lactic acid, citric acid, and malic acid. Similarly, wheat *ABCG1*/*PDR1* is upregulated under high calcium and aluminum conditions as well as by *Fusarium gramineum* infection and application of the virulence factor deoxynivalenol (Shang et al. 2009).

11.3 ABCG/WBC Half-Transporters

Although originally named for the *Drosophila* WBC half-transporters that form functional heterodimers, some WBC-like ABCG half-transporters such as the mammalian ABCG1 and ABCG4 lipid/sterol transporters appear to form homodimers. However, coexpression of mammalian ABCG1 with a catalytically inactive form of ABCG4, ABCG4-(K-M), abolished overall ATPase activity, suggesting that heterodimerization is possible and may be the default state of these transporters (Cserepes et al. 2004). Another characterized mammalian member of this subfamily, ABCG2, is expressed in the harderian gland, where it mediates the transport of protoporphyrin IX conjugates (Jonker et al. 2007).

In plants, five ABCG/WBC transporters have been partially characterized to date: *Arabidopsis* ABCG12 (CER5), ABCG11 (DSO), and ABCG25, *Gossypium hirsutum* ABCG1 (GhWBC1), and *Nicotiana tabacum* ABCG1 (NtWBC1). *Arabidopsis* ABCG25 forms a homodimer and mediates ABA efflux. *Arabidopsis* ABCG12 appears to export wax monomers from epidermal cells, and disruption of this transporter activity causes abnormal accumulation of lipids in wax-secreting

cells (Pighin et al. 2004). The wax load and surface wax crystals on *abcg12* (*cer5)* mutant stems are reduced, but not abolished altogether. Since the total epidermal wax quantities are not reduced in *abcg12*, this indicates that the mutant is impaired in export of wax monomers, but not wax biosynthesis. *ABCG12* is expressed in all organs, and the rather mild phenotype suggests that another transporter is able to partially perform a similar function in wax secretion.

The *abcg11* (*dso)* mutant shows interorgan fusion, a decrease in epicuticular wax load, and misshapen epidermal cells and stomata (Panikashvili et al. 2007; Luo et al. 2007; Bird et al. 2007). The different composition of both wax and cutin in the *dso* mutant suggests that ABCG11 transports both wax and cutin monomers. ABCG11/DSO plays an important role in forming the cuticula, and thus, in plant defense. Expression of *ABCG11* is restricted to the epidermis, and the ABCB11 protein localizes to the PM on the cuticular side of the cell. *ABCG11* expression is also upregulated by abscisic treatment, wounding, and salt stress (Panikashvili et al. 2007). The *dso* knockout mutant is more susceptible to high salinity in comparison to the wild type (Panikashvili et al. 2007).

Several other WBC transporters from other plant species have been partially described. However, their exact function is not known. The ABCG1 transporter in cotton appears to function in fiber elongation, as the *abcg1* mutant has significantly shorter fibers, and overexpression of *ABCG1* in *Arabidopsis* leads to shortened siliques (Zhu et al. 2003). It is not clear whether this indicates a role in transport of hormones or structural compounds. *ABCG1* from *N. tabacum* is exclusively expressed in the stigma, suggesting a role in reproduction, but remains otherwise uncharacterized (Otsu et al. 2004).

12 Conclusions

Despite the high conservation of the functional units of plasma membrane ABC transporters, they can differ largely in their linear and three-dimensional arrangement, membrane localization, transport directionality, mechanisms of ATP hydrolysis, and substrate specificity. Although the basic mechanism of ABC transporters involves hydrolysis of ATP to drive transport, the details of how this is translated into substrate translocation can differ. Asymmetric ATP-binding sites, reverse organization of the TMDs and NBFs, as well as differences in TMD barrel structure contribute to the differences in overall structure of ABC subfamilies. With respect to transport function, the effects of these differences are largely unknown. For example, the reverse organization of ABCG transporters has unknown impacts on their three-dimensional protein structure and function. Thus, knowledge about the structure and function of members of each ABC transporter subfamily needs to be gathered in order to make more general statements about their respective mechanism.

However, the substrate specificity of ABC transporters also appears to differ within each subfamily, suggesting that no extreme alteration in the protein structure

is necessary to change substrate-binding affinities or even transport directionality. Mutational analysis can provide valuable information about the physiological function and potential substrate-binding sites. Mutational analyses indicate that hotspots localized to discrete locations in the three-dimensional structures of the proteins account for substrate specificity, thus indicating that the protein structure cannot solely be analyzed based on the linear amino acid sequence in order to understand functionality. Sequence analyses that integrate structural comparison are likely to generate more meaningful hypotheses for future site-directed mutagenesis and domain swapping studies.

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Hormone Transport

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Abstract Hormone transport in plants is a fundamental process governing all aspects of plant development. Identification and functional analysis of plasma membrane proteins, which regulate the import and export of plant hormones, is a requirement if we are to develop a systems biology level understanding of these processes. For several plant hormones, the characterization of the transporters has produced remarkable steps towards this goal, in particular for auxin transport, where the synthesis of mathematical and laboratory approaches is now being realised. We review current data on the proteins involved in phytohormone transport including discussions of their identity, functional interactions, and kinetic parameters. In particular, for auxin transporters we discuss how expression systems enable us to dissect out the transport mechanisms, and how data from such studies can be combined with mathematical and computational models to develop a systems-based perspective of auxin transport.

1 Introduction

Plant hormones (phytohormones) have been the subject of intensive investigations since the 1920s. The focus of this chapter is our understanding of the transport mechanisms for these hormones across the plant plasma membrane. For some of these hormones, we are now in a position to understand the long-distance transport, the cellular uptake, intracellular signalling and the cellular efflux processes.

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However, for many others we are still someway short of even identifying the major players in these events. We present descriptions of the transport of four hormones, namely auxins, cytokinins (CK), abscisic acid (ABA) and brassinosteroids (BR). As the identity of transporters (or indeed receptors) for gibberellic acid (GA) remains unknown, we do not discuss this hormone further except to note that as a weak hydrophobic organic acid (pK of 4.0) it is likely that there is significant diffusion across the plasma membrane in addition to any direct transport process. We also exclude gaseous ethylene from our discussion as it seems unlikely to require membrane-bound transporters. Similarly, we do not discuss jasmonate transport in any detail for the reason that transport of jasmonate is best understood in terms of organelle, rather than plasma membrane transport. Simply, we would remind the reader that the ATP-binding cassette (ABC) transporter COMATOSE [AtABCD1; (Verrier et al. 2008)] is peroxisomally localised in Arabidopsis and is proposed to act as an exporter of jasmonate precursors into the peroxisome prior to rounds of β-oxidation to complete the jasmonic acid synthesis pathway (Theodoulou et al. 2005).

Understanding the transport of hormones at a molecular level can contribute to a systems biology level understanding of plant hormones transport. Such complexity of phytohormone transport requires diverse information on the component processes. Firstly, we need to determine the localization of the transporters, right down to the cellular polarity of the transporters at the individual cell in the case of understanding cell-to-cell transport [e.g. (Muller et al. 1998; Swarup et al. 2001; Vieten et al. 2005; Wisniewska et al. 2006)]. Secondly, we need to determine how transporters are regulated, either by alterations in the sub-cellular localization, or by direct functional regulation [e.g. (Geldner et al. 2003; Paciorek et al. 2005; Bouchard et al. 2006; Dharmasiri et al. 2006; Jaillais et al. 2006; Lee and Cho 2006; Blakeslee et al. 2007)]. Thirdly, we need to determine the function of the individual transport proteins themselves, in terms of their substrate specificities and transport parameters. Fourthly, we need to combine all relevant data from such studies in order to produce and validate computer models of phytohormone flux and their role in plant development. Specifically, we include two sections which we hope will stimulate interest: the first is the use of heterologous expression systems to provide quantitative data related to hormone transporters, and the second is the use of computer modelling approaches directed towards understanding and predicting phytohormone gradient generation and maintenance.

2 Known Transport Proteins for Phytohormones

2.1 Transporters for Auxins

Goldsmith's chemiosmotic hypothesis (Goldsmith 1977), developed to explain experimental data on auxin movement through plant cells (Rubery and Sheldrake 1974; Raven 1975), identified the need for specific auxin influx and efflux carriers.

Possible genes that coded for auxin carriers were first identified in the 1980s by creating mutants defective in tropisms or stimulus-response that could be altered or phenocopied by application of auxin analogues or auxin transport inhibitors (Maher and Martindale 1980). The *aux1* mutant, showing an agravitropic phenotype that could be rescued by the membrane-permeable auxin 1-naphthylacetic acid (1-NAA) but not the membrane-impermeable 2-4 dichlorophenoxyacetic acid (2,4-D), led to the cloning of the AUX1 gene (Bennett et al. 1996) and subsequent identification of an entire family of auxin influx carriers, the AUX–LAX family [Table 1; (Swarup et al. 2008)]. AUX1 typifies the family, and is a plasma membrane polypeptide of 485 amino acids, with a predicted molecular mass of 54.1 kDa and a predicted topology of 11 membrane spanning helices (Swarup et al. 2004). This topology has been partially confirmed by taking advantage of the pH gradient across the plasma membrane and pH-sensitive YFP-fusion constructs of AUX1 (Swarup et al. 2004). Sequence analysis of the AUX–LAX proteins places them as members of the amino acid/auxin permease (AAAP) family which transport amino acids or their derivatives and have a predicted proton symport mechanism (Young et al. 1999; Kerr and Bennett 2007). AUX1 has been confirmed as a true influx carrier by determination of auxin import into *Xenopus* oocytes heterologously expressing AUX1 [see below and (Yang et al. 2006)].

Although the Arabidopsis LAX proteins remain to be as well characterised [with the exception of LAX3 (Swarup et al. 2008)], there is a strong conservation of amino acids, with between 73 and 82% identity, indicative of a common biochemical function (Parry et al. 2001). LAX3 has been shown directly to encode an auxin efflux carrier, to localize to the plasma membrane and to be expressed in the central stele and in small groups of cortical and epidermal cells overlaying lateral root primordia (Swarup et al. 2008) where it promotes the emergence of lateral roots. LAX1 expression has been observed in the L1 layer of the shoot meristem where it functions in phyllotaxis (Bainbridge et al. 2008).

The PIN family of auxin efflux carriers, so called because the *pin-formed1* mutant displays a bare, pin-like inflorescence lacking flowers (Haughn and Somerville 1988), were first identified as possible auxin transport proteins when plants treated with polar auxin transport inhibitors showed the pin-like phenotype and reduced transport of radio-labelled indole-3-acetic acid (IAA) in the inflorescence (Okada et al. 1991). Since then, many members of the PIN family have been identified through gene cloning [(Gälweiler et al. 1998; Luschnig et al. 1998; Muller et al. 1998; Utsuno et al. 1998) and see Table 1], and several Arabidopsis PINs (PIN1, PIN2, PIN3, PIN4 and PIN7) have been implicated in auxin transport that influences, tropic growth responses, root meristem patterning and early embryo development (Vieten et al. 2005, 2007). PIN proteins are more diverse in size, being 350–650 amino acids in length, and have a predicted molecular mass of 40–70 kDa. The predicted topology is ten transmembrane helices, with two groups of five helices at each terminus, separated by a large hydrophilic loop of approximately 300 amino acids that is presumably intracellular, and whose absence in some PINs (e.g. Arabidopsis PIN5 and PIN8) is responsible for the diversity in primary sequence length (Kerr and Bennett 2007). The tissue distribution and polarity of

Table 1 Auxin influx and efflux carrier genes identified in common model plants

	Arabidopsis thaliana	*Medicago truncatula*	*Oryza sativa*	*Physcomitrella patens*	*Populus trichocarpa*	*Zea mays*
AUX1/LAX	4	5	5	5	8	4–5
PIN	8	13	13–16	3	16	11
ABCB1[a]		ABD33400.2 (43%)	OsABCB22;75%	EDQ56030 (ABCB16; 56%)	n/d	AAR00316 (ZmPgp1; 46%)
ABCB4[a]		ABD33400.2 (41%)	OsABCB4; 56%	EDQ51051 (ABCB13; 57%)	CAE47098 (28%)	AAR00316 (ZmPgp1; 42%)
ABCB19[a]		ABD33400.2 (43%)	OsABCB14;82%	EDQ56030 (ABCB16; 58%)	BAA94511 (30%)	AAR00316 (ZmPgp1; 51%)

n/d none determined, to date

[a]For ABCB-type transporters, it is currently difficult to distinguish auxin transporters from those involved in transport of other substrates on the basis of the primary sequence. Thus, for each of the proposed Arabidopsis ABC-type auxin transporters the closest homologue, as identified by BLAST searches, is given with a GenBank accession code or protein annotation, together with the highest local percentage identity

PIN expression is a subject beyond the scope of the current chapter and the reader is directed to (Paponov et al. 2005; Vieten et al. 2005; Wisniewska et al. 2006) and references therein.

The ABC transporter family includes a subset (ABCB/MDR/PGP), which has been implicated in auxin export. In plants, full length ABCB proteins [using the revised nomenclature of (Verrier et al. 2008)] are over 1,200 amino acids residues in length and have a molecular weight of 125–150 kDa. The full length protein can be considered as consisting of two homologous halves, each half comprising a transmembrane domain followed by a cytoplasmic nucleotide-binding/ATP-hydrolysis site that are joined by a flexible linker region. The function of ABCB proteins is believed to be as ATP-hydrolysis-driven, amphipathic anion transporters (Rea et al. 2003). There are three members of the Arabidopsis ABCB family that, owing to observed abnormalities in root development, including growth, lateral-root formation and gravitropic response, have been postulated to be auxin transporters in plants, viz ABCB1, ABCB4 and ABCB19. Mutants in *Atpgp1* (encoding ABCB1) and *Atmdr1* (encoding ABCB19) were observed to show reduced growth and auxin transport implicating ABCB transporters in auxin transport (Noh et al. 2001, 2003). More recently, ABCB4 has also been shown to have a mutant phenotype suggestive of auxin transport defects, although (as discussed below) the direction of auxin transport is contentious (Santelia et al. 2005; Terasaka et al. 2005; Cho et al. 2007). Tissue expression patterns of ABCB1, B19 and B4 are also consistent with a role in root auxin transport, being strongly expressed in mature root tissue, the epidermis of the elongation zone and the root apex [see e.g. (Bandyopadhyay et al. 2007)]. To date, the ABCB auxin transporters have not been shown to have polar plasma membrane localization, except in newly divided vascular cells (Blakeslee et al. 2007).

Dynamic redistribution of PIN and AUX/LAX auxin transporters occurs to redirect polar auxin flow and mediate developmental processes. This is achieved via vesicular trafficking of the proteins between the plasma membrane and endosomes. Influx and efflux carriers have been shown to be sensitive to endomembrane transport inhibitors brefeldin A and endosidin1 (Geldner et al. 2001, 2003; Robert et al. 2008). The effects of brefeldin A, at least for PIN1 in roots, are mediated by the ADP ribosylation factor GTP exchange factor (ARF-GEF) GNOM that functions in coated vesicle assembly (Steinmann et al. 1999; Geldner et al. 2003). Other components of vesicle trafficking that have been implicated in PIN regulation include the sorting nexin SNX1 that functions in retrograde membrane protein trafficking (Jaillais et al. 2006), the ARF-GTPase activating factor SCARFACE (Sieburth et al. 2006), and the BFA-insensitive ARF-GEF protein GNOM-like 1 that mediates ER to Golgi trafficking (Jürgen et al. 2001; Teh and Moore 2007). The colossin-like scaffolding protein BIG and components of the actin cytoskeleton have also been shown to function in PIN protein trafficking [reviewed in (Vieten et al. 2007)].

Several other levels of regulation of auxin transporters are documented (Titapiwatanakun and Murphy 2008) and these are considered only briefly here. Protein:protein interactions may control both initial targeting and the stabilisation

of auxin transporters. For example, AUX1 requires the ER-located accessory protein AXR4 for its transport to the plasma membrane (Dharmasiri et al. 2006), and PIN1 localisation within detergent-resistant micro-domains of the plasma membrane is mediated by interaction with ABCB19 (Titapiwatanakun et al. 2009). Post-translational modification may also modulate both activity and localization – the Ser/Thr kinase PINOID (PID) has been shown to rapidly mediate alterations in the polarity of PIN1, 2 and 4 (Friml et al. 2004), although in roots *pid* mutants perturb PIN2 trafficking rather than polarity (Sukumar et al. 2009). Finally, the sterol composition of membranes has been implicated with auxin transporter polarity (Kleine-Vehn et al. 2006; Men et al. 2008) and stability (Titapiwatanakun et al. 2009). It is clear that the mechanisms regulating the polar transport machinery are complex and, although substantial advances in our understanding of it have been made, there is still a huge amount that remains unclear.

2.2 Cytokinin Transporters

Auxin transport is well characterized compared to that of other phytohormones. For cytokinin (CK), putative uptake systems for nucleobase and nucleoside derivatives have been identified and characterized in heterologous yeast expression systems (Gillissen et al. 2000; Burkle et al. 2003; Hirose et al. 2005; Sun et al. 2005). Cytokinins are known to be translocated in the xylem and phloem and, therefore, exporters responsible for loading of synthesized CKs into the long-distance transport stream, as well as importers responsible for the selective uptake of CKs into target tissues, have been postulated (Sakakibara 2006). Complementation studies of a yeast mutant defective in the common uptake of bases with an Arabidopsis cDNA expression library enabled the identification of a single cDNA capable of restoring growth on adenine (Gillissen et al. 2000). This transporter, AtPUP1 (*pu*rine *p*ermease), was later shown to be capable of mediating CK nucleobase import into Arabidopsis suspension cells as well as into *S. cerevisiae* expressing recombinant AtPUP1. Two related members of this family (at least 15 genes in Arabidopsis) were apparently either less functional (AtPUP2) or non-functional (AtPUP3), although the expression and plasma membrane targeting of the recombinant proteins was not compared (Burkle et al. 2003). A candidate gene approach in rice to identify transporters for nucleoside CKs, which are the major cytokinins in the xylem, identified a member of the equilibrative nucleoside transporter (OsENT2) (Hirose et al. 2005). An equivalent gene from Arabidopsis (AtENT8) was identified through a screen for suppressors of a CK overproduction mutation. AtENT8 and the related AtENT3 were implicated in CK riboside uptake in plant although the reduction in CK uptake in mutant plants was relatively small (Sun et al. 2005). Additionally, an alternative study found no evidence for CK transport by AtENT4, 6 and 7 (Wormit et al. 2004). However, it does seem that CKs are transported by proteins responsible for nucleobase and nucleoside transport. Although low affinity

constants for transport (K_M) were reported for AtPUPs when expressed in yeast (in the low micromolar range), they are likely to be physiologically relevant transporters of CKs as the uptake kinetics of cytokinins into cultured Arabidopsis cells is multiphasic with a low-affinity contribution (Cedzich et al. 2008). PUP genes are a subset of the drug metabolite transport superfamily [transporter classification database; TCDB 2.A.7 (Saier et al. 2006)], whilst ENT genes have the TCDB entry 2.A.57 (Saier et al. 2006). The presence of many PUP and ENT genes in higher plant species (Gillissen et al. 2000; Hirose et al. 2005) means that much future research will be required to determine the true substrates of each transporter, and the exact role in plant physiology.

2.3 Abscisic Acid Transporters

The past 2 years has seen an explosion of data identifying, validating and occasionally rejecting putative components of the ABA signalling pathway. An exciting culmination of this has been the positive identification of members of the PYR (*pyrabactin resistant*) protein family as being receptors for ABA, with an accompanying avalanche of structural biology papers detailing the mechanisms of ligand binding and downstream signalling. The ABA receptor story, together with the false dawns, has been recently critically reviewed (Cutler et al. 2010), and the reader is referred there for citations to the primary literature.

Two recent studies, published back-to-back, now provide tantalising evidence of the efflux of ABA from sites of synthesis, and its uptake into target cells (Kang et al. 2010; Kuromori et al. 2010). Remarkably, these transport processes, with opposite directionality, are proposed to be carried out by related members of the 28-member-strong (in Arabidopsis) ABCG family of transporters, adding further evidence to the argument that eukaryotic ABC proteins are capable not just of efflux, but also of import (Lee et al. 2008). Abscisic acid transporters were identified by either candidate screening of AtABCG knock-out mutants for defects in germination and stomatal regulation (Kang et al. 2010), or by shotgun screening for ABA-sensitive germination phenotypes (Kuromori et al. 2010). Two identified genes encoded the transporters AtABCG25, proposed to mediate ABA efflux from sites of synthesis (Kuromori et al. 2010), and AtABCG40, proposed to mediate ABA influx into guard cells (Kang et al. 2010). GFP/YFP fusions of AtABCG25/40 proteins in plants are consistent with both proteins being localized to the plasma membrane – in agreement with an involvement in inter-cellular transport process (Kang et al. 2010; Kuromori et al. 2010).

Both transporters have been studied following expression in heterologous systems and the characterization of them as ATP-hydrolysis-dependent, stereoisomer-selective transporters has been confirmed (Kang et al. 2010; Kuromori et al. 2010). However, the pH dependence of transport has only been addressed for AtABCG40, and there are many remaining questions, such as the kinetics of the transport process, quantitative analysis of substrate:transporter binding and identification

of transport regulators, that further studies will need to address. In addition, as AtABCG25 is a so-called "half-transporter" (McDevitt et al. 2009), the identification of whether it functions as a homodimer, or heterodimer to effect ABA transport remains a key question. What is evident is that the years 2008–2010 have been *anni mirabiles* for the ABA field and there is doubtless much more to come.

2.4 Brassinosteroid Transport

Although brassinosteroids (BR) exert long-distance effects through "cross-talk" with other phytohormones, they are not believed to travel long distances themselves (Symons et al. 2008). However, short-range transport of brassinosteroids including cellular efflux systems is suggested by the presence of extracellular receptors (Gendron and Wang 2007; Li and Jin 2007). However, to date, the only candidate proteins for binding and delivering brassinosteroids to the extracellular face of the membrane are members of the pathogenesis-related (PR) 10 family of proteins. Betv1, the major allergen in birch tree pollen and the best-studied PR-10, can bind a diverse array of hydrophobic molecules, including brassinosteroids and the cytokinin kinetin (Markovic-Housley et al. 2003; Koistinen et al. 2005). Betv1 displays structural changes, that are pH and membrane-interaction dependent (Mogensen et al. 2007), can permeabilize membranes (Mogensen et al. 2007), and has been speculated as a potential delivery agent for BRs into the cytoplasm (Markovic-Housley et al. 2003; Symons et al. 2008). However, no definitive evidence of this function has been forthcoming, and other protein families, such as the A or G classes of ABC transporters, have also been suggested as putative BR transporters (Symons et al. 2008).

2.5 Heterologous Expression of Phytohormone Transporters

The characterization of specific phytohormone transport proteins has, to date, centred almost exclusively on the auxin transporters. But what is the preferred way to characterize the substrate:transporter interaction? Approaches can either be based upon homologous or heterologous expression systems. Our perspective is that if attempting to study specific auxin transporters it is worth trying to remove as much of the background of "auxin interaction" that could interfere with analysis of the data through use of heterologous systems. That is not to say that expression of auxin transporters in plant cells does not make a significant contribution to our understanding. Indeed, both PINs and AtABCB proteins have been analysed after expression in tobacco (BY2) cells (Petrasek et al. 2006; Cho et al. 2007), and this data is explored further below. For now, we briefly outline the heterologous expression systems that may be utilised for analysis of phytohormone transporter function.

Perhaps, the most unequivocal data obtained, to date, for auxin transport comes from *Xenopus* oocytes injected with cDNA for AUX1 and LAX3 (Yang et al. 2006; Swarup et al. 2008). The advantages of the system are the ability to measure transport of auxin into (or, presumably, out of) the cell, and the ease of confirmation that the transporter is localized to the cell surface (Yang et al. 2006). The major disadvantages are: the specialization required to undertake the study, the lack of a cell wall equivalent to that in plants, sensitivity to pH gradients equivalent to those seen in plants, and the impossibility of scaling up, in instances where purified protein is a requirement. Where transport measurements in cells are the priority, but the availability of the *Xenopus* system is restricted, the most common alternatives are yeasts (see below for details) and mammalian cells transfected with plasmids encoding auxin transporters. We have demonstrated that both HEK293T (an embryonic kidney cell line) and U2OS (an osteosarcoma cell line) can express AUX1 and LAX3, that the protein is targeted to the cell membrane and that import of auxin can be measured upon incubation of the cells in the presence of an auxin and pH gradient (Swarup et al. 2008). Similar results were demonstrated for expression of AUX1 in HeLa cells (Blakeslee et al. 2007). However, the approach is not without problems. We, and others, acknowledge that the background transport of auxin by organic anion (OAT) transporters and other proteins in such systems is high and, again, the cost of scaling up the system is prohibitive.

Why would we wish to "scale up" expression? For many membrane transporters, purification has enabled even more detailed characterization and in the long-term of course the structures of plant hormone transporters are an important goal. The priorities in scaling up expression are primarily related to cost-effectiveness – four systems are readily accessible. The first three are yeast expression systems. As a eukaryote, one can reasonably expect that *S. cerevisiae*, *S. pombe*, or *P. pastoris* (a commonly employed methylotrophic yeast expression system) will be able to express and post-translationally modify phytohormone transporters and direct them appropriately to the plasma membrane. Studies of PINs and AtABCBs expressed in *S. cerevisiae* and *S. pombe* are discussed below. We have attempted AUX1 expression in *P. pastoris* but have achieved highly variable results, depending on the position and the nature of the epitope tag in the protein (Carrier et al. 2009). A further system that more laboratories are now able to access, if higher level of expression is required, is a baculovirus expression vector (BEV) system (technology available from several companies including Merck/Novagen, Invitrogen and BD-Clontech). Infection of insect cells (which require little more than orbital incubators at 27–29°C to culture them) with recombinant viruses encoding auxin transporters is associated with high level expression that is easily scaled up (Carrier et al. 2008). Historically, many laboratories were dissuaded from using a BEV system because of the long lead time to generate a suitable infectious virus. Many technological improvements have cut this initial step down to just 4 weeks, even in novice hands. The major caveat for BEV systems is that the infection of insect cells by virus renders the cells highly porous, particularly in the later stages of infection when expression is typically greatest. Thus, direct measurements of transport are problematic, although in unrelated transport systems, the use of inside

out vesicles prepared from such cells has enabled transport studies to be performed [e.g. (Zelcer et al. 2003)].

Several other heterologous systems could in principle be tested for transporter expression, both in terms of low-level expression that would be appropriate for transport assays and in terms of scalability should purification be required. A *Lactococcus lactis* system has not proved amenable for AUX–LAX expression (Carrier et al. 2009), although it has been used successfully to express several other eukaryotic membrane transport proteins (Janvilisri et al. 2003; Kunji et al. 2005; Monne et al. 2005). Other prokaryotes, such as the *E. coli* strains C41/C43 have been used to express membrane proteins but reports on their success for eukaryotic proteins are infrequent (Miroux and Walker 1996).

2.6 Functional Characterization of Auxin Transporters

The work of several groups in the last 2–3 years has allowed the auxin transport community to shed rather non-descript terms such as "facilitator" and be increasingly confident that AUX–LAX, PINs and ABCBs are genuine transporters of auxin. For the AUX–LAX family, expression in *Xenopus* oocytes has revealed that AUX1 and LAX3 can both import auxin in a pH- and IAA concentration-dependent fashion (Yang et al. 2006; Swarup et al. 2008). For AUX1, the preferred substrate was clearly shown to be the anionic form of the weak acid, with net transport maximal at external pH of 5.5–6.5 (Yang et al. 2006). Both transporters displayed an apparent K_M for auxin in the high nanomolar range (Yang et al. 2006; Swarup et al. 2008) and, for AUX1, competition studies suggested that the substrate specificity of the protein was well approximated in the heterologous system. AUX1 transport studies have been complemented by direct determination of AUX1:IAA interaction in ligand-binding assays (Carrier et al. 2008). The data are comparable, showing that the K_D for auxin binding is in the low micromolar range and that the preferred substrate is anionic IAA^- (Carrier et al. 2008). Notably, both auxin transport and auxin-binding studies have produced data in heterologous systems that is compatible with classical plant cell suspension studies (Delbarre et al. 1996; Yang et al. 2006; Carrier et al. 2008). This compatibility gives confidence that mutant forms of AUX–LAX proteins can now be studied in detail to reveal the molecular mechanism of the auxin transport defect, and that quantitative data can be obtained from these systems and used in computational approaches (see below).

PIN protein catalysed auxin efflux has been studied by several groups, all using Arabidopsis PINs. Homologous systems (cultured Arabidopsis cells in suspension), plant heterologous systems (tobacco BY2 cells) and other eukaryotic systems (transfected human and yeast cells) have all been employed to characterize PIN function (Petrasek et al. 2003, 2006; Lee and Cho 2006). Despite the clear evidence that PINs 1-4, 6 and 7 are all functional auxin exporters (including export of 1-NAA acid and 2,4-D), there are some detractions. The substrate specificity of PINs may not be preserved in some heterologous systems – for example, benzoic acid was

transported in *S. cerevisiae* studies (Petrasek et al. 2006), and none of the presented data currently provides mathematical data describing either an apparent K_M for auxin transport, or apparent K_D for auxin binding. However, more recent studies in *S. pombe* (discussed below) offer prospects for future characterization.

Functional studies on ABCB proteins present some additional problems that AUX–LAX and PIN studies are relatively protected from. Primarily, the common heterologous expression systems contain multiple ABC transporters with diverse specificity, including pleiotropic drug resistance (PDR) and multidrug resistance (MDR) pumps. These pumps have a very broad substrate specificity towards hydrophobic compounds and auxins are potential substrates of numerous endogenous ABC transporters in yeast and mammalian cells. Despite this, several studies have demonstrated that ABCB1 and ABCB19 efflux auxin when they are expressed in heterologous systems (HeLa cells and *S. cerevisiae* deficient in PDR-type ABC transporters) (Geisler et al. 2005; Bouchard et al. 2006; Petrasek et al. 2006). Again, the substrate specificity is not as expected (with benzoic acid being also transported). As discussed below, interactions of ABCB transporters with other proteins may be required to confer correct specificity. For ABCB4, contention remains as to the direction of transport; if the protein does function as an importer of auxin (Santelia et al. 2005; Terasaka et al. 2005), then this has implications for the entire eukaryotic ABC transporter community, as to date only a couple of other eukaryotic ABC proteins have been demonstrated to be importers (Lee et al. 2008; Kang et al. 2010) although others have been proffered as possibilities (Molday 2007). However, a third study of ABCB4 has postulated a more conventional export direction (Cho et al. 2007). Again, it remains possible that yet-to-be revealed protein:protein interactions may govern the transport direction. Finally, we note that expression of ABCB4 in *S. pombe* was consistent with a bi-directional transport activity dependent on substrate concentration (Yang and Murphy 2009), Comparison with other recently described bi-directional transport proteins in plants is warranted (Dundar and Bush 2009).

One possible detraction of heterologous expression studies is the extent to which they are able to recapitulate and explain findings which shed light on the specific protein:protein interactions that may regulate auxin transporter stability, localization and function (Bandyopadhyay et al. 2007; Mravec et al. 2008). For AUX1, it is known that the endoplasmic reticulum accessory protein AXR4 is a requirement for correct plasma membrane targeting (Dharmasiri et al. 2006). In heterologous systems, the presence of this auxiliary protein seems to be unnecessary to recapitulate the substrate specificity of AUX1 (Yang et al. 2006; Carrier et al. 2008) although co-expression of AUX1 and AXR4 does influence the oligomeric state of the transporter (Carrier, Abu Bakar and Kerr, unpublished observations). For AtABCBs and PINs, the situation appears be different as there is co-localization of ABCB1 and ABCB19 with PIN1 and PIN2 at the plasma membrane (Blakeslee et al. 2007; Titapiwatanakun et al. 2009). This co-localization is backed up by co-immunoprecipitation and yeast two-hybrid studies demonstrating a physical interaction between the ABC transporter and the PIN protein (Blakeslee et al. 2007). The functional consequences of these interactions have been considered

by co-expression studies and both auxin transport rate and substrate specificity are altered (Blakeslee et al. 2007). Additional interactions between ABCB proteins and immunophilin-like proteins (Geisler et al. 2003; Bouchard et al. 2006; Bailly et al. 2008) may further increase the complexity that heterologous co-expression methods have to unravel in order to provide a clear picture of auxin transporter function and regulation.

What would address some of these concerns is an expression system for which surface expression of all major classes of auxin transporter is possible, and which demonstrates auxin transport with the expected substrate/inhibitor profile. Recently, a fission yeast (*S. pombe*) expression system has been described that broadly satisfies these two criteria (Yang and Murphy 2009). Firstly, the expression of several PINs, ABCBs and AUX1 was validated by western blotting, and subsequently the ability of these proteins to transport IAA was confirmed by accumulation assays. The substrate specificity (for the PINs) was also improved compared to studies in *S. cerevisiae* (Petrasek et al. 2006). The ability of this system to mirror the important protein:protein interactions that drive auxin transporter function and localization can now be evaluated (Blakeslee et al. 2007; Yang and Murphy 2009).

2.7 *Computational and Mathematical Approaches to Understanding Phytohormone Transport*

In this section, we examine the relationship between experiments conducted in the lab and experiments conducted on computers. This relationship is exemplified in systems biology, and we begin by briefly summarising the aims and methodology of this emerging field. We then briefly review current systems-level models of hormone transport, and discuss how parameterisation of such models is necessary to build accurate models of hormone transport.

Systems biology aims to understand biological systems at the system level (Alon 2006; Di Ventura et al. 2006; Szallasi et al. 2006), and involves researchers from the biological, computer and mathematical sciences. Its research methodology follows an iterative cycle (Kitano 2002): in vivo or in vitro experimentation leads to hypotheses about biological phenomena, which are encapsulated in models, and data on biological phenomena, which are used to validate the models. These models result in predictions about the biological phenomena, which are experimentally tested, leading to further data and refined hypotheses and models.

Two approaches exist to modelling biological phenomena: computational and mathematical modelling, each with their own advantages and disadvantages (Fisher and Henzinger 2007). Here, we can only briefly summarise some key differences, and direct the reader to a number of excellent papers for a more detailed discussion (Wolkenhauer et al. 2004; Fisher and Henzinger 2007). At their heart, these approaches differ in terms of how they represent the biological system in question. Mathematical models represent biological systems as sets of differential equations expressing relationships between quantities and how they change over time (known

as *denotational* semantics). Conversely, computational models represent biological systems as a collection of entities along with the rules of interaction between these entities (known as *operational* semantics).

In relation to plant hormones, the bulk of modelling efforts to date have focussed on auxin transport and its role in phenomena such as gravitropism, phyllotaxis and venation patterning. A number of recent, comprehensive reviews of these models already exist (Mjolsness 2006; Heisler and Jonsson 2007; Kramer 2008), so here we will briefly summarise the characteristics of these models. The majority of models are tissue-level, that is, they focus on the flow of auxin through a region of tissue composed of a number of cells, in either two dimensions [for example, (Kramer 2004; Rolland-Lagan and Prusinkiewicz 2005; Barbier de Reuille et al. 2006; Grieneisen et al. 2007)] or less often in three dimensions [for example, (Swarup et al. 2005)]. Often, different tissue layers, such as endodermis and cortex are modelled. Cells are either modelled as single compartments or as apoplastic and cytoplasmic compartments, the latter of which sometimes contains a separate vacuole compartment. Each of these compartments is either treated as spatially homogeneous, or subdivided into a number of sub-volumes in order to allow for more modelling of processes such as intracellular diffusion. The majority of models, to date, have been numerically solved mathematical models.

Auxin transport models generally incorporate a subset of the following auxin-related processes: apoplast and cytoplasmic diffusion, membrane diffusion, carrier-mediated influx and carrier-mediated efflux. In order to construct accurate models of these processes, the analogous biological processes need to be characterised sufficiently through the establishment of a number of key parameters. For auxin, these parameter include: for apoplast and cytoplasm diffusion (Kramer 2006), the diffusion coefficient; for membrane diffusion, the membrane permeability coefficient; for carrier-mediated transport, transporter efficiency; and a number of other parameters including membrane potentials, and auxin disassociation, association, synthesis and degradation rates (Kramer and Bennett 2006). Generally, for both mathematical and computational models, there is a fairly straightforward relationship between biochemical parameters such as K_M and K_D and modelling parameters such as carrier efficiency. While a small number of these parameters have been established in vitro, the majority of these parameters are still unknown in vivo, and in many cases have to be estimated from a partial picture of the underlying biochemistry or results in heterologous systems. To further complicate things, many of these parameters are pH-dependant and therefore need to be established for a range of pH values. This fact makes the establishment of a complete set of biologically validated parameters both time-consuming and experimentally challenging. A key area of future collaboration between modellers, biochemists and plant biologists will be in the establishment of the in vitro and in vivo values of these parameters.

As we have discussed, it is often time-consuming and experimentally challenging or infeasible to establish all of the parameters necessary to build a complete model. A principled approach and current area of research is automated parameter estimation (Tsai and Wang 2005; Zi and Klipp 2006). Also known as the inverse problem, this approach takes a set of experimental data and a partially

parameterised model and seeks to determine the values of the unknown parameters so that the model output most closely fits experimental observations. Parameter estimation problems are often noisy, non-linear, multimodal (have local optima) and computationally expensive, and a wide range of techniques have been developed to address these characteristics (Banga 2008). For small and medium size problems, classical linear and non-linear optimisation techniques have been used to successfully solve a wide range of problems. However, when the size of the model becomes larger, as is the case with many system biology models, these approaches become computationally expensive. Furthermore, classical optimisation approaches generally perform poorly on noisy, multimodal problems. To efficiently solve such problems, which are common in systems biology, a range of modern optimisation approaches, such as evolutionary algorithms and simulated annealing, have been developed (Moles et al. 2003). These latter approaches have been used to estimate parameters in, for example, biochemical pathways (Modchang et al. 2008), and an interesting line of future research will be their application to transporter kinetics.

3 Conclusion

The cloning of plant hormone transporters, coupled with powerful in vivo approaches determining cellular localization and redistribution, and in vitro studies of individual transporters have made it possible to understand component pieces of the hormone transport jigsaw. The future challenge is to realise how these components interact so as to be able to understand the system level basis of hormone transport. From a modelling perspective, we have seen that computational and mathematical models offer their own strengths and weaknesses, and an important area of future research will be the development of frameworks, which allow the efficient combination of both mathematical and computational models to form integrative multi-scale models of this biological system.

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Section III
Signal Transduction at the Plasma Membrane

Plant Hormone Perception at the Plasma Membrane

Sona Pandey

Abstract The plasma membrane is the perception site for multiple signals including hormones in both plants and animals. Research done in the past decade has identified receptors and perception mechanisms of the majority of plant hormones. Molecular genetic evidence now exists for PM perception of three of the plant hormones: brassinosteroids, cytokinins and abscisic acid. It is interesting to note that three distinct PM-localized perception and signal transduction modules exist for these three hormones. Brassinosteroids are perceived by PM-localized leucine rich repeat receptor-like kinases (LRR-RLKs), cytokinins by a two-component phosphorelay module similar to the prokaryotic two-component system, and abscisic acid by an atypical G-protein coupled signaling pathway.

1 Introduction

The plasma membrane (PM) is a dynamic, multisensory barrier between the inside and outside of a cell. The PM efficiently perceives and processes an astonishing array of signals to maintain cell integrity and viability. Biochemical studies have predicted the PM as a site of plant hormone perception. Recent studies, however, have identified both PM-localized and unexpectedly, cytoplasmic receptors for plant hormones. In retrospect, this seems logical as unlike animal hormones, plant hormones are produced and sensed in almost every cell type and they act both as endogenous and exogenous signals. This chapter discusses the signal perception of plant hormones at the PM. Plant hormones for which PM-localized perception systems do not exist or have not yet been identified and signals other than plant hormones that are perceived at the PM are not the focus of this chapter.

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The identification of receptor(s) and elucidation of their perception mechanisms is central to our understanding of the diverse processes regulated by plant hormones. The plant hormones auxin and gibberellic acid have soluble receptors (Dharmasiri et al. 2005; Kepinski and Leyser 2005; Ueguchi-Tanaka et al. 2005) and ethylene receptors are localized to the endoplasmic reticulum (Yoo et al. 2009). The PM-localized receptors have been identified for brassinosteroids (BRs, Li and Chory 1997) and cytokinins (To and Kieber 2008) and very recently for abscisic acid (ABA), which also has additional cytosolic receptors (Ma et al. 2009; Pandey et al. 2009; Park et al. 2009). While the specifics of signal perception and transduction for BRs and cytokinins have been worked out in great detail, analysis of the perception mechanisms of ABA is still in its infancy. The following discussion provides a glimpse of the diversity of signaling mechanisms plants exploit and which has possibly contributed to their evolutionary success while maintaining a sessile life style.

2 Brassinosteroids

Brassinosteroids were discovered in 1979 as steroid compounds from *Brassica napus* pollen that affected stem elongation (Grove et al. 1979). BRs are plant-specific polyhydroxylated steroids that regulate almost every phase of plant growth and development including overall morphology, photomorphogenesis, vascular differentiation, flowering, germination, seed size and stress responses (Clouse and Sasse 1998; Asami et al. 2005). BRs are perceived at the PM which is unique to plants as animal steroid hormone receptors are nuclear-localized, ligand-activated transcription factors (Bogan et al. 1998).

The use of extensive molecular genetics in combination with elegant biochemical studies has elucidated a nearly complete sequence of events from BR perception at the PM to signal transduction through the cytoplasm to the nucleus and eventual changes in the levels of effector genes and proteins (Vert et al. 2005; Belkhadir and Chory 2006). A series of serine/threonine phosphorylation events are involved in the perception and transduction of BR signals. The first event involves the receptor kinases BRI1 and BAK1 and their regulators at the PM; the second, the glycogen synthase kinase like protein BIN2 in the cytosol; and the third, the nuclear phosphoprotein transcription factors BES1 and BZR1 which bind BR-responsive gene promoters in their dephosphorylated state. The elucidation of BR signal perception and transduction mechanisms has greatly benefitted from the presence of a relatively easily quantifiable whole plant phenotype where varying degrees of plant dwarfism are directly related to the strength of the signal (e.g., strong *bri1* mutants are extreme dwarfs and weak mutants are semidwarfs) which has aided effective reverse and forward genetic screens (summarized in Table 1). In addition, expression of the BR biosynthetic genes *CPD* and *DWF4* and the phosphorylation status of a BR-dependent transcription factor BES1 serve as excellent molecular markers for the study of BR signal transduction.

Table 1 BR signaling components

Gene	Identification screen	Protein	Localization	Phenotypes
Brassinosteroid insensitive 1 (BRI1)	EMS mutagenesis for BL insensitivity in signaling pathways	LRR-RLK, S/T protein kinase activity	PM	Strong alleles: complete BL insensitivity, severely dwarf plants, weak alleles: semi dwarf plants
BRI1 associated kinase 1 (BAK1)	Dominant genetic suppressor of weak *bri1* in activation tagged lines, and BRI1 interacting proteins in yeast-2-hybrid	LRR-RLK, S/T protein kinase activity	PM	*bak1-1 D*: enhances weak *bri1* phenotype, BAK1 overexpression: BR hypersensitive plants
BRI1 kinase inhibitor 1 (BKI1)	BRI1 interacting protein in yeast-2-hybrid	Unknown, can be phosphorylated by BRI1	PM, cytosol	Lower BKI1 level by RNAi: BR hypersensitive plants, BKI1 overexpression: phenotypes similar to weak *bri1* alleles
BR signaling kinases (BSKs)	Proteomics analysis of BL regulated phosphoproteins at PM	Protein kinases of receptor-like cytoplasmic kinase (RLCK) family	PM	Overexpression of *BSK3* in a strong *bri1* background suppresses *bri1* phenotype
Brassinosteroid insensitive 2 (BIN2)	EMS mutagenesis, gain-of-function, semi dominant mutation, *bri1* like phenotype	Glycogen synthase 3 (GSK3)-like kinase, S/T kinase activity	Cytosol, nucleus	BIN2 gain-of-function mutants: severely dwarf plants, triple knock-out loss of function mutant (*BIN 2* and two close homologs): constitutive BR-response
BRI1 suppressor 1 (BSU1)	Dominant genetic suppressor of weak *bri1* in activation tagged lines	Kelch repeat containing, S/T phosphatase	Nucleus	Expression of *BSU1* in *bin2* or *bri1* background suppresses their phenotypes, dominant gain-of-function mutation accumulates dephosphorylated BES1
BRI1-EMS suppressor 1 (BES1)	Suppression screen of weak *bri1* mutant, dominant, gain-of-function mutation	Transcription factor, phosphoprotein	Cytosol, nucleus	Gain-of-function *bes1* mutant: suppresses weak *bri1* phenotype, BL hypersensitive, BES1-RNAi: weak *bri1* phenotype
Brassinazol resistant 1 (BZR1)	Insensitivity to BL biosynthesis inhibitor brassinazol, gain-of-function, dominant mutation	Transcription factor, phosphoprotein	Cytosol, nucleus	*bzr1-1D*: BR hypersensitive in dark, weak *bri1*-like phenotype in light

2.1 The PM-Localized Components of BR Signaling

Brassinosteroid insensitive 1 (BRI1) is localized to the PM and is the primary BR receptor. The *BRI1* gene was first identified in a mutant screen for brassinolide (BL, the most active brassinosteroid) insensitivity. A subsequent search of Arabidopsis EMS mutants for defects in BR signaling but not in biosynthesis identified 18 different alleles with lesions in a single gene, *BRI1* (Li and Chory 1997). BR signaling mutants exhibit dwarfism, dark green, rounded and curled leaves, and other developmental defects similar to BR biosynthetic mutants. However, unlike BR biosynthetic mutants, BR-insensitive mutants cannot be rescued by the exogenous application of BL. *BRI1* encodes a leucine rich repeat receptor-like kinase (LRR-RLK) with an extracellular domain containing 24 LRRs and a 70 amino acid island domain. A single transmembrane region connects the extracellular region of the protein to its intracellular region that has a serine/threonine (S/T) protein kinase domain and a short C-terminal extension that plays a regulatory role in kinase activation (Vert et al. 2005).

The receptor-like structure and BL-insensitivity of *bri1* mutants suggested BR receptor function, and this activity was subsequently corroborated by both, genetic and biochemical experiments. The first evidence for the role of BRI1 in BL sensing came from using a chimeric protein containing the extracellular and transmembrane domains of BRI1 and the intracellular S/T-kinase domain of Xa21 receptor-like kinase. The Xa21 RLK is involved in disease resistance in rice where it mediates generation of hypersensitive response (HR) when challenged with an incompatible pathogen *Xanthomonas Oryzae pv. oryzae* (He et al. 2000). HR can generally be quantified by measuring the expression of specific disease resistance genes. It was reasoned that if the chimeric protein would be active, a signal specific to the sensor domain (in this case BL) would generate a response specific to the kinase domain (in this case, the expression of disease resistance marker genes). Treatment of rice suspension culture cells expressing the chimeric protein with BL indeed resulted in the expression of disease resistance genes, confirming the role of BRI1 extracellular domain in sensing BL (He et al. 2000). In additional experiments, radiolabeled BL-binding was detected in the microsomal fractions expressing BRI1, overexpression of BRI1 resulted in more BL-binding sites in the isolated microsomes, and the BRI1 antibodies could immunoprecipitate the BL binding-activity, signifying the role of BRI1 in BR-perception (Wang ZY et al. 2001b). Further experiments confirmed direct binding of affinity-labeled BL to the extracellular 70 amino acid domain of BRI1 (Kinoshita et al. 2005). The C-terminal of BRI1 is autoinhibitory as proteins with C-terminal deletion are hyperactive kinases in vitro and show BL-hypersensitive phenotypes when expressed in plants. BL-binding causes autophosphorylation of BRI1 leading to a change in protein conformation that releases the inhibitory effects of the C-terminus and begins signal transduction (Wang et al. 2005). BL-binding also promotes BRI1 dimerization (Russinova et al. 2004; Wang et al. 2005). Three additional BRI-like proteins (BRL1-3) are present in the Arabidopsis

genome and these proteins are proposed to act redundantly with BRI1 in specific signaling pathways (Caño-Delgado et al. 2004; Zhou et al. 2004).

Genetic and biochemical studies have shown that three other proteins, BAK1, BKI1 and BSK1 (and its homologs) are PM localized and are involved in the regulation of BRI1 activity via phosphorylation-dependent changes (Fig. 1). *BAK1* encodes an RLK with five LRRs and a cytoplasmic kinase domain. BAK1 was identified separately in a genetic screen for a dominant genetic suppressor of *bri1* using activation-tagged lines and in a BRI1 yeast two-hybrid interaction screen (Nam and Li 2002; Li et al. 2002). A dominant *bak1* mutation (*bak1-1D*) enhances the weak *bri1* phenotype, whereas overexpression of *BAK1* makes plants mildly hypersensitive to BL, suggesting that it is a positive regulator of BR signaling. BAK1 is also PM-localized. The presence of BL facilitates the interaction between BRI1 and BAK1 (Li et al. 2002). BAK1 is autophosphorylated in response to BL and both BRI1 and BAK1 have S/T kinase activity towards each other. BAK1 does not directly bind BL and is proposed to be a coreceptor, acting with BRI1 to perceive the BL signal (Li et al. 2002; Nam and Li 2002).

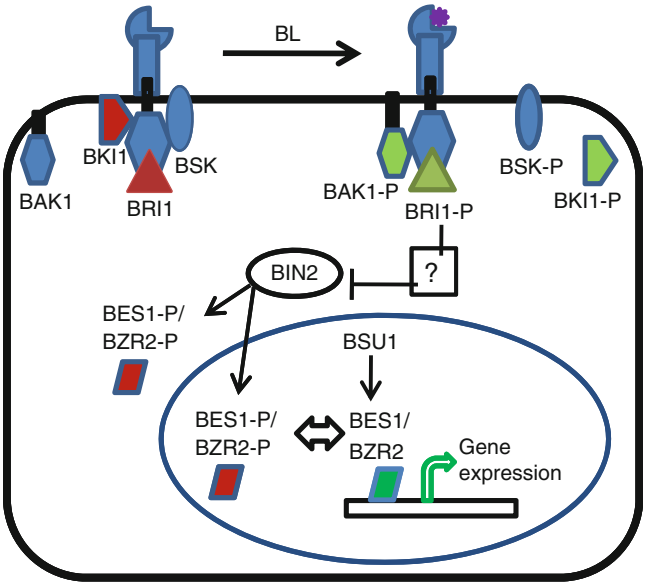

Fig. 1 Brassinosteroid signal perception and transduction mechanism: Brassinolide (BL) is perceived at the PM by BRI1. BL binding causes autophosphorylation of BRI1 at its C-terminus, release of autoinhibition and phosphorylation of associated regulatory proteins BKI1 and BSKs. Phosphorylated BKI1 and BSKs dissociate from BRI1 and phosphorylated BRI1 associates with phosphorylated BAK1. Active BRI1 inhibits the activity of BIN2 via an unknown mechanism (represented by "?"). Bin2 phosphorylates the transcription factors BES1 and BZR1. These transcription factors bind to the promoters of the BR-responsive genes in their dephosphorylated states. A nuclear phosphatase BSU1 is involved along with Bin2 to determine the phosphorylation status and by extension transcription of BR-responsive genes

The BKI1 protein was identified in an yeast two hybrid screen with a BRI1 kinase domain as bait (Wang and Chory 2006). The C-terminus of BKI1 interacts with the intracellular domain of BRI1 both in vitro and in planta. Genetic and biochemical evidence indicate that BKI1 is a negative regulator of BR signaling. Plants expressing lower levels of BKI1 have longer hypocotyls and exhibit hypersensitivity to exogenously applied BL, whereas overexpression of BKI1 results in smaller plants with rounder leaves, similar to weak *bri1* alleles. BKI1 is typically localized both at the PM and in the cytosol but undergoes a signal-dependent relocalization. BL causes rapid dissociation of BKI1 from the PM and this process requires the presence of an active BRI1 kinase. BKI1 can be phosphorylated by BRI1 in vitro and it inhibits the interaction between BRI1 and BAK1 (Wang and Chory 2006). The fate of BKI1 after dissociation from the PM and its potential downstream roles are still unknown.

The BSK proteins, BSK1 and 2, were identified in a proteomics experiment as PM-localized proteins that show BR-dependent changes in phosphorylation (Tang et al. 2008). The Arabidopsis genome has ten additional BSK protein kinases and the proteins possibly act redundantly to regulate BL signaling. The BSK proteins have an N-terminal kinase domain and C-terminal tetratricopeptide repeat domain usually involved in protein–protein interactions. BSK1 is localized to the PM and its PM localization does not change upon BL-treatment. BSK1 and 3 interact with BRI1 in vivo and, in contrast to the BRI1-BAK1 interaction, the presence of BL inhibits their interaction with BRI1. BSK1 and 3 are phosphorylated by BRI1 under in vitro assay conditions. Genetic evidence suggests that BSKs are positive regulators of BR signaling as overexpression of BSK3 in a strong *bri1-116* background suppresses its phenotype (Tang et al. 2008).

2.2 The Cytosolic and Nuclear Components of BR Signaling

BIN2 is a GSK3-like S/T kinase that is a downstream component in BR signaling and was identified in the original genetic screen of BR insensitive mutants (Li et al. 2001; Li and Nam 2002; Fig. 1). The original *bin2* allele was isolated as a gain-of-function, semidominant mutation that displayed phenotypes similar to *bri1*. *bin2* is allelic to two previously described mutants *dwarf12* (Choe et al. 2002) and *ultracurvata 1*(*ucu1*, Pérez-Pérez et al. 2002). BIN2 is a negative regulator of BR signaling. Biochemical analyses have confirmed that BIN2 is phosphorylated and exhibits a BL-dependent kinase activity; however it is not a direct substrate of BRI1, BAK1, BKI1 or BSK proteins. The *bin2* gain-of-function mutation results in a hyperactive kinase. Introduction of this mutant version of *BIN2* into the wild-type plants recapitulates the phenotype of *bin2* gain-of-function mutation. Additionally, overexpression of *BIN2* in a weak *bri1* background results in two types of phenotypes; plants with high level of BIN2 are severely dwarf and plants that show cosuppression of endogenous *BIN2* are almost wild type, confirming the role of BIN2 as a negative regulator of BR signaling (Li and Nam 2002).

These observations were further corroborated by the analysis of loss-of-function mutants. Arabidopsis has ten GSK genes and *BIN2* along with two other genes forms a small subfamily of GSK3-II. Single loss-of function *bin2* mutants are essentially wild type; however, the triple knock-out mutants of all GSK3-II members display a constitutive BR-response (Vert and Chory 2006).

Two highly similar transcription factors BES1/BZR2 and BZR1 are the direct downstream targets of BIN2. BES1 and BZR1 bind to distinct regions of the promoters of BR-responsive genes to culminate the BR signal transduction pathway. The roles of these proteins in BR signaling were identified by genetic screens. BES1 was identified in a suppression screen of a weak *bri1* mutant as a dominant, gain-of-function mutation (Yin et al. 2002). BZR1 was identified as a dominant negative mutation that causes insensitivity to brassinazol, a specific inhibitor of BR biosynthesis (Wang et al. 2002). BES1 and BZR1 are highly similar (88% identical), nuclear-localized phosphoproteins with 25 conserved sites of GSK3 kinase phosphorylation. Interestingly, the mutations uncovered by these two entirely different genetic screens map to the same amino acid change in both proteins (He et al. 2002; Wang et al. 2002; Yin et al. 2002). Genetic analysis revealed that BES1 and BZR1 are epistatic to BIN2. Both BES1 and BZR1 interact with BIN2 in vitro and in vivo, and BIN2 efficiently phosphorylates BES1 and BZR1 (He et al. 2002, 2005; Yin et al. 2002, 2005). Phosphorylated BES1 and BZR1 do not bind to the promoters of BR responsive genes. Thus, in the presence of an active BIN2, BR-responsive gene expression is inhibited, whereas the absence of BIN2 activity causes accumulation of dephosphorylated BES1 and thereby activation of gene transcription.

Another nuclear localized protein, *bri1*-suppressor 1 (BSU1), is also involved in phosphorylation-dependent BR signal transduction in the nucleus (Mora-García et al. 2004). BSU1 is an S/T, Kelch repeat-containing phosphatase and is a member of a small gene family. Expression of BSU1 in *bin2* background restores the phenotype of *bin2* mutants, suggesting that it either acts at the level of or is epistatic to BIN2. BSU1 dominant gain-of-function mutants accumulate excessive amounts of dephosphorylated BES1 in the nucleus, suggesting that BSU1 dephosphorylates BES1 in vivo. Consequently, it is possible that in the nucleus, a balance between BIN2 and BSU1 activities determine the phosphorylation status of BES1 and similar transcription factors that ultimately determines the gene expression changes in response to BRs.

2.3 *Proposed Mechanism of the Action of BR Perception and Signal Transduction*

Based on the data available to date, the following mechanism of BL perception and signal transduction has been proposed (Fig. 1). BRI1 activity is maintained at a basal level by its own inhibitory C-terminus and by its interaction with BKI1 and

BSK proteins. Upon BL perception, a change in protein conformation causes autophosphorylation and activation of BRI1 and release of autoinhibition followed by BKI1 and BSK phosphorylation. Phosphorylated BKI1 and BSK proteins dissociate from BRI1, and BKI1 relocates from the PM to the cytosol. BKI1 dissociation possibly allows interaction of BAK1 and BRI1. Phosphorylated BRI1, BAK1, BKI1 and BSKs then transduce the signal via inhibition of BIN2; however, the exact molecular mechanism of information relay from the BR receptor complex at the PM to BIN2 in the cytosol is not yet known. Neither BRI1 nor other known BRI1 interacting proteins directly interact with BIN2.

Under normal conditions, BIN2, in combination with BSU1 phosphatase in the nucleus, determines the level of dephosphorylated BES1 and BZR1. BL binding to BRI1 causes inhibition of BIN2 activity, leading to the accumulation of active, dephosphorylated BES1 and BZR1 in the nucleus. Dephosphorylated BES1 and BZR1 bind to distinct sequences on the promoters of BR responsive genes to control transcription.

Regulation of BIN2 activity remains the obvious unanswered question in the BR signal transduction pathway. Identification of BIN2 interacting protein(s) that acts between BRI1 and BIN2 will be helpful to understand how the signal perception at the PM is linked to the signal transduction. Additionally, since the triple knock-out mutants of *BIN2* and its homologs have obvious phenotypes, genetic screens in this mutant background may also help identify yet undiscovered signaling proteins involved in BR perception and signal transduction.

3 Cytokinin

Cytokinins were discovered as compounds affecting cell division in tobacco tissue culture by C. O. Miller and coworkers in the 1950s (Miller et al. 1955). Cytokinins are adenine derivatives, typically synthesized by isoprenylation of ADP or ATP by the enzyme ADP/ATP-isoprenyate transferase (Kakimoto 2003). At least four different chemical modifications of the classic adenine rings are classified as cytokinins, of which *trans*-zeatin (tZ) and isopentenyladenine (iP) are considered biologically active based on their receptor-binding activities (Yamada et al. 2001; Spíchal et al. 2004). Cytokinins have been shown to regulate almost all aspect of plant growth and development including cell division, root elongation, shoot-apical meristem (SAM) formation, apical dominance, senescence and circadian rhythms (Kakimoto 2003; Sakakibara 2006). Recent studies have also identified important roles for cytokinins in legume nodulation (Murray et al. 2007; Tirichine et al. 2007) and plant biotic and abiotic stress tolerance (Walters and McRoberts 2006; Tran et al. 2007).

Cytokinins are perceived by a two-component system (Kakimoto 2003; To and Kieber 2008). This system utilizes a series of alternating histidine (His) and aspartate (Asp) phosphorylation reactions to sense and transduce a signal. In its simplest form the system consists of two components, a histidine kinase that has a

sensor domain and a response regulator that has a receiver domain. Ligand binding to the sensor domain of the histidine kinase results in autophosphorylation of a conserved His residue. This phosphorylated, active kinase allows transfer of a phosphoryl group to the conserved Asp residue on the receiver domain of the response regulator. The response regulator typically potentiates gene-transcription to initiate outputs of the signal transduction pathway (Klumpp and Krieglstein 2002). This basic mechanism is loosely conserved from bacteria and yeast to plants and the comparisons to this mechanism in other systems has tremendously helped to identify the repertoire of cytokinin signaling components by homology-based searches.

In Arabidopsis, there are three main players in the perception and transduction of cytokinins (Kiba et al. 2005); the sensor histidine kinases (*A*rabidopsis *H*istidine *K*inase, AHKs), the intermediary phosphotransfer proteins (*A*rabidopsis *H*istidine *P*hosphor-transfer proteins, AHPs) and the response regulators (*A*rabidopsis *R*esponse *R*egulators, ARRs). Each of these classes of proteins is a member of a multigene family. Recent work has also indentified additional components such as *c*ytokinin *r*esponse *f*actors as a part of the signaling network; however, they are not involved in direct phosphorelay events.

3.1 The PM-Localized Components of Cytokinin Signaling

The Arabidopsis genome contains 11 HKs, 3 of which, AHK2, AHK3 and AHK4/CRE1/WOL, function as cytokinin receptors (To and Kieber 2008). The proteins are similar to the hybrid sensor kinases of bacteria as the single protein has a ligand-binding extracellular domain, a HK domain as well as a receiver domain (Fig. 2). The extracellular region of AHK2, 3 and 4 contains the *c*yclase/*H*is kinase-*as*sociated *e*xtracellular (CHASE) domain; typical of sensory HKs (Anantharaman and Aravind 2001). HKs that lack CHASE domain are not involved in cytokinin perception (Kakimoto 2003).

The AHK4/CRE1/WOL gene was first identified as a regulator of root morphogenesis and vascular differentiation. Mutation in *AHK4* resulted into short aberrant roots (WOODEN LEG, *WOL*) that had fewer cells and excessive xylem differentiation (Mähönen et al. 2000). Its role in sensing cytokinins was discovered by a genetic screen designed to isolate cytokinin resistant mutants in a tissue culture system. Researchers discovered a mutation in the same gene makes calli completely insensitive to exogenous cytokinins (CYTOKININ RESPONSIVE *CRE1,* Inoue et al. 2001). AHK2 and AHK3 genes were identified as homologs of CRE1/AHK4 gene. The AHK4 gene is primarily expressed in root vasculatures, whereas the AHK2 and AHK3 genes are mostly expressed in aerial parts of the plant (Higuchi et al. 2004; Nishimura et al. 2004). The following lines of evidence established these genes as bona fide cytokinin receptors: (1) a mutation in the osmosensing histidine kinase gene (*SLN1*) in yeast is lethal due to the absence of a phosphorelay mechanism. Heterologous expression of *AHK4* genes

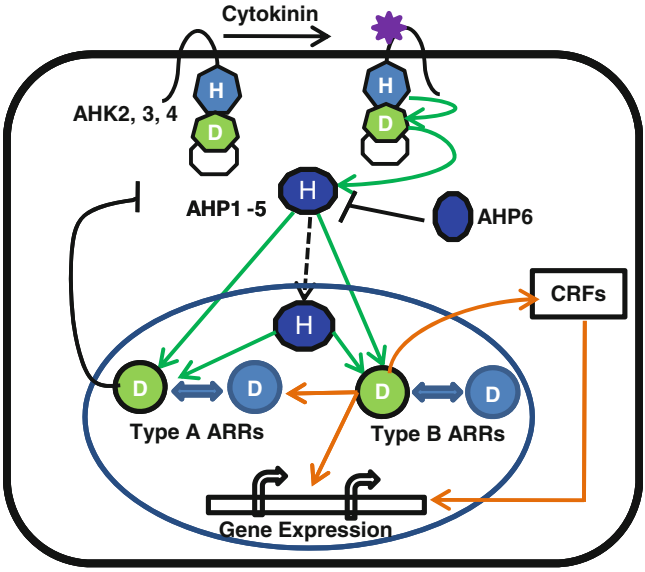

Fig. 2 Cytokinin signal perception and transduction mechanism: Cytokinin is perceived at the PM by the family of receptors AHK2, 3 and 4. Cytokinin-binding starts a His to Asp phosphorelay from the receptor at the PM via the intermediary proteins AHPs in the cytosol to the response regulators in the nucleus. The phosphorylated response regulators determine the transcription of cytokinin-regulated genes. *Green arrows* represent phosphorelay of His and Asp. *Orange arrows* represent transcriptional activation

in *Δsln1* yeast restores viability only in the presence of biologically active cytokinins. Moreover, introduction of a mutant version of *AHK4* that lacks the conserved His residue in the sensor kinase domain or the conserved Asp residue in the receiver domain fails to restore viability (Inoue et al. 2001; Ueguchi et al. 2001); (2) membrane fractions obtained from yeast expressing AHK4 or bacteria expressing AHK4 or AHK3 show binding with biologically active cytokinins at a physiologically relevant concentration (Yamada et al. 2001; Spíchal et al. 2004); (3) the AHK4 protein is involved in a cytokinin-dependent bidirectional phosphorelay. In the presence of cytokinin, AHK4 phosphorylates AHPs, whereas it dephosphorylates them in the absence of cytokinins. AHK2 and AKH3 are also involved in phosphorylation of AHPs, but the dephosphorylation (phosphatase) activity seems to be unique to AHK4 (Mähönen et al. 2006a, b); (4) a mutation in the CHASE domain of AHK4 renders the protein insensitive to cytokinin and converts it into a constitutively active phosphatase (Mähönen et al. 2006b); (5) overexpression of individual AHK proteins in Arabidopsis protoplasts makes the protoplasts hypersensitive to cytokinins (Hwang and Sheen 2001); and (6) T-DNA insertional mutants of individual AHK2, AHK3 or AHK4 display partial cytokinin insensitivity, whereas the *ahk2ahk3ahk4* triple mutant is almost completely insensitive to cytokinins (Higuchi et al. 2004; Nishimura et al. 2004; Riefler et al. 2006).

3.2 *The Cytosolic and Nuclear Components of Cytokinin Signaling*

3.2.1 The Phosphotransfer Proteins

Arabidopsis contains five conventional AHPs (AHP1–5) that have the conserved His residue for phosphotransfer and one pseudo-AHP (AHP6) that lacks this conserved amino acid (Suzuki et al. 2000; Fig. 2). The AHPs were first identified by screening of an Arabidopsis cDNA library with YPD1 cDNA that encodes a His phosphotransfer (HPt) protein in yeast (Miyata et al. 1998). *In silico* analysis of Arabidopsis (and other sequenced genomes) subsequently identified the whole inventory of these proteins. The AHP genes have overlapping expression patterns, with AHP4 showing the lowest level of expression, overall (Tanaka et al. 2004). A series of biochemical, cell-biological and molecular-genetic experiments established the AHPs proteins as an intermediary between the sensor AHKs and ARRs. The AHP genes (*AHP1–3*) can complement yeast mutant defective in YPD phosphotransfer protein (Miyata et al. 1998). The AHP proteins (AHP1–3) interact both with the upstream receptor AHK4 and with multiple downstream effector ARRs in Y2H assays (Yamada et al. 1998; Imamura et al. 1999; Urao et al. 2000). The proteins are involved in phosphorelay of information, as they are phosphorylated by AHKs in a cytokinin-dependent manner. Moreover, the AHP proteins phosphorylate both type-A and type-B ARRs, their direct downstream targets (Imamura et al. 1998, 1999; Tanaka et al. 2004; Mähönen et al. 2006b; Fig. 2). Localization studies with GFP-tagged AHP proteins demonstrated that the proteins normally localize to the cytoplasm but undergo a cytokinin-dependent relocalization to the nucleus (Hwang and Sheen 2001). Genetic evidence with T-DNA insertion mutants further substantiated these findings. As expected from their overlapping expression patterns and highly similar protein sequences, the single mutants essentially exhibit wild-type sensitivities to cytokinin, but the triple, quadruple and quintuple mutants show increasing degrees of cytokinin insensitivity in various phenotypic assays (Hutchison et al. 2006). Additionally, ectopic expression of AHP2 gene results in plants exhibiting some degree of cytokinin hypersensitivity (Suzuki et al. 2002). These data thus confirm that the AHP1–5 proteins are positive regulators of cytokinin signaling. Interestingly, the pseudo-AHP protein (AHP6) that cannot contribute to phosphorelay of information due to lack of conserved His residue is a negative regulator of cytokinin signaling (Mähönen et al. 2006a). The gene was identified as a suppressor of AHK4/WOL gene. The expression of AHP6 gene is down-regulated by cytokinins.

3.2.2 The Response Regulators

The type-A and type-B ARRs represent the final components of the cytokinin phosphorelay machinery (Kakimoto 2003). The ARR proteins have been divided

into three subgroups; type-A ARRs (10 members, ARR3–ARR9 and ARR15–ARR17); type-B ARRs (11 members, ARR1, ARR2, ARR10–ARR14 and ARR18–ARR21) and type-C ARRs (2 members, ARR22 and ARR24). In addition there are nine proteins described as Arabidopsis pseudo-response regulators (APRRs). The APRRs lack the conserved Asp residue required for phosphorylation. The type-A ARRs have short C-termini and their expression is rapidly up-regulated by cytokinin treatment. The type-B ARRs are cytokinin-dependent transcriptional activators and have DNA-binding and trans-activating domains at their C-termini. The type-B ARRs activate transcription of cytokinin-regulated genes, including that of type-A ARRs and cytokinin response factors. The type-C ARRs are involved in regulating circadian rhythms, whereas almost nothing is known of the functions of APRRs (To and Kieber 2008).

The type-A ARRs were identified on the basis of their sequence similarity to the cyanobacterium *Synechocystis* response regulators and by differential display screening of cytokinin-treated plants for genes showing altered expression (Brandstatter and Kieber 1998; Imamura et al. 1998). The type-A ARRs are primary cytokinin-response genes as they exhibit significant up-regulation in response to cytokinin treatment (Brandstatter and Kieber 1998; D'Agostino et al. 2000). Biochemical and genetic evidence confirm their roles as response regulators. The ARRs exhibit the phosphoryl group acceptance in vitro and in vivo in biochemical assays (Hwang and Sheen 2001; Sakai et al. 2001). In addition, a subset of ARRs physically interacts with AHPs (Tanaka et al. 2004; Mähönen et al. 2006b). The type-A ARRs are negative regulators of cytokinin signaling (Kiba et al. 2003; To et al. 2004). T-DNA knock-out mutants in single Type-A ARR genes have wild-type sensitivities to cytokinin, but higher order mutations, including the *arr3,4,5,6,8,9* sextuple mutant, exhibit varying degrees of cytokinin hypersensitivity in physiological assays (To et al. 2004;). Expectedly, overexpression of individual ARR genes causes cytokinin hyposensitivity. The negative regulation of cytokinin sensitivity by type-A ARRs is dependent on the presence of conserved phosphorylation of Asp as wild-type but not mutant versions of these genes can complement the *arr3,4,5,6* mutant (To et al. 2004, 2007).

The type-B ARRs are transcriptional activators and are positive regulators of cytokinin signal (Mason et al. 2005; Argyros et al. 2008). Similar to higher order mutants in type-A ARRs, the multiple type-B ARR mutants also exhibit phenotypes in various cytokinin signaling pathways. The *arr1,10,12* triple mutant is insensitive to cytokinin in root elongation and callus induction assays and is compromised in cytokinin-induced type-A ARR expression (Imamura et al. 2003; Tajima et al. 2004; Mason et al. 2005; Yokoyama et al. 2007; Ishida et al. 2008).

In addition to these two classes of response regulators, the cytokinin signaling pathway also includes a class of AP2-like transcription factors (Rashotte et al. 2003, 2006). The transcripts of these genes are rapidly up-regulated by cytokinins and their protein products undergo a cytokinin-induced relocalization to the nucleus (Rashotte et al. 2006). These cytokinin response factors also act redundantly and affect transcription of cytokinin-induced genes, some of which are also induced by type-B ARRs (Rashotte et al. 2006).

3.3 Proposed Mechanism of Action of Cytokinin Perception and Signal Transduction

Direct ligand-binding and mutagenesis studies have confirmed that cytokinin binds to the CHASE domain of plasma membrane-localized receptors AHK2, 3 and 4 and initiates a His-Asp-His-Asp phosphorelay event (Fig. 2). Cytokinin-binding leads to autophosphorylation of the conserved His residue on the kinase domain followed by an intramolecular phosphotransfer event where the phosphoryl group is transferred from the conserved His residue on the kinase domain to the Asp residue of the receiver domain of the AHK proteins. The next step is the first intermolecular phosphotransfer reaction. The conserved His on authentic AHPs in the cytoplasm receives the phosphoryl group from AHKs. The phosphorylated AHPs relocate to the nucleus and carry out the final phosphotransfer reaction by transferring the phosphoryl group to the conserved Asp on type-A and type-B ARRs. The phosphorylated, active type-A ARRs transduce downstream signaling pathways via yet undiscovered signaling components. The type-A ARRs are also involved in feedback regulation of cytokinin signaling. Additionally, at least a subset of phosphorylated type-A ARRs also control protein degradation as the phosphorylated ARR5, 6 and 7 are more stable than their dephosphorylated forms.

Phosphorylation is required for type-B ARR activity. In the absence of cytokinins, the dephosphorylated receiver domain acts as a repressor of the transcriptional activator domain of type-B ARRs. Upon phosphotransfer from AHPs on ASP of the receiver domain, the repression is released. Active type-B ARRs induce transcription of cytokinin-activated genes, including those of type-A ARRs. Some of the targets of type-B ARRs are also activated by cytokinin response factors.

4 Abscisic Acid

ABA was discovered in the early 1960s by different groups studying abscission in cotton and lupine fruits, and bud dormancy in woody plants (Addicott et al. 1968). Since then, the roles of ABA have become obvious in both physiological and adaptive responses of plants (Christmann et al. 2006). The major pathways regulated by ABA in plants include seed development, dormancy and germination, leaf senescence, inhibition of root length, transition between vegetative to reproductive phase, and stomatal responses (Finkelstein et al. 2002, 2008; Verslues and Zhu 2005; Chinnusamy et al. 2004, 2008). In addition, ABA is defined as the "stress hormone" in plants given its role during abiotic stress conditions, such as drought, cold and high salt, as well as biotic stress (Fujita et al. 2006; Seki et al. 2007; Asselbergh et al. 2008) responses of plants. Owing to its importance as a regulator of so many diverse processes, a number of distinct signaling modules have been identified that transduce the ABA signal (Li et al. 2006).

ABA perception and signaling seem to be much more complex than the other plant hormones. There has been a long-standing debate whether extracellular, PM-localized or soluble receptors are involved in sensing ABA. Electrophysiological and biochemical studies have suggested the presence of extracellular ABA perception sites (Allan and Trewavas 1994; Anderson et al. 1994; Gilroy and Jones 1994). Use of anti-idiotypic-ABA antibodies or affinity labeled-ABA (biotinylated ABA) in conjunction with the fluorescence-labeled avidin has visually demonstrated the presence of ABA binding sites at the plasma membrane of stomatal guard cells (Pedron et al. 1998; Yamazaki et al. 2003; Kitahata et al. 2005). Conversely, ABA microinjected directly into the cytoplasm had an effect on stomatal responses suggesting the presence of intracellular ABA perception sites (Schwartz et al. 1994). The recent discovery of multiple ABA-receptors localized both at the PM and in the cytosol has finally provided the molecular tools to start dissecting the multiple ABA signal perception and transduction modules.

Multiple lines of evidence have linked the PM-perception of ABA to the heterotrimeric G-protein signaling pathways in plants. Heterotrimeric G-proteins are signal transducers in all metazoans (Offermanns 2003). In the classic mode of G-protein signaling, ligand binding to a PM-localized G-protein coupled receptor (GPCR) causes activation of Gα subunit by a GDP-to-GTP exchange reaction. The active, GTP-bound Gα dissociates from the GPCR and the Gβγ dimer. GTP-bound Gα and Gβγ interact with multiple downstream effectors to transduce the signal. The Gα has an intrinsic GTPase activity, which hydrolyzes the bound GTP back to GDP and restores the Gαβγ trimer at the PM in association with the GPCR (Cabrera-Vera et al. 2003).

Biochemical studies have shown that ABA perception at the PM of the cereal aleurone cells, stomatal guard cells and Arabidopsis suspension culture cells involves changes in Ca^{2+} level and activation of phospholipase D (Gilroy and Jones 1994), both known effectors of G-protein signaling. Additionally, ABA added extracellularly affects PLD activity and this activation is directly regulated by the presence of GTP/GDP (Gilroy and Jones 1994). Molecular genetic studies have further corroborated these studies as G-proteins regulate almost all aspects of ABA-signaling. Arabidopsis mutants lacking Gα and/or Gβ subunits show hypersensitivity to ABA in seed germination and early seedling growth and development and hyposensitivity to ABA in stomatal response assay, suggesting complex, combinatorial, cell type-specific regulation of ABA signaling by G-proteins (Wang et al. 2001a; Pandey et al. 2006). These data imply the presence of a PM-localized, ABA-sensing module coupled to the G-protein signaling pathway.

The first report of a PM-localized ABA receptor identified a protein designated as G-protein coupled receptor 2 (GCR2, Liu et al. 2007). However, the molecular identity of the protein as a GPCR as well as its role in ABA- signaling has been equivocal (Gao et al. 2007; Johnston et al. 2007a; Guo et al. 2008; Illingworth et al. 2008; Risk et al. 2009). The reported ABA-related phenotypes of the *gcr2* mutants are weak to absent. The protein bears no structural hallmarks of a GPCR (instead it shows significant similarity to bacterial lanthionine synthase C).

A new class of G-proteins, comprised of two highly homologous proteins that have GPCR-like topology and inherent G-protein activity (*G*PCR-*t*ype-*G* proteins GTG1 and 2), has recently been identified as ABA receptors (Pandey et al. 2009). The GTG proteins were identified by an *in silico* analysis using vertebrate GPCRs as query. The Arabidopsis GTG proteins show high sequence homology to a class of orphan GPCR, GPR89. The homologs of GTG proteins are present across phyla. The GTG proteins are ubiquitously expressed and localize to the PM in transient protoplast transfection assays. As their name suggests, the GTG proteins have two distinct activities. The purified GTG proteins exhibit specific- and reversible GTP/GDP-binding and GTPase-activity, similar to signaling G-proteins. In addition, the GTG proteins interact with the sole canonical G-protein α subunit of Arabidopsis, GPA1, similar to canonical GPCRs. Mutants lacking both GTG genes exhibit hyposensitivity to ABA in all classic physiological assays. The GTG proteins bind ABA in a saturable, stereo-specific manner at relevant physiological concentrations. The GTP/GDP-bound state of GTG proteins seems to determine the efficiency of ABA binding as the presence of GDP facilitates ABA-binding. Interestingly, the interaction of GTG proteins with GPA1 has important physiological consequences as this interaction inhibits the GTPase activity of GTG proteins.

The following characteristics of GTG proteins substantiate their role as ABA-receptors: (1) the GTG proteins have receptor-like topology and PM-localization; (2) the purified proteins exhibit saturable, highly specific ABA-binding; (3) the mutants lacking both *GTG* genes are ABA hyposensitive in classic ABA-responses; (4) the *GTG* genes are ubiquitously expressed, a requirement given the role of ABA in almost all parts of the plant; and (5) ABA has no effect on the expression of *GTG* transcripts. Moreover, existence of GTG proteins in two different conformations (GTP-bound and GDP-bound), and a conformation-dependent ABA binding, provides a possible structural mechanism for their action.

The details of the mechanism of action of GTG proteins are not known at this time nor are their immediate downstream pathways. However, based on the available data in conjunction with GPA1, the following hypothetical model has been proposed (Fig. 3). Arabidopsis GPA1 mostly stays in its GTP-bound conformation, given its very slow GTPase activity (Johnston et al. 2007b). GTP-bound GPA1 inhibits the GTPase-activity of GTG proteins, maintaining them in their GTP-bound conformation, which binds ABA less efficiently. This model thus suggests that GTP-bound GPA1 down-regulates ABA-binding and ABA-signal propagation. By extension, plants lacking the GTG receptors should be less sensitive to ABA, whereas plants lacking GPA1 should be hypersensitive to ABA as more GTG proteins will be available in their GDP-bound confirmation in such mutants. All these data are inherently consistent with the known phenotypes of *gtg1 gtg2* mutants (ABA hyposensitive) and *gpa1* mutants (ABA-hypersensitive, except for the stomatal phenotypes). This model proposes that a dynamic equilibrium between the GTP-/GDP-bound states of the GTG proteins and GPA1 determines ABA-perception and signaling in WT plants.

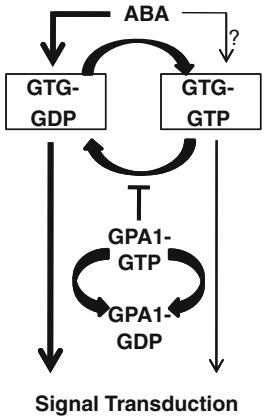

Fig. 3 A model for the proposed mechanism of action of GTG proteins. The PM-localized GTG proteins bind ABA more efficiently in their GDP-bound conformation. GPA1 in its GTP-bound form maintains the GTG proteins in their GTP-bound conformation, thereby negatively regulating the signal perception and transduction. The absence of GTG proteins makes plants hyposensitive to ABA whereas in the absence of GPA1 the negative regulation is released, resulting into ABA hypersensitive plants

In comparison to the signal transduction pathways in BR and cytokinin signaling, we know nothing of the mechanism of signal transduction by GTG proteins. Questions such as where does ABA bind to the GTG proteins, what conformational changes take place in the protein after ABA binding, what are the immediate downstream effectors of GTG proteins and how does the signal transduction by GTG proteins fit in the context of the overall ABA signaling in plants remain to be answered to establish the signal transduction pathways of the GTG proteins.

5 Conclusion

Identification of receptors for major plant hormones in the past 10–12 years has added greatly to our knowledge of how plants sense and transduce a signal. This research has also reinforced the idea that plants have evolved multiple mechanisms to sustain a stationary life style. In almost all cases, the receptors and effectors are encoded by multigene families that act redundantly to provide functional plasticity in signal perception. Multiple unanswered questions remain, especially in ABA signal perception and signal transduction. Additionally, almost all of information available to date is from the model plant *Arabidopsis thaliana*. Although the receptors and majority of effector proteins seem to be conserved where analyses have been performed on other plant species, specific yet unidentified differences may be present that need to be explored e.g., the role of cytokinin receptor AHK4/CRE1 during nodulation (Murray et al. 2007; Tirichine et al. 2007) and perception of systemin by BRI1 homologs in tomato (Szekeres 2003). In addition, since more than one receptor modules have been identified for ABA, how the two modules interact to generate and regulate the overall effects of ABA will also be an area of active future research.

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Light Sensing at the Plasma Membrane

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Abstract Light is one of the most important environmental factors controlling plant development and its perception is achieved through a suite of photoreceptor proteins. Like photoreceptors associated with our vision, plant photosensors can detect the presence, intensity, direction and color of light, and in turn, utilize this information to direct their growth accordingly. To date, four different types of photoreceptors have been identified in plants, namely the phytochromes, cryptochromes, phototropins and members of the Zeitlupe (ZTL) family. Phytochromes are photoreversible red/far-red photoreceptors whereas cryptochromes, phototropins and members of the ZTL family specifically absorb UV-A/blue wavelengths. Plants also respond to UV-B and green light, but the photosensors responsible for their detection remain elusive. A summary of light-regulated events that are known to occur at the plant plasma membrane will be discussed, as will the biochemical and photochemical properties of the photoreceptor proteins involved.

1 Phototropin Blue-Light Receptors

1.1 Phototropin Activity and Biological Functions

Like many aspects of plant receptor signaling, much of our understanding of phototropin function has come from genetic analysis of the model plant *Arabidopsis thaliana*. Arabidopsis contains two phototropins (PHOT1 and PHOT2) that have overlapping and distinct functions (Christie 2007). More specifically, PHOT1 and PHOT2 have been shown to act redundantly in regulating phototropism

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(Sakai et al. 2001), stomatal opening (Kinoshita et al. 2001), chloroplast accumulation movement to low light intensities (Kagawa et al. 2001) and leaf expansion (Sakamoto and Briggs 2002; Inoue et al. 2008a), but exhibit different light sensitivities (Sakai et al. 2001). Whilst PHOT1 mediates the rapid inhibition of hypocotyl growth upon transfer of dark-grown seedlings to light (Folta and Spalding 2001), PHOT2 is solely responsible for chloroplast avoidance movement at high light intensities (Kagawa et al. 2001) and for the accumulation of chloroplasts at the bottom of cells in darkness (Suetsugu et al. 2005). Ultimately, the afore-mentioned phototropin-mediated processes serve to optimize the photosynthetic efficiency of plants and promote growth under weak light conditions (Takemiya et al. 2005). More recently, a role for PHOT2 in regulating blue light-dependent nuclear positioning in Arabidopsis has been reported (Iwabuchi et al. 2007). Phototropins are also found in lower plants, including the unicellular green alga *Chlamydomonas reinhardtii* (Huang et al. 2002). A phototropin in *Chlamydomonas* regulates the algal sexual lifecycle in response to blue light (Huang and Beck 2003). However, despite their functional differences the mode of action of higher and lower plant phototropins appears to be highly conserved (Onodera et al. 2005).

Among the plant photoreceptors identified, phototropins are predominantly localized at the PM (Sakamoto and Briggs 2002; Kong et al. 2006). Indeed, discovery of this photoreceptor family was facilitated by their inherent biochemistry involving blue light-induced receptor autophosphorylation, which is readily detectable in membrane extracts prepared from a variety of plant species (Fig. 1a; Briggs et al. 2001). Although hydrophilic proteins, phototropins are tightly associated with the plasma membrane (PM) under noninductive conditions (Sakamoto and Briggs 2002; Kong et al. 2006). The exact nature underlying their association

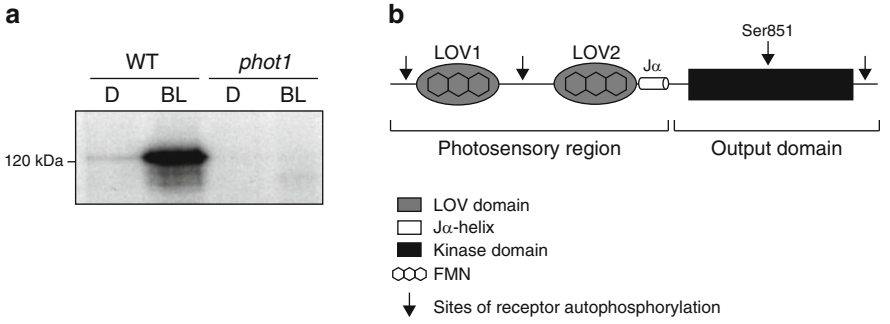

Fig. 1 Kinase activity and protein structure of plant phototropins. (**a**) Autoradiograph showing blue light-activated autophosphorylation of phot1 in extracts prepared from wild-type dark-grown Arabidopsis seedlings (WT) or from mutant seedlings lacking phot1 (*phot1*). Membrane proteins were prepared under dim red light and given a mock irradiation (D for dark) or a pulse of blue light (BL) prior to the addition of radiolabelled ATP. Proteins were separated on a polyacrylamide gel and exposed to autoradiography. (**b**) Cartoon illustrating the domain structure of phototropin blue light receptors. Relative positions of the phosphorylation sites of Arabidopsis phot1, including serine residue 851 that is required for receptor signaling are indicated

with the PM is not known, but may involve some mode of posttranslational modification within the C-terminal region of the protein (Kong et al. 2006). Upon irradiation, a fraction of PHOT1 is rapidly released from the PM in Arabidopsis (Sakamoto and Briggs 2002) whereas PHOT2 associates with the Golgi apparatus (Kong et al. 2006). At present, the biological significance of this partial light-induced internalization is not fully understood, but may represent a form of phototropin signaling (Wan et al. 2008).

1.2 LOV Domains and Blue Light Sensing

The structure of plant phototropins can be separated into two parts: a N-terminal photosensory input region coupled to a C-terminal effector or output region that contains a classic serine/threonine kinase motif (Fig. 1b). The N-terminal region comprises two so-called LOV domains each of which bind the vitamin-B derived cofactor flavin mononucleotide (FMN) as a blue light-absorbing chromophore (Christie et al. 1998, 1999). LOV domains exhibit protein sequence homology to motifs found in a diverse range of eukaryotic and prokaryotic proteins involved in sensing Light, Oxygen, or Voltage, hence the acronym LOV (Huala et al. 1997). Protein crystallography has shown that the LOV domain consists primarily of five antiparallel β-sheets and two α-helices, binding the FMN tightly inside an enclosed structure (Crosson and Moffat 2001).

LOV domains expressed and purified from *Escherichia coli* are yellow in color owing to their bound flavin cofactor and are photochemically active in solution as monitored by absorbance or fluorescence spectroscopy (Salomon et al. 2000; Swartz et al. 2001; Kasahara et al. 2002). In darkness, LOV domains bind FMN noncovalently forming a spectral species, LOV_{447}, absorbing maximally near 447 nm. Irradiation of the domain induces the formation of a covalent bond between the C(4a) carbon of the FMN and the sulphur atom of a nearby, conserved cysteine residue within the domain (Salomon et al. 2000; Swartz et al. 2001). Formation of this so-called cysteinyl adduct occurs within microseconds of illumination and produces a spectral species, LOV_{390}, absorbing maximally near 390 nm (Salomon et al. 2000; Swartz et al. 2001). Formation of LOV_{390} is fully reversible in darkness and represents the active signaling state that leads to photoreceptor activation (Salomon et al. 2000; Swartz et al. 2001). To date, it is still a mystery as to why the phototropins contain two LOV photosensors, namely LOV1 and LOV2 (Fig. 1b). Photochemically active LOV2 is necessary for phototropin function (Christie et al. 2002; Cho et al. 2007), while the presence and photochemical reactivity of LOV1 has been shown to be dispensable (Christie et al. 2002; Cho et al. 2007; Sullivan et al. 2008). However, LOV1 has been proposed to mediate receptor dimerization (Salomon et al. 2004; Nakasako et al. 2008) and/or modulate the photochemical reactivity of LOV2 (Matsuoka and Tokutomi 2005).

1.3 Phototropin Activation and Phosphorylation

It is now generally viewed that the phototropin LOV2 domain functions as a repressor of the C-terminal kinase domain in the dark and that this mode of repression is alleviated upon photoexcitation, resulting in receptor autophosphorylation throughout the protein (Christie 2007; Matsuoka et al. 2007; Tokutomi et al. 2008). Photoexcitation of LOV2 leads to displacement of an α-helix from the surface of the domain (Harper et al. 2003) and unfolding of this α-helix, designated Jα, results in activation of the C-terminal kinase domain (Harper et al. 2004). Protein rearrangements within the central β-sheet scaffold have also been reported to play a role in propagating the photochemical signal generated within LOV2 domain (Nozaki et al. 2004; Iwata et al. 2005; Jones et al. 2007) to bring about protein changes at the surface necessary for activation of the C-terminal kinase domain and autophosphorylation of specific serine/threonine residues (Fig. 1b; Inoue et al. 2008b; Sullivan et al. 2008). Light-activated phototropin can return to its nonphosphorylated state upon incubation in darkness (Briggs et al. 2001). This recovery process involves dephosphorylation of the receptor by an as yet unidentified protein phosphatase.

Autophosphorylation at a conserved serine residue (Ser851) within the kinase domain of Arabidopsis PHOT1 is essential for receptor signaling (Inoue et al. 2008b). Phosphorylation of the equivalent site is known to enhance the activity and promote substrate recognition in other kinases such as protein kinase A (Tokutomi et al. 2008). Although several sites of phototropin autophosphorylation have been mapped upstream of LOV2 (Inoue et al. 2008b; Sullivan et al. 2008), there is still no information as to their functional consequences. A truncated version of Arabidopsis PHOT1 comprising only LOV2 and the C-terminal kinase domain is functionally active in transgenic Arabidopsis lacking both PHOT1 and PHOT2, implying that phosphorylation of sites upstream of LOV2 is not required for receptor signaling (Sullivan et al. 2008). However, autophosphorylation of PHOT1 is known to mediate binding of 14-3-3 proteins (Kinoshita et al. 2003), key regulators of protein function in eukaryotes that preferentially bind to phosphoserine/threonine-containing motifs. Mutation of the phosphorylation sites required for 14-3-3 binding, located in the peptide region between LOV1 and LOV2, do not appear to perturb the functionality of Arabidopsis phot1 (Inoue et al. 2008b). Therefore, the biological significance of 14-3-3 binding to phototropins awaits further investigation.

1.4 Phototropin Signaling at the PM

Several rapid signaling events have been reported to occur at the PM following phototropin activation. For example, proton extrusion is essential for stomatal opening and is known to involve activation of the PM H^+-ATPase (Shimazaki

et al. 2007). Phototropin-mediated activation of the guard cell H^+-ATPase involves phosphorylation of the H^+ATPase and subsequent 14-3-3 binding. However, the fungal toxin fusicoccin has been shown to induce phosphorylation of the H^+-ATPase in the absence of PHOT1 and PHOT2, implying that the guard cell H^+-ATPase is not a direct substrate for phototropin kinase activity (Ueno et al. 2005).

Electrophysiological techniques have also been used to identify the presence of a phototropin-regulated Ca^{2+} channel at the PM in Arabidopsis (Babourina et al. 2002; Stoelzle et al. 2003). Several studies employing different experimental approaches have shown that phototropin activation leads to an increase in cytosolic Ca^{2+} concentrations (Baum et al. 1999; Babourina et al. 2002; Harada et al. 2003; Stoelzle et al. 2003). Pharmacological analysis using the membrane-permeable calcium-specific chelator BAPTA indicates that the rapid, blue light-induced increase in cytosolic Ca^{2+} observed in dark-grown seedlings is associated with the PHOT1-mediated rapid growth-inhibition response that occurs in seedlings emerging from the soil (Folta et al. 2003). In this regard, PHOT1 one has recently been reported to negatively regulate the expression of an inositol polyphosphate 5-phosphatase gene, which modulates the inhibition of hypocotyl growth via changes in cytosolic Ca^{2+} (Chen et al. 2008).

So far, no endogenous substrate for phototropin kinase activity has been identified other than the receptors themselves. Nonetheless, a number of phototropin-interacting proteins have been isolated. Nonphototropic hypocotyl 3 (NPH3) is a novel PM-associated protein that directly interacts with phot1 (Motchoulski and Liscum 1999). NPH3 is thought to serve as a protein scaffold to assemble components of a phototropin receptor complex. Arabidopsis mutants lacking NPH3 fail to show phototropism, demonstrating that NPH3 is essential for this response (Motchoulski and Liscum 1999). In addition, NPH3 is required for optimal leaf positioning and leaf flattening in Arabidopsis (Inoue et al. 2008a). Interestingly, photoactivation of phot1 leads to dephosphorylation of NPH3, a signaling process that has been linked to the onset of phototropic curvature (Pedmale and Liscum 2007). However, mutation of potential NPH3 phosphorylation sites does affect its phototropic function (Tsuchida-Mayama et al. 2008), raising further questions as to the biological significance of NPH3 dephosphorylation.

Phototropism ultimately results from an increase in growth on the shaded side of the stem owing to an accumulation of the plant growth hormone auxin (Iino 2006). As light passes through the stem, it becomes progressively diffracted thereby generating a gradient of phototropin activation across the organ, the highest level of activity occurring on the irradiated side. Formation of this biochemical gradient underlies the directionality of phototropic response (Salomon et al. 1997). Presently, little information is available regarding how a differential stimulation of phototropin activity across the stem results in an accumulation of auxin on the shaded side of the stem, but this process is likely due to effects on the localization and activity of specific PM proteins required for auxin transport (Friml et al. 2002; Blakeslee et al. 2004; Noh et al. 2003; Nagashima et al. 2008a).

2 Additional Plant Blue Light Receptors

A second LOV-containing photoreceptor family has recently been characterized in higher plants. Arabidopsis contains three single LOV-containing proteins known as the Zeitlupe (ZTL) family that play important roles in regulating targeted degradation of components associated with circadian clock function and flowering in a light-dependent manner (Kim et al. 2007; Sawa et al. 2007). In addition, cryptochromes (CRY1 and CRY2), a separate family of flavin-based photoreceptors that share homology with photolyase DNA repair enzymes (Cashmore et al. 1999), are largely responsible for plant photomorphogenesis under blue light. Cryptochromes control various aspects of development including cotyledon expansion, hypocotyl growth inhibition, transcriptional regulation and the synthesis of pigments such as chlorophyll and anthocyanin (Li and Yang 2006). Together with the phytochromes, cryptochromes also act to reset the circadian clock and to control the onset of flowering (Yanovsky and Kay 2002).

Like the phototropins, activation of ZTL and cryptochrome photoreceptors is initiated through photoexcitation of their flavin chromophores (Imaizumi et al. 2003; Banerjee et al. 2007). While members of both these photoreceptor families localize to the cytosol and/or nucleus (Cashmore et al. 1999; Kiyosue and Wada 2000; Yasuhara et al. 2004; Fukamatsu et al. 2005), more recent evidence indicates that, at least for CRY1, photoactivation can influence events at the PM. A modified version of CRY1 that is constitutively localized to the nucleus in Arabidopsis can activate anion channel activity at the PM (Wu and Spalding 2007), which plays a role in mediating the slow phase of hypocotyl growth inhibition under blue light (Folta and Spalding 2001). Moreover, anion channel activation by CRY1 occurs within seconds (Folta and Spalding 2001), implying that the signal communication process involved is too rapid to include changes in gene expression. Light-driven redox changes or ion fluxes could conceivably connect these nuclear and PM activities (Wu and Spalding 2007).

3 Phytochrome Red/Far-Red Light Receptors

Phytochromes, like the cryptochromes control many aspects of plant development and growth. Arabidopsis contains five phytochromes (PHYA–E) that mainly absorb red and far-red (FR) wavelengths of light via a covalently attached linear tetrapyrole chromophore that is synthesized from heme (Chen et al. 2004). Phytochromes interconvert between red and FR absorbing forms referred to as Pr and Pfr, where the Pfr form is considered to be the active form because many physiological responses are promoted by red light (Chen et al. 2004). Phytochromes are able to sense the ratio of red and far-red light and use this information to monitor spectral qualities such as sunset and sunrise or as an indicator of shading.

Although the majority of phytochrome action is thought to stem from its light-driven nuclear import from the cytosol (Fankhauser and Chen 2008), there are

reported examples whereby phytochrome can function from the cytosol. For instance, the effects of polarized red light imply that phytochrome in the moss *Ceratodon purpureus* acts at the cell periphery (Hartmann et al. 1983, Esch et al. 1999), whilst being localized predominantly in the cytosol (Lamparter et al. 1995). In Arabidopsis, nuclear accumulation of PHYA depends on two regulatory components known as FR-elongated hypocotyl (FHY1) and FHL (FHY1-like) (Hiltbrunner et al. 2006). Mutants lacking both FHY1 and FHL retain the PHYA-mediated enhancement of blue light-induced phototropism (Rösler et al. 2007) that is typically observed in dark-grown Arabidopsis seedlings preirradiated with red light (Whippo and Hangarter 2003). Photoactivation of PHYA therefore modulates phototropin receptor signaling initiated at the PM. Signal integration between these photosensory pathways appears to involve the PM associated protein Phytochrome Kinase Substrate 1 (PKS1), which plays a role in the establishment of phototropic curvatures and is known to interact with both PHYA and PHOT1 (Fankhauser et al. 1999; Lariguet et al. 2006). Cryptochromes also function to modulate phototropic curvatures in Arabidopsis (Whippo and Hangarter 2003; Kang et al. 2008). Recent studies indicate that the effects of phytochrome and cryptochrome on differential hypocotyl growth are mediated, at least in part, through an alteration in the protein levels of ATP-binding Cassette B19 (ABCB19) (Nagashima et al. 2008b), an integral membrane-bound protein associated with auxin transport (Titapiwatanakun and Murphy 2008).

In some plants species, including the fern *Adiantum capillus-veneris*, phototropism and chloroplast movement is induced by red light as well as blue. *Adiantum* contains a novel dual red/blue light-sensing photoreceptor known as neochrome, comprising a red light-absorbing phytochrome photosensory domain fused to the N-terminus of an entire phototropin receptor (Nozue et al. 1998). The presence of such a hybrid photoreceptor is proposed to enhance light sensitivity and aid the prevalence of species such as ferns in low light conditions typically found under the canopy of dense forests. Intriguingly, when expressed in Arabidopsis, *Adiantum* neochrome elicits both red and blue light-induced phototropism in the absence of the phototropins (Kanegae et al. 2006), implying that these photoreceptors share a common mode of action.

4 UV-B and Green Light

Irradiation with UV-B has profound effects on plant growth and development. Fortunately, the earth is surrounded by a stratospheric ozone layer that completely filters out UV-C and absorbs a great proportion of the UV-B irradiation. Exposure to high levels of UV-B is associated with melanomas, eye damage and immunosuppression in animals, whereas inhibition of phototaxis has been observed in ciliates and impaired growth, DNA damage, and photooxidation of membrane lipids and photosynthetic pigments has been observed in plants. Low levels of UV-B are known to trigger various plant adaptive responses that offer protection

against the potential harmful effects of UV-B and promote survival under stress conditions (Jenkins and Brown 2007). In this regard, UV-resistance 8 (UVR8) appears to play a key role in mediating the effects of UV-B in Arabidopsis (Brown et al. 2005), where its mode of action originates from the nucleus (Kaiserli and Jenkins 2007). Whether UVR8 itself, or some other protein functions as a UV-B photoreceptor is yet to be resolved. In animal cells, UV-B irradiation is known to influence the activity of growth factor receptor tyrosine kinases (RTKs) at the PM (Devary et al. 1992) and the use of RTK inhibitors has been shown to block UV-responses (Sachsenmaier et al. 1994). By analogy, recent evidence suggests that membrane-bound receptor kinases for brassinosteroid phytohormones are involved in UV-B signaling in plants. Specifically, mutants defective in brassinosteroid sensing and synthesis show impaired induction of some UV-B-regulated genes (Sävenstrand et al. 2004). Further studies are now required to clarify the role of brassinosteroids in UV-B signaling.

In addition to UV-B wavelengths, green light also has discrete effects on plant biology and the photosensory mechanisms involved are now being elucidated (Folta and Maruhnich 2007). Phytochromes and cryptochromes absorb green light in addition to red and blue wavelengths, but their efficiency in processing this spectral quality is poor by comparison. In the case of cryptochromes, green light has been shown to negate their photoactivation by blue light owing to the flavin photochemistry inherent to these photoreceptors (Banerjee et al. 2007). Furthermore, green light-induced photoresponses have been documented in the absence of these receptors (Folta and Maruhnich 2007). To date, the molecular identity of a potential green light receptor in higher plants remains elusive. Potential candidates for green light-absorbing chromophores have been suggested including derivatives of flavin (Folta and Maruhnich 2007) and zeaxanthin, a carotenoid that has been implicated in the blue-green photoreversibility observed for stomatal opening (Frechilla et al. 2000). Cyanobacteriochromes are a newly recognized group of photoreceptors that are distinct relatives of phytochrome, but are only found in cyanobacteria. Notably, CcaS from *Synechocystis* undergoes reversible photoconversion between green and red light-absorbing forms that contrasts with the red/far-red photoreversibilty of phytochromes (Hirose et al. 2008). Thus, CcaS and related proteins (Narikawa et al. 2008) represent recognized green light receptors that are members of the expanded phytochrome superfamily.

5 Conclusions

Phototropins represent the predominant photosensory system at the PM in higher plants given the nature of their subcellular localization. Yet, as discussed, other photodetection systems localized to the nucleus or cytosol are also capable of instigating biochemical events at the plant PM. Whether the list of plant photoreceptors is now complete awaits further investigation. However, evidence is emerging to suggest that other photoreceptor candidates may exist. For instance,

G-protein coupled receptor 1 (GCR1), an integral membrane protein that bares homology to photoreceptors involved in animal vision, has recently been shown to be required for the blue light-induction of gene expression in dark-grown Arabidopsis seedlings (Warpeha et al. 2007). The physiological role of CRY3 in Arabidopsis is presently not known, despite being well characterized at the structural and photochemical level (Huang et al. 2006). In contrast to CRY1 and CRY2, CRY3 exhibits DNA photolyase activity (Selby and Sancar 2006) and is known to localize to both chloroplasts and mitochondria (Kleine et al. 2003). A protein with homology to type-1 aquaporins has been identified in the membranes of *Cucurbita pepo* hypocotyls whose flavin binding capacity is influenced by blue light (Lorenz et al. 2003). Arabidopsis also contains a unique LOV-containing protein that is unrelated to the phototropins or the ZTL family, yet the function of this protein remains unknown (Christie 2007), as is the molecular identity of a potential UV-B photoreceptor. Further investigation of these photosensory systems will undoubtedly yield exciting advances in the years to come.

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The Hull of Fame: Lipid Signaling in the Plasma Membrane

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Abstract As the outermost barrier to the apoplast, the plasma membrane is critical for sensing and propagating signals that arise at the cell surface. Plasma membrane lipids are important mediators of signaling and the phosphoinositides (PIs), and sphingolipids are the major classes of lipids implicated in plant signaling. In this chapter, we will summarize the major findings on plant PI signaling and on the emerging signaling role of sphingolipids.

1 The Role of the Plasma Membrane in Cell Signaling

As the outermost membrane of the cell, the plasma membrane plays a critical role as a barrier to the external environment and in sensing both physical and chemical changes that occur at the surface of the cell. The plasma membrane is comprised of a lipid bilayer and associated integral and peripheral proteins. The distribution of lipids and proteins in the membrane is dynamic and nonuniform, giving rise to discrete regions or microdomains, which may serve signaling or other functions. The continuity of the plasma membrane with cytoplasmic organelles, such as the ER, the vacuole, and its close association with the cytoskeleton further defines membrane heterogeneity and provides additional interfaces for cellular signaling. It is hard therefore to view the functions of the plasma membrane independently from endomembranes.

The plasma membrane lipids in plants that have a signaling function fall into two classes, the phosphoinositides (PIs) and the sphingolipids. Between these two classes, we have a more comprehensive picture of the PI signaling pathway.

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1.1 The Phosphoinositide Pathway

The PI pathway is well conserved among eukaryotic cells. Although the PIs are a minor component of the cellular phospholipids, they play a major role in the cellular regulation of cytoskeletal dynamics, membrane trafficking, and signaling (Janmey and Lindberg 2004; Balla 2006; Di Paolo and De Camilli 2006; Krauss and Haucke 2007). Long considered the precursors of the second messengers for signaling, it is becoming evident that the phospholipids, themselves, are important for signaling (Raucher et al. 2000; Meijer and Munnik 2003; Heilmann 2009). The functional diversity of the PIs comes in part from the multiple phosphorylated isomers arising from the specific phosphorylation of the six hydroxyls on the inositol ring. Furthermore, the ability of the PIs and their derived soluble inositol phosphates to permeate both hydrophobic and hydrophilic environments provides a means of both sensing and propagating a signal (Stevenson et al. 2000). PIs are derived from phosphatidylinositol (PtdIns) by phosphorylation of the inositol ring of the lipid-headgroup (Stevenson et al. 2000; Meijer and Munnik 2003). Hydroxyl-functions at positions D3, D4, and D5 and the ensuing combinations give rise to a whole family of structurally related lipids, which include PtdIns-monophosphates (PtdIns3P, PtdIns4P, and PtdIns5P), PtdIns-bisphosphates (PtdIns(3,4)P_2, PtdIns(3,5)P_2, and PtdIns(4,5)P_2), and PtdIns-trisphosphate (PtdIns(3,4,5)P_3), which has not been detected in plant cells to date (Boss et al. 2006).

In the canonical PI signaling pathway of the plasma membrane, PtdIns is sequentially phosphorylated, first to PtdIns4P and then PtdIns(4,5)P_2 by specific lipid kinase enzymes. In response to stimulation, phospholipase C (PLC) is activated to hydrolyze PtdIns(4,5)P_2 giving rise to the second messengers diacylglycerol and the soluble InsP_3. While many of the players in this pathway are conserved in plants, there are important differences between animal and plant lipid signaling (Boss et al. 2008; Munnik and Testerink 2009). One of the earliest differences detected was the low abundance of PtdInsP_2 compared with PtdInsP in plants, in the order of 1:10 or 1:20 in plant cells compared with 1:1 or 1:2 in animal cells (Boss et al. 2006). Additionally, plants lack the DAG-activated protein kinase C, an important part of the signaling cascade in mammalian cells. In contrast, DAG is rapidly converted to phosphatidic acid (PtdOH) via DAG kinase in plants, and PtdOH is emerging as an important signaling molecule in its own right (Testerink and Munnik 2005; Wang et al. 2006). There are also differences in the composition and numbers of gene families encoding the critical enzymes that synthesize and hydrolyze the PI lipids (i.e., PI 4-kinase, PIP 5-kinase, and PLC) in plants compared to the mammalian counterparts (Mueller-Roeber and Pical 2002). Another important feature is that, at present, a gene encoding a canonical InsP_3 receptor has not been identified in plants. However, both InsP_3 and InsP_6 have been shown to trigger the release of Ca^{2+} from intracellular stores (Krinke et al. 2007). Figure 1 depicts a model of PI-mediated signaling with the key players implicated in plant signaling shown in bold.

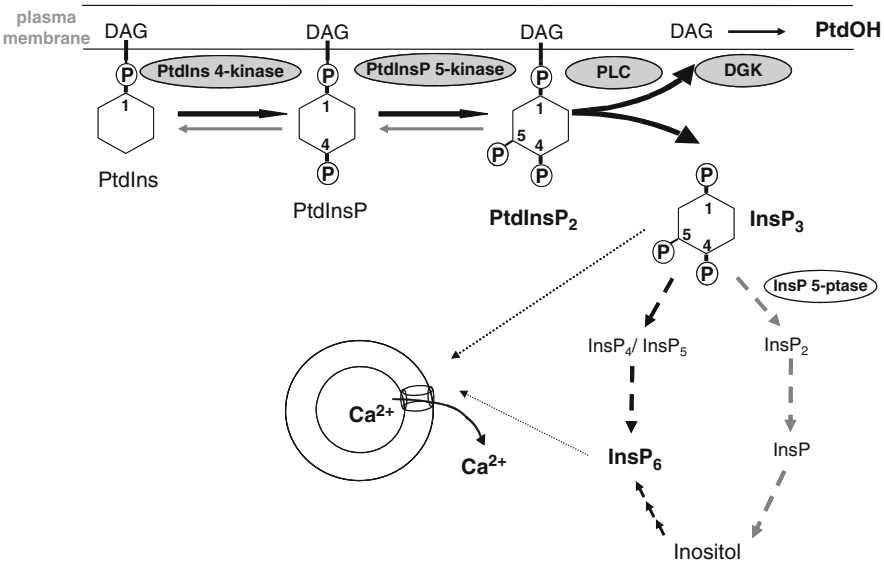

Fig. 1 Model of the phosphoinositide pathway in plants. *Solid arrows* denote major steps in the PI pathway (kinases, *black* and phosphatases, *gray*). *Dashed arrows* indicate routes of $InsP_3$ turnover (kinases, *black* and phosphatases, *gray*). *Ovals* denote enzymes in the pathway and key intermediates that have been implicated in signaling in plants are in *bold*. Abbreviations are as follows: *PtdIns* phosphatidylinositol, *PtdInsP* phosphatidylinositol 4-phosphate, *PtdInsP*$_2$ phosphatidylinositol(4,5) bisphosphate, *DAG* diacylglycerol, *PtdOH* phosphatidic acid, *InsP*$_3$ inositol (1,4,5) trisphosphate, *InsP*$_6$ inositol hexakisphosphate

2 PIs and the Plasma Membrane

The detection of the plasma membrane-associated PIs in plants has not been easy. In vivo labeling with ^{32}P Pi or [^{3}H] inositol (Cho and Boss 1995) has been used extensively in different plant systems to monitor temporal changes in lipids. For detecting spatial distribution, immunolocalization of fixed tissue has had some success. Braun et al. (1999) showed that PtdInsP_2 epitopes were localized to the apical plasma membrane microdomains of growing pollen tube and root hair tips. Visualizing PIs in living cells has been greatly advanced by the use of fluorescence-tagged specific lipid binding domains(Várnai and Balla 1998). This technique has been adapted to plants (Vermeer et al. 2006, 2009; van Leeuwen et al. 2007) and has greatly aided the understanding of PI-distribution in plant cells (Heilmann 2009). Using fluorescence-tagged Pleckstrin homology (PH)-domains of the human FAPP1 (family A PtdIns4P adaptor protein) PtdIns4P was visualized in plant plasma membranes (Thole et al. 2008). Additionally, the plasma membrane association of PtdIns(4,5)P_2 in plant cells was demonstrated using the PH domain of the human PLCδ1 in growing pollen tubes (Kost et al. 1999; Dowd et al. 2006;

Helling et al. 2006; Ischebeck et al. 2008), root hairs (Vincent et al. 2005), and guard cells (Lee et al. 2007).

2.1 Interaction of Soluble PI-Pathway Enzymes with the Plasma Membrane

Several of the reactions contributing to the biosynthesis or degradation of PIs are located at the plasma membrane, and plasma membrane-enriched fractions from plant cells display the highest biochemical activity of the major enzymes in the PI pathway, including PtdIns 4-kinases (PI4Ks), PtdIns4P 5-kinases (PIP5Ks), and PLCs. It is therefore surprising that these enzymes are actually not integral membrane proteins but rather soluble proteins that peripherally associate with cellular membranes. Molecular characterization of the *Arabidopsis* PI4K, PIP5K, and PLC isoforms and their domain structure have revealed that while the plant enzymes share significant homology with their mammalian counterparts, particularly with respect to the catalytic region, many of the plant enzymes contain additional unique domains that may play roles in membrane localization (Mueller-Roeber and Pical 2002). The plant PI4Kα enzyme contains a PH domain, which binds PtdIns4P, the product of the enzyme reaction, and may be involved in effecting localization (Stevenson-Paulik et al. 2003; Boss et al. 2006). The second class of plant PtdIns 4-kinases, PI4Kβ, lacks the PH domain but instead contains a *p*lant-specific *P*I4K *c*harged (PPC) domain, which binds different PIs and is involved in membrane association (Lou et al. 2006). In addition, the major subfamily of plant PIP5Ks contain multiple copies of a *m*embrane *o*ccupation and *r*ecognition *n*exus (MORN) domain, which is a unique feature conserved among plant enzymes and not seen in the mammalian PtdIns4P 5-kinases. MORN motifs are found in animal and plant proteins, which are involved in mediating protein-to-membrane contacts, such as junctophilins (Takeshima et al. 2000), the MORN1 protein from *Toxoplasma gondii* (Gubbels et al. 2006), and the accumulation and replication of chloroplasts 3 (ARC3) protein from *Arabidopsis* (Shimada et al. 2004; Maple et al. 2007). The series of hydrophobic and basic amino acids in the MORN motifs are thought to bind to the membrane by a combination of hydrophobic and electrostatic interactions in a manner similar to the N terminus of *m*yristoylated *a*lanine *r*ich *C-k*inase *s*ubstrate MARCKS (McLaughlin and Murray 2005). Recent studies have revealed that the MORN domain of *Arabidopsis* PIP5K1 binds to both $PtdInsP_2$ (the product of its reaction) and PtdOH (Im et al. 2007a). This interaction with membrane-associated lipids is a potential means of regulating enzyme activity as well as localization (Boss et al. 2008).

Additional insight on how enzymes of the PI pathway might associate with membranes has come from the analysis of the 3D structure of a related mammalian protein. For instance, the membrane-facing side of the mammalian PIP5K II β is rich in basic residues, allowing electrostatic interaction with anionic phospholipids (Rao et al. 1998; Burden et al. 1999) and is devoid of residues, which could sterically obstruct the interaction with a planar membrane surface (Rao et al. 1998).

Structural modeling of the catalytic portions of *Arabidopsis* PI4P 5-kinases based on the known crystal structure of the human PIP-kinase II beta (Rao et al. 1998) suggests that the plant enzymes share the structural adaptations proposed to facilitate protein-membrane interactions (Ingo Heilmann, unpublished data).

The biophysical properties of a membrane are defined by its lipid composition, which is affected by both the presence of certain lipid classes as well as the nature of the associated fatty acids (Mukherjee et al. 1999; Mukherjee and Maxfield 2000; Ahyayauch et al. 2006), which represent the actual hydrophobic core of the lipid bilayer. Several of the enzymes of PI-metabolism, including invertebrate PIP5Ks and PLC (Carricaburu and Fournier 2001) and mammalian PI-phosphatases (Schmid et al. 2004), exhibit specificities not only for certain lipid head-groups but can also discriminate between their substrates with regard to the associated fatty acids.

Recent evidence from the plant field indicates that there are differences in the fatty acid composition associated with stress-induced PIs compared with PIs that are constitutively present within cells (König et al. 2007; Heilmann 2008), and that PIs with distinct fatty acid profiles may serve distinct functions within plant cells (König et al. 2007; Heilmann 2008). In support of this concept, it has been reported that the two PtdIns synthases of Arabidopsis have distinct preferences for cytidinediphosphodiacylglycerol substrates with polyunsaturated or more saturated substrates (Löfke et al. 2008), thus possibly giving rise to pools of PtdIns that are differentiated on the basis of their associated fatty acids. Other studies suggest that catalytic function of the PI-specific PLC from *Bacillus cereus* is critically dependent on membrane curvature (Ahyayauch et al. 2005), a factor defined by the lipid composition of the membrane, especially regarding nonsubstrate lipids (Ahyayauch et al. 2005, 2006). These observations suggest that PI-biosynthesis and breakdown may be regulated in time and space by the characteristics of local membrane lipid composition, and that such regulation results in spatially restricted local microdomains of PI-formation (Cho et al. 2006). The PIs are, therefore, not evenly distributed across a membrane but rather appear in hot-spots that may be generated in a dynamic fashion according to cellular requirements. It has recently been shown that PtdIns(4,5)P_2 in fact forms microdomains in the plasma membrane of plant cells (Furt et al. 2010).

PIs are involved in many processes in plant cells via the generation of second messengers, the regulation of membrane trafficking (Thole and Nielsen 2008), and cytoskeletal dynamics (Drøbak et al. 2004; Staiger and Blanchoin 2006). The following paragraphs summarize how PIs regulate some key aspects of plant function including the development and the regulation of cell polarity as well as the adaptation to stress conditions.

3 PIs and the Regulation of Tip Growth

Polarized or tip growth occurs in rapidly elongating plant cells such as root hairs and pollen tubes and requires a tip-directed transport of secretory vesicles. This process is highly regulated and involves a tip-focused Ca^{2+} gradient, as well as the

complex interplay of Rac-Rop GTPases, the actin cytoskeleton, and PIs associated with endomembranes and the plasma membrane (Kost 2008; Lee and Yang 2008). A fascinating picture of plasma membrane-associated PIs and their role in regulating tip growth in plant cells has emerged from several recent publications.

The localization of $PtdInsP_2$ to the apical plasma membrane of pollen tubes was described in some early work (Braun et al. 1999; Kost et al. 1999), and a Rac homolog that localizes to the apical plasma membrane was shown to coimmunoprecipitate with a PIP5K activity, suggesting a mechanism for generation of $PtdInsP_2$. More recently, plant PI-PLCs have been implicated in maintaining an apical domain enriched in $PtdInsP_2$ in growing pollen tips. In tobacco pollen tubes, YFP-PLC3 was found to accumulate at the flanks of the pollen tube tip plasma membrane (Helling et al. 2006). Furthermore, the expression of an inactive form of PI-PLC1 in petunia pollen tubes resulted in disorganization of the actin cytoskeleton, disruption of the tip-focused Ca^{2+} gradient, and a loss of polar growth (Dowd et al. 2006). Related work has identified specific PIP5K isoforms that are involved in generating the apical $PtdInsP_2$ in Arabidopsis pollen tubes (Ischebeck et al. 2008; Sousa et al. 2008). Fluorescence-tagged AtPIPK4 and AtPIPK5 enzymes were shown to colocalize with the plasma membrane $PtdInsP_2$ microdomain of the pollen tube tip. T-DNA insertion lines of both these genes had reduced pollen germination and defects in pollen tube growth (Ischebeck et al. 2008; Sousa et al. 2008). Similarly, heterologous overexpression of *PIP5K4* or *PIP5K5* resulted in increased apical pectin deposition, multiple tube branching and other severe morphological changes (Ischebeck et al. 2008). The data strongly suggest that maintaining $PtdInsP_2$ levels at the pollen tube tip is critical for correct membrane trafficking and secretion and that the distribution of the enzymes responsible for both generating and hydrolyzing this lipid is highly regulated.

Both PtdIns4P and $PtdIns(4,5)P_2$ play a role in root hair development. PI-4K β1 (one of the enzymes responsible for generating PtdIns4P) along with its effector protein RabA4b (a Rab GTPase) were shown to be required for proper root hair development (Preuss et al. 2006). RabA4b is involved in polarized membrane trafficking and tip-restricted expansion. Additionally, a root hair defective mutant *rhd4-1*, which has short, bulgy roots and altered RabA4b dynamics, was found to encode a PtdIns4P phosphatase AtSAC7 (Thole et al. 2008). While PtdIns4P is found primarily in a tip-localized plasma membrane domain in wild type root hairs, higher levels of PtdIns4P were associated with internal membranes in the *rhd4-1* mutant (Thole et al. 2008; Vermeer et al. 2009). Only actively growing root hairs exhibit the tip-localized plasma membrane distribution of PtdIns4P (Vermeer et al. 2009), suggesting that regulation of PtIns4P is a key step in polarized growth of root hairs. In addition to PtIns4P, $PtdIns(4,5)P_2$ is also critical for root hair development and elongation. A tip-localized $PtdIns(4,5)P_2$ gradient was observed in wild type plasma membranes but was lost in the lipid transfer protein mutant *AtSfh1p*, which exhibits aberrant root hair morphology (Vincent et al. 2005). A specific *Arabidopsis* PIP5K isoform, PIP5K3, has been shown to localize to the plasma membrane of root hair cells (Kusano et al. 2008; Stenzel et al. 2008); the *pipk5k3* mutant has reduced levels of $PtdIns(4,5)P_2$ and has shorter root hairs. The mutant phenotype

could be complemented by ectopic expression of PIP5K3 in root hairs and PtdIns(4,5)P_2 levels restored to normal (Stenzel et al. 2008). PtdInsP_2-hydrolyzing phosphatases may be involved in regulating PtdInsP_2 levels in the root; the PI phosphatase mutant *sac9* accumulated higher PtdIns(4,5)P_2 levels in root tissue and showed decreased primary and lateral root growth (Williams et al. 2005).

It is clear that generating and maintaining a tip-localized distribution of PtdIns(4,5)P_2 is critical for polarized growth in both pollen tubes and root hairs and that the concerted action of specific lipid kinases, PLC, and lipid phosphatases is essential for this regulation.

4 PI Control of Ion Channels

Another important function of PIs in eukaryotic cells is the control of membrane-integral ion channels and transporters that are localized at the plasma membrane. In mammalian cells, a number of membrane transport proteins are regulated by PIs, (particularly PtdIns(4,5)P_2), including inward rectifying K^+ channels, voltage-gated Ca^{2+} channels, transient receptor potential channels (TRPs), as well as Ca pumps and Na^+/Ca^{2+} exchangers (Gamper and Shapiro 2007; Huang 2007; Nilius et al. 2008). The abundance of the lipids is under dynamic control and changes rapidly in response to exogenous signals, thus providing the means to both rapidly and specifically control plasma membrane localized transporters.

In plants, early work suggested that the plasma membrane H^+-ATPase is activated in vitro by PtdIns(4,5)P_2 (Memon et al. 1989). More recent reports provide evidence of ion channel regulation by PIs in plants. Liu et al. (2005) demonstrated that three types of plant shaker type K^+ channels, the inward rectifying KAT1 and LKT1, as well as the outward rectifying SKOR could be activated to different degrees by the addition of PtdIns-monophosphates or PtdIns(4,5)P_2. PtdIns(4,5)P_2 has also been proposed to play a role in the inactivation of slow anion channels in guard cells during stomatal opening (Lee et al. 2007). These two studies relied on the addition of exogenous phospholipids to excised patches or guard cell protoplasts. Using tobacco protoplasts genetically manipulated to have either high or low plasma membrane levels of PtdIns(4,5)P_2, Ma et al. (2009) showed that K^+ efflux activity was inversely related to plasma membrane PtdIns(4,5)P_2 levels. Decreasing the high PtdInsP_2 levels by pretreatment with ABA or by neutralizing the pH increased the activity of the *N. tabacum* outward rectifying K^+ channel, ORK. PtdIns(4,5)P_2 levels were manipulated by the heterologous expression of either the human type1 PIPKα to overproduce PtdIns(4,5)P_2 (Im et al. 2007a, b) or the human inositol polyphosphate 5-phosphatase. (InsP 5-ptase) to decrease basal InsP_3 and PtdIns(4,5)P_2 (Perera et al. 2002). The 'InsP 5-ptase tobacco cells had higher K^+ efflux, an observation which is consistent with the fact that transgenic *Arabidopsis* plants expressing InsP 5-ptase lose less water and are more drought-tolerant compared to wild type plants (Perera et al. 2008). Additionally, disruption of the

PIP5K isoform AtPIP5K4 led to reduced stomatal opening in the light, and this phenotype could be rescued by exogenous addition of $PtdInsP_2$ (Lee et al. 2007).

5 PIs and Plant Stress Responses

It has been known for many years that the levels of particular PIs and $InsP_3$ change in plant cells with application of various biotic and abiotic stresses (Cho et al. 1993; Crain and Yueh 1995; Dove et al. 1997; Heilmann et al. 1999; Perera et al. 1999; Pical et al. 1999; Drøbak and Watkins 2000; DeWald et al. 2001; Meijer and Munnik 2003), leading to the hypothesis that PIs have roles in signal transduction events during the adaptation to stress conditions. Many reports of rapid and transient changes in PIs have been documented in different plant species in response to hyperosmotic and salt stress (Cho et al. 1993; Heilmann et al. 1999; Pical et al. 1999; Drøbak and Watkins 2000; DeWald et al. 2001), and some of the lipid changes have been shown to be associated with the plasma membrane (Heilmann et al. 1999, 2001; Perera et al. 1999, 2002). A transient increase in $PtdIns(4,5)P_2$ has been considered to be a substrate for PLC, generating $InsP_3$ and DAG and acting as part of the osmotic signaling cascade leading to Ca^{2+} release from internal stores (DeWald et al. 2001). Since there does not appear to be a canonical mammalian type $InsP_3$ receptor in plants, there is some debate whether or not $InsP_3$ by itself represents a bioactive signal or whether downstream metabolites of $InsP_3$ serve signaling functions in plants (Mosblech et al. 2008; Munnik and Testerink 2009). It is also conceivable that the removal or hydrolysis of $PtdIns(4,5)P_2$ itself acts as a signal. Some recent studies on the characterization of fatty acid species associated with the PIs have shed light on this. As already mentioned above, $PtdIns(4,5)P_2$ transiently increasing in *Arabidopsis* leaves upon exposure to hyperosmotic stress was found to contain increased proportions of unsaturated fatty acids compared with $PtdIns(4,5)P_2$ isolated from nonstressed plants. The concomitant production of $InsP_3$ was accompanied by increased production of unsaturated DAG and unsaturated species of its immediate phosphorylation product, PtdOH (König et al. 2007). These data suggest that part of the unsaturated $PtdIns(4,5)P_2$ formed upon hyperosmotic stress may be hydrolyzed by PLC (Heilmann 2008), resulting in $InsP_3$ production and Ca^{2+}-release (DeWald et al. 2001).

More recently, stress-induced $PtdIns(4,5)P_2$ has been visualized at the plasma membrane of plant cells (van Leeuwen et al. 2007; König et al. 2008). The dynamic nature of this response is further underscored by the observation that in response to salt stress, $PtdIns(4,5)P_2$ increases transiently in the plasma membrane, and at later time points, $PtdIns(4,5)P_2$ was found to be associated with clathrin-coated vesicles (CCVs). Furthermore, the vesicle coat protein, clathrin, was shown to rapidly relocate from the cytosol to the plasma membrane upon salt stress (König et al. 2008), possibly as an endocytotic mechanism. Additionally, in tobacco cells, PIP5K, which was originally associated with the plasma membrane relocated to endomembranes upon osmotic stress (Im et al. 2007a, b). Clearly, the combined observations suggest that PIs are required at the plasma membrane of cells

experiencing stress conditions. However, apart from the potential $InsP_3$-mediated Ca^{2+} release and ensuing signal transduction, not much is known of the downstream consequences of changes in PIs during adaptation to stress. Identification of interacting partners of the PIs will be an exciting area of research in the future that might further elucidate the physiological roles of PIs in plant stress adaptation.

PtdOH is another important signaling molecule in plant stress responses (Testerink and Munnik 2005; Wang 2005; Wang et al. 2006). PtdOH may be generated via the PLC-mediated pathway as outlined in Fig. 1. In addition, PtdOH is generated from the structural phospholipids, phosphatidlycholine (PtdCho), phosphatidylethanolamine (PtdEtn), and phosphatidylglycerol (PtdGro) by phospholipase D (PLD) (Wang 2005; Wang et al. 2006). In contrast to the single family of plant PI-specific PLCs, plants contain six subfamilies of PLDs, each containing multiple isoforms (Wang et al. 2006). Many different abiotic and biotic stresses have been shown to trigger an increase in PtdOH in plants (Testerink and Munnik 2005; Wang et al. 2006). In some instances, PtdOH is further converted to DGPP, which may attenuate the PtdOH signal or serve a separate signaling function (van Schooten et al. 2006). In many stress responses, the generation of PtdOH occurs via both PLC and PLD pathways; the initial increase of PtdOH in response to fungal elicitation was shown to be through the PLC pathway (de Jong et al. 2004). Furthermore, with cold treatment, PtdOH levels increased within 10 min and ~80% of the PtdOH produced was via the PLC-mediated pathway (Ruelland et al. 2002). PtdOH and PLDs are involved in regulating guard cell responses (Wang et al. 2006), chilling stress and cold tolerance (Testerink and Munnik 2005; Wang et al. 2006), and plant defense responses (Bargmann and Munnik 2006).

As well as exhibiting rapid increases in response to stress, PtdOH may be involved in membrane tethering and regulation of enzymes. PtdOH target proteins such as AtPDK1 (Anthony et al. 2004), a CDPK from carrot (Farmer and Choi 1999), and AtPIP5K1 (Perera et al. 2005; Im et al. 2007a, b) are activated by the interaction. Interestingly, binding to PtdOH inactivates ABI1, a negative regulator of ABA signaling (Zhang et al. 2004), as well as CTR1, a negative regulator of ethylene responses (Testerink et al. 2007, 2008), thereby implicating PtdOH in activating stress hormone signaling. PtdOH may also influence actin dynamics by regulating the actin-binding protein AtCP (Huang et al. 2006). The variety of PI metabolites and the signaling molecules derived from them suggests that their interconversion is subject to tight control. Access of signaling lipids to target proteins or to enzymes mediating their conversion or breakdown might be limited by restricted lateral mobility within the membrane (Zappel and Panstruga 2008).

6 Sphingolipids in Plant Signaling

Lipid rafts are plasma membrane microdomains, which have been identified in both plant and animal cells. Lipid rafts are enriched in sterols and sphingolipids, and proteins associated with lipid rafts are involved in cellular signaling and trafficking.

In plants, the lipid composition and proteome of these microdomains have been described in several recent papers (Grennan 2007; Zappel and Panstruga 2008) and chapter "Lipids of the Plant Plasma Membrane" by Furt. While the concept of "lipid rafts" enriched in sphingolipids and sterols is intriguing, current methods to enrich and biochemically analyze "lipid rafts" rely on the insolubility of the membrane domains in certain detergents (the so-called "detergent-insoluble membranes," DIMs). This criterion is largely artificial, and it must be noted that DIMs prepared might not represent any single relevant biological structure. From proteomic analysis, it is obvious that DIMs do in fact hold a particular set of associated proteins, the functions of which to date remain largely unknown (Morel et al. 2006). Also, microdomains might not all be detergent-insoluble, raising the question whether a comprehensive study of membrane microdomains is currently feasible. Nonetheless, it has become clear in recent years that sphingolipids and sterols represent important signaling factors with profound effects on the physiology of plants.

Reports on the roles of sterols in plant microdomain formation are still scarce. However, a number of reports indicate that sterol-containing plasma membrane microdomains are involved in the mediation of polar growth or cell polarity. For instance, sterols have been implicated in the polar distribution of pin-formed (PIN) auxin efflux carriers in roots (Men et al. 2008), which are centrally involved in the control of plant growth. In petunia pollen tubes, an involvement of oxysterol-binding proteins in polar growth signaling has been proposed (Skirpan et al. 2006). Importantly, it was demonstrated that fluorescence-tagged oxysterol-binding proteins decorated a spotted pattern in the apical plasma membrane of petunia pollen tubes (Skirpan et al. 2006), suggesting the presence of sterol-containing lipid microdomains. Recent findings of enzymes associated with cell wall biosynthesis in DIM preparations from poplar (Bessueille et al. 2009) may suggest that part of the secretory machinery of plant cells is associated with "lipid raft"-like microdomains.

Sphingolipids are synthesized in the ER from serine and palmitoyl-CoA and are involved in maintaining the structural integrity of membranes in all eukaryote cells (Lynch and Dunn 2004; Boss et al. 2008). While membrane-predominant glycolipids have two fatty-acyl chains, the unique feature of sphingolipids is the presence of sphingoid long-chain base (LCB shinganin) that is linked to fatty-acyl chain (ceramide). Ceramide can be incorporated into complex sphingolipids, phosphosphingolipids such as sphingomyelin, which has phosphocholin as a head group or glycosphingolipids such as glucosylceramide (GlcCer), and glycosylated inositol-phosphoceramide (GIPC), which have glucose or inositol phosphate as a head group (Vesper et al. 1999; Markham and Jaworski 2007). In plants, GlcCer is the predominant sphingolipid (~5–30 mol% of total lipids) in the plasma membrane (Warnecke and Heinz 2003; Lynch and Dunn 2004). The presence of GlcCer in the plasma membrane has been implicated in regulating membrane permeability in relation to many stresses including drought, toxic metals, cold, and freezing tolerance (Lynch and Dunn 2004).

Sphingolipids and their metabolites including sphingoid long-chain bases (LCB), phosphorylated LCBs, and ceramides (Cers) have signaling functions in

membrane-associated cellular processes in animals, including cell cycle arrest, apoptosis, senescence, and stress responses (Zhang and Kiechle 2004; Schenck et al. 2007; Hannun and Obeid 2008). Fungal toxins have been shown to inhibit sphinganine acyltransferase (SAT), resulting in increased LCBs and programmed cell death (PCD) (Abbas et al. 1994; Wright et al. 2003). Furthermore, Cer has been implicated in PCD in plants, similar to animals (Liang et al. 2003; Townley et al. 2005; Chen et al. 2008). The tomato Asc1 gene is involved in Cer synthesis and when overexpressed in a sensitive tomato cultivar conferred enhanced toxin resistance and prevented the accumulation of LCBs (Brandwagt et al. 2000, 2002; Spassieva et al. 2002). In addition, both *Arabidopsis ACD11* and *ACD5* mutants, which lack ceramide kinase activity, displayed enhanced PCD (Brodersen et al. 2002; Liang et al. 2003). These results suggest that proper control of LCB and Cer levels in plants may be important for defense and PCD responses, although currently the mechanism of this regulation is unknown.

One of the most extensively studied sphingolipids is sphingosine-1-phosphate (S1P), which has been implicated as a signaling molecule in mammalian and plant cells (Spiegel and Milstein 2002; Lynch and Dunn 2004). In mammals, S1P interacts with a family of specific cell surface G-protein-coupled receptors (GPCRs) and acts as an intracellular second messenger to regulate Ca^{2+} homeostasis, cell growth, and survival (Spiegel and Milstein 2003). In plants, a role for S1P has been proposed in guard cell signaling (Ng et al. 2001; Ng and Hetherington 2001; Coursol et al. 2003; Pandey and Assmann 2004; Coursol et al. 2005). Exogenous addition of S1P to guard cells generated cytosolic Ca^{2+} transients and induced stomatal closure (Ng et al. 2001). ABA-induced stomatal closure was attenuated by adding an inhibitor of sphingosine kinase, SphK (Ng et al. 2001). Furthermore, unlike the response of WT protoplasts, ion channels in the protoplasts of *gpa1*knockout plants lacking the heterotrimeric G-protein a-subunit did not respond to the exogenous addition of S1P, suggesting that G-proteins are downstream of S1P in ABA signaling (Coursol et al. 2003). In addition to S1P, phytosphingosine-1-phosphate also regulates stomatal closure (Coursol et al. 2005).

S1P is synthesized by SphK, which phosphorylates S1P from sphingosine, and cellular levels are tightly regulated. S1P can be dephosphorylated by specific S1P phosphohydrolase or converted to phosphoethanolamine and hexadecenal by S1P lyase, which are further metabolized into glycerophospholipids and PtdEtn, respectively (Le Stunff et al. 2004). However, genes involved in S1P regulation have not been well studied. To understand S1P functions in plants, Tsegaye et al. (2007) characterized an S1P lyase mutant that exhibits accumulation of S1P. There was no effect on plant growth and development; however, mutant plants were sensitive to the sphingolipid inhibitor, fumonisin B1. Studies have shown that SphK activity can be increased by ABA in the guard cell (Coursol et al. 2003) and that this activity resides predominantly in intracellular membranes (Coursol et al. 2005). Because of the hydrophobic nature of lipid secondary messengers such as S1P, their localization and redistribution upon signals in the cell could have impact on their downstream effectors (Wattenberg et al. 2006). In this regard, it is interesting to know where and how genes involved in S1P regulation would be localized. Several

studies in animal systems have shown that SphK activity is regulated by translocation to the plasma membrane that is accomplished by binding acidic phospholipids such as phosphatidyl serine (PtdSer), PtdIns, and PtdOH and that results in enhanced SphK activity in vitro (Liu et al. 2000; Pitson et al. 2000; Delon et al. 2004). Furthermore, the membrane association of SphK may be regulated by PKC (Johnson et al. 2002), the extracellular signal-regulated kinase (ERK) (Pitson et al. 2003), or platelet-derived growth factor (Pitson et al. 2005) via phosphorylation. These studies suggest that relocalization of SphK1 to plasma membrane could be an important determinant of its signaling function for distinct signaling effect.

In contrast to mammalian SphK, most of SphK activity in plants is associated with membrane fraction, and two SphK1 and SphK2 are found in mitochondria and tonoplast, respectively (Marion et al. 2008; Worrall et al. 2008). The involvement of SphK in guard cell ABA signaling and in the control of germination has been recently investigated using genetic approaches to increase S1P levels in plant cells by overexpression of SphK1 and RNAi of S1P phosphohydrolase and S1P lyase (Worrall et al. 2008). However, it is unclear whether the plant enzymes relocalize to the plasma membrane and whether lipid binding or posttranslational modification plays a role in regulating the plant enzymes. The plant SphK1 phosphorylates not only sphingosine but also long-chain sphingoid bases including phytosphingosine (Worrall et al. 2008). Sphingosine and S1P are very minor components of sphingolipids in higher plant (Dunn et al. 2004; Imai and Nishiura 2005). To define the role of S1P, Michaelson et al. (2009) characterized a sphingolipid D4-desaturase mutant (which lacked sphingosine and S1P) and found no effect on the drought stress response of the mutant (Michaelson et al. 2009). The authors suggest that this particular LCB1-P either does not play a dominant role in the drought stress or is functionally redundant. Other LCBs and LCB phosphates (LCBPs), such as delta8-desaturated LCBs (Ryan et al. 2007) and phytosphingosine (Xiong et al. 2008), are emerging as important modulators of plant processes. Increases in relative amounts of *cis* C-8 unsaturated LCBs have been shown to enhance aluminum tolerance in *Arabidopsis thaliana* (Ryan et al. 2007). Xiong et al. (2008) have challenged either isolated nuclei or intact tobacco cell suspension cultures with different LCB derivatives and discovered that dihydroxy-LCBS elicited $\Delta[Ca^{2+}]_{nuc}$ in both systems. The presence of nuclear LCBs is intriguing and suggests the possibility of interaction with calcium-based signaling pathways in the nucleus.

7 Conclusions

As outlined in this chapter, the plasma membrane-associated lipids are clearly involved in the regulation of numerous physiological processes in plants. Recent advances in technologies for isolating and quantifying the plant lipidome (Welti and Wang 2004) and the sphingolipidome (Markham et al. 2006; Markham and Jaworski 2007), visualizing the lipids in real time as well as the judicious use of mutants have opened up the field of lipid signaling. The potential crosstalk between

the PI and sphingolipid pathways and the potential of nuclear cycles for these lipids are exciting new areas of research. As the outermost barrier to the apoplastic environment, the plasma membrane is responsible for delivering multiple signals to the cell. We anticipate that more information on the complex regulation of plasma membrane signaling and the downstream physiological responses in plants will be revealed in the future.

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Plasma Membrane and Abiotic Stress

Bronwyn J. Barkla and Omar Pantoja

Abstract Environmental factors exert influence on nearly every aspect of plant function throughout its life cycle. In response to changing and often unfavorable conditions, stress perception in plants initiates signal transduction events that lead to expression of specific stress-related genes and generation of stress-protecting metabolites. Some of these responses are evidently adaptive and lead to changes that increase the chance of survival under adverse conditions, while others are symptoms of stress injury and are pathological in nature. As stressful abiotic environmental conditions can range from exposure to drought, salinity, cold, freezing, high temperature, anoxia, high light intensity, and nutrient imbalance, a complex and overlapping network of molecular machinery must regulate plant responses to these conditions.

The plasma membrane (PM) of the plant cell acts as an important barrier that separates and shields the cell from its environment. However, the PM is also the site of sensors that interpret environmental conditions and transduce signals to other sites on the membrane, inside the cell, and distal portions of the plant to provide for direct and rapid responses to changing environmental conditions. PM sensors can respond directly to alleviate a stress condition, signal secondary changes at the membrane, or activate signaling cascades that potentiate tertiary changes in stress-regulated gene expression.

1 Plasma Membrane Abiotic Stress Sensing

There is abundant information regarding plant abiotic stress-inducible signaling cascades and gene expression, but much less is known about plant abiotic stress sensors. Abiotic stress sensors in bacteria, cyanobacteria, and the yeast *Saccharomyces*

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cereviseae are primarily made up of receptor kinase two- or multicomponent systems consisting of a sensory histidine kinase linked directly or indirectly via a phosphorelay molecule to a response regulator (Murata and Los 2006; Lohrmann and Harter 2002). In some particular cases, the response regulator may also function directly as a transcription factor, thus eliminating the requirement for intermediate signaling cascades (Lohrmann and Harter 2002).

In *Arabidopsis thaliana*, accumulating evidence suggests that two members of the histidine kinase family are involved in osmosensing: AHK1, a protein that detects changes in water status and desiccation (Wohlbach et al. 2008), and CRE1/AHK4, which responds to hyperosmotic stress-related changes in turgor pressure in the presence of cytokinin. Both putative sensors complement a deletion mutant of the yeast osmosensing histidine kinase SLN1 (Reiser et al. 2003; Urao et al. 1999), and *AHK1* can act as a positive regulator in osmotic stress signaling in Arabidopsis plants (Tran et al. 2007). While the assumption can be made that these sensors are PM-localized, it has not been confirmed experimentally, and neither protein has appeared in PM proteomic analyses. Moreover, AHK5, a related histidine kinase that responds to H_2O_2, has been shown to localize to both the cytoplasm and the PM in Arabidopsis (Desikan et al. 2008), and recently, the histidine kinase subfamily of ETR ethylene receptors were shown to be targeted to the endomembrane network and did not localize to the PM (Grefen et al. 2008). Two other related histidine kinase members have also been implicated in abiotic stress sensing; *AHK2* was shown to be regulated by abscisic acid (ABA), osmotic stress, and salt stress, while *AHK3* was shown to be induced by salt and cold stress (Wohlbach et al. 2008).

Other possible abiotic stress sensors at the plant PM are the receptor-like kinases, an unusually large family of proteins that consist of an extracellular receptor domain, a single transmembrane domain, and an intracellular serine/threonine kinase domain. While expression of the genes encoding these kinases has been shown to be regulated by different abiotic stress signals, there is no evidence of a direct role of these kinases in environmental sensing (Tamura et al. 2003). Rather, the evidence points to a more likely role in hormone signaling and host–pathogen recognition (Morris and Walker 2003).

Sensing of abiotic stress may not be limited to protein kinases. In yeast and mammals, PM proteins have been identified, which not only function as transporters but also act as sensors and signal relay proteins (Özcan et al. 1998; Diez-Sampedro et al. 2003). Interestingly, these proteins have unusually long C-terminal cytoplasmic domains, a trait shared by the Arabidopsis PM Na^+/H^+ exchanger SOS1/NHX7, which has been proposed to be an Na^+ sensor (Zhu 2002). However, as of yet, a sensor function has not been clearly demonstrated for any of these proteins. Evidence that SOS1 is a downstream target of a CBL–CIPK calcium-sensing network (see below) suggests that SOS1 is not likely to function as a primary salt stress sensor.

Some insights into the role of the SOS1 C-terminus have come from a yeast two-hybrid screen which detected that the cytoplasmic tail portion binds RCD1/CEO1 (Katiyar-Agarwal et al. 2006), a salt and oxidative stress-responsive nuclear protein that interacts with transcription factors such as STO and DREB2A (Belles-Boix

et al. 2000). Under stress conditions, RCD1/CEO1 localizes to both the nucleus and cytoplasm, thus suggesting it may relay a signal between the two compartments (Katiyar-Agarwal et al. 2006).

Cold, drought, and salt stress can induce production of the phytohormone/plant growth regulator ABA, which itself may be sensed at the PM. Candidate PM proteins for sensing and relaying the ABA signal are two proteins, *At*GTG1 and *At*GTG2 (Pandey et al. 2009). While these proteins show homology to a human Golgi-localized anion channel (Maeda et al. 2008), they share some properties of G protein-coupled receptors (GPCRs), as both proteins interacted with *At*GPA1, a G-protein α subunit, itself localized to the PM by a lipid anchor (Temple and Jones 2007). As would be expected for loss of ABA sensing, mutants lacking both proteins were shown to be hyposensitive to ABA and impaired in upstream ABA signaling responses. It was reported that *At*GTG1 and *At*GTG2 bind ABA with high affinity (Pandey et al. 2009); however, a reevaluation of ABA-binding assays cautions the interpretation of these studies under conditions of suboptimal protein purification (Risk et al. 2009). The proteins are widely expressed in most tissues, although neither *At*GTG1 nor *At*GTG2 are transcriptionally regulated by abiotic stress (Pandey et al. 2009). ABA perception at the PM in turn regulates a wide variety of plant physiological and developmental processes that have been well-studied, especially as they pertain to guard cell physiology. These include the production of reactive oxygen species (ROS), increases in cytosolic free Ca^{2+} levels ($[Ca^{2+}]_i$), protein kinase cascades, activation and inhibition of PM channels, including anion channels, and K^+ influx and K^+ efflux channels; some of which are PM delimited events (Fan et al. 2004).

Perception of most abiotic stresses results in the generation of calcium signals within the plant cell, which, in turn, elicit distinct concentration-dependent responses that depend on the duration, oscillation, and location of the calcium signal (McAinsh and Pittman 2009). In plants, the calcineurin B-like protein (CBL) family represents a unique group of small calcium-binding proteins that act as calcium sensors. They play a key role in decoding calcium transients by interacting with and regulating members of a family of serine/threonine protein kinases known as CBL-interacting protein kinases (CIPKs) and generating a localized signaling cascade that results in phosphorylation of specific target proteins (Batistic and Kudla 2009; Luan 2009). Unique calcium signatures are obtained by the programmed mixing and matching of CBL proteins with CIPKs at specific locations throughout the cell, which include the PM (Kim et al. 2000). PM localization of particular CBL proteins occurs via lipid modifications, as has been demonstrated for both CBL4 and CBL1 (Ishitani et al. 2000; Batistic et al. 2008). Loss-of-function studies of PM CBLs highlight their role in plant abiotic stress tolerance. Disruption of *CBL4*/*SOS3* in Arabidopsis results in plants that are hypersensitive to salt (Shi et al. 1999), disruption of *CBL1* results in plants that are hypersensitive to drought, high salt, and hyperosmotic stress (Albrecht et al. 2003; Cheong et al. 2003), and disruption of *CBL9* results in plants that are hypersensitive to ABA, salt, and osmotic stress (Pandey et al. 2004). Expression profiling also shows the importance of these sensors in abiotic stress response. In rice, expression analysis of ten *CBL* family members identified only two members, which showed no changes under any stress conditions,

with most showing regulation by multiple stresses, and many containing stress-related *cis*-elements in their promotor (Gu et al. 2008).

2 PM Targets of Abiotic Stress Signals

Many targets of stress signaling mechanisms are localized to the PM. These PM delimited events mediate regulation of membrane-resident channels, transporters, pumps, and other target proteins. Recruitment of CIPK family members to the PM is thought to be orchestrated by specific CBL proteins, as the CIPK protein has no membrane spanning domains and no recognizable lipid modification sites (Batistic et al. 2008). Rather, a C-terminal regulatory domain of CIPK contains a 24-amino acid conserved NAF/FISL motif that mediates its interaction with the CBL protein by binding to a hydrophobic crevice (Akaboshi et al. 2008). Moreover, this domain exerts autoinhibitory effects on the kinase activity of the CIPK protein when in the unbound formation (Guo et al. 2001). CBL binding and subsequent targeting of CIPK's to the PM allows for activation of the CIPK and subsequent phosphorylation of membrane-associated substrates. Many members of the CIPK family have been shown to be regulated at the level of transcription by one or more abiotic stress condition (Pandey 2008). In rice, of the 27 *CIPK* genes detected, 20 are regulated by salt, osmotic stress, low temperature, ABA, nutrient deficiency, or a combination of these (Xiang et al. 2007).

The physiological roles of several CBL–CIPK-target networks at the PM have been established in Arabidopsis, and these have been implicated in abiotic stress tolerance. The first elucidation of a CBL–CIPK-target interaction in plants was obtained from a genetic screen for salt overly sensitive mutants in Arabidopsis (Zhu et al. 1998) and resulted in the cloning of three genes (SOS1, SOS2, and SOS3), which are all required for salt tolerance. Analysis of these genes determined that the interaction of CBL4/SOS3 with CIPK24/SOS2 directed the kinase to the PM where it was shown to stimulate the activity of the Na^+/H^+ exchanger NHX7/SOS1 resulting in removal of excess cytoplasmic Na^+ (Qiu et al. 2002). Reconstitution of the pathway in yeast confirmed that CIPK24 phosphorylated the NHX7/SOS1 protein in vitro (Quintero et al. 2002), and a phosphoproteomic study of the PM identified a putative phosphorylation site on the long hydrophilic C-terminal of the NHX7/SOS1 protein (Nühse et al. 2004).

The PM K^+-uptake channel AKT1 has also been shown to be a target protein for phosphorylation by a CBL/CIPK signaling network involved in regulating the protein under K^+-deficiency. Through combined genetic, biochemical, and electrophysiological studies, it was shown that two CBL proteins, CBL1 and CBL9, interacted independently with CIPK23 at the PM to phosphorylate and activate inward K^+ currents through AKT1 (Xu et al. 2006). This multistep signaling cascade also includes the 2C-type protein phosphatase AIP1, which acts as a signaling terminator in conjunction with CIPK23 to dephosphorylate the AKT1 protein and thereby deactivate the K^+-channel (Lee et al. 2007). Both *cipk23* and *cbl1*/*cbl9*

double mutants are sensitive to low K^+, while, conversely, overexpression of *CIPK23, CBL1*, or *CBL9* resulted in plants that accumulated significantly more K^+ than wild type and were hypertolerant to K^+ deficiency (Xu et al. 2006). Moreover, *CIPK23* expression increased under low K^+ growth conditions, although this was not seen with either *CBL1* or *CBL9* (Xu et al. 2006; Cheong et al. 2007). Interestingly, *cipk23* mutants are drought-tolerant and show reduced transpirational water loss and ABA hypersensitivity (Cheong et al. 2007). The complexity of this network was further demonstrated by Lee et al. (2007), who showed that AKT1 interacts with two additional CIPKs, CIPK6 and CIPK16, in addition to CIPK23, and that interaction with these CIPKs results in activation of inward K^+ currents, albeit with reduced magnitude. No physiological function has been ascribed to the binding of CIPK6 or CIPK16 to the AKT1 to date.

A third, well characterized CBL–CIPK-target network localized to the PM is one resulting in the phosphorylation and subsequent negative regulation of an Arabidopsis P-type H^+-ATPase, AHA2, which results in perturbations of apoplastic pH (Fuglsang et al. 2007). CBL2/SCaBP1 and CIPK11/PKS5 have been implicated as upstream mediators of this signaling cascade. Studies established that CIPK11/PKS5 phosphorylated AHA2 in vitro, with the putative phosphorylation site localized to a serine residue in the C-terminal. Yeast reconstitution of the CBL2–CIPK11–AHA2 pathway confirmed the phosphorylation and negative regulation of AHA2 and showed that phosphorylation-dependent changes in AHA2 structure destabilized the binding of a 14-3-3 protein (Fuglsang et al. 2007). However, phosphoproteomics studies have not detected an AHA2 phosphopeptide (Nühse et al. 2004), to corroborate these in vitro studies. Also, while differential regulation of *CIPK11*/*PKS5* transcripts have been observed following treatment with NaCl, drought, ABA, and glucose, *cipk11*/*pks5* mutant plants did not show any phenotypic defect under these conditions (Fuglsang et al. 2007), making it difficult to assign a physiological role in abiotic stress tolerance to the signaling pathway. Nevertheless, some insight can be gained from the observation that *cipk11*/*pks5* mutants are more tolerant to high extracellular pH due to the ability of the deregulated AHA2 to acidify the extracellular space (Fuglsang et al. 2007).

PM transporters could function directly as relay mechanisms or effectors during the adaptive response of plant cells to stress conditions, mainly in cases where ions or metabolites must be transported across the membrane in order to maintain cell homeostasis and to remove toxic ions. One well-studied, but still controversial, mechanism implicated in adaptation to salinity stress is mediated by HKT transporters that have been variously identified as K^+ and/or Na^+ transport systems at the PM (Schachtman and Schroeder 1994; Rubio et al. 1995; Horie et al. 2001; Golldack et al. 2002; Rodríguez-Navarro and Rubio 2006). These transporters belong to a small family with only one member in Arabidopsis and nine members in rice (Garciadeblás et al. 2003). Initial characterization of HKT1;1 from Arabidopsis and HKT2;1 from rice demonstrated a large Na^+-selective transport activity relative to K^+ when expressed in yeast and Xenopus oocytes (Uozumi et al. 2000; Horie et al. 2001; Garciadeblás et al. 2003). However, Golldack et al. (2002) showed that HKT2;1 is competent in the transport of both K^+ and Na^+. More recent

studies have demonstrated that HKT1;1 from Arabidopsis and its closest homolog SKC1 (HKT1;5) in rice function by removing Na^+ from the xylem sap in the root, thus reducing both long distance Na^+ transport and Na^+ accumulation in the leaves (Ren et al. 2005; Sunarpi et al. 2005; Horie et al. 2007). *SKC1* is linked with a QTL in rice that is associated with the maintenance of K^+ content in the shoot under salt stress conditions (Ren et al. 2005). The *SKC1*/*HKT1;5* locus encodes a functional Na^+-selective transporter, whose activity was shown to be K^+-independent. Contrary to expectations, the isoform encoded by the *NSKC1* gene from the salt-resistant Nona Bokra rice variety was more active than KSKC1 from salt-sensitive Koshihikari variety (Ren et al. 2005). This difference in transport activity was attributed to four amino acid changes that exist in the sequence between the two rice varieties (Ren et al. 2005). GUS expression analyses demonstrated that *SKC1* was mainly present in the xylem parenchyma cells surrounding the xylem vessels, where it could serve in the removal of Na^+ from the xylem sap. Consistent with these results and supporting a direct role of HKT transporters in salt tolerance, Ren et al. (2005) reported lower levels of Na^+ in the xylem and shoot tissues upon salt stress in salt-tolerant Nona Bokra as compared to the salt-sensitive Koshihikari. Similar observations have been made with the homologous HKT1;1 transporter from Arabidopsis (Sunarpi et al. 2005). HKT1;1 is expressed mainly in the cells surrounding the xylem vessels, with higher expression observed in the root than in the shoot. Analyses of three different mutants of *HKT1*, *athkt1-3* in the Wassilewskija (Ws) background and *athkt1-1*, *athkt1* (disruption allele FN1148; Gong et al. 2004) in the Columbia background, showed clear increases in the Na^+ content of the xylem sap under control and Na^+ stress conditions, indicating a role of *At*HKT1;1 in lowering xylem Na^+ content (Sunarpi et al. 2005). Associated with these changes, Sunarpi et al. (2005) also reported a decrease in Na^+ levels in the phloem sap of the three different *hkt1;1* mutants, thus confirming an earlier observation reported by Berthomieu et al. (2003) for a partial loss-of-function *hkt1;1* allele, *sas 2-1*.

The decrease in Na^+ phloem content observed in the different *hkt1;1* mutants has been used to postulate that *Arabidopsis* HKT1;1 participates in the recirculation of Na^+ from the shoot to the root via the phloem, thus decreasing the toxic effects of Na^+ in shoot tissues. Further confirmation of the role of HKT1;1 in xylem Na^+ removal was provided by its localization to the PM of leaf xylem parenchyma cells by immunoelectron microscopy. This confirmed previous work that showed PM localization of *Mesembryanthemum crystallinum* HKT1;1 (Su et al. 2003). However, as expression of Arabidopsis *HKT1;1* was induced by 30 mM NaCl or KCl and equimolar concentrations of mannitol or sorbitol, HKT1 transporters may also play an additional role in osmotic adaptation (Sunarpi et al. 2005).

3 Stomatal Guard Cells and Stress Responses at the PM

Studies of stomatal guard cell physiology have played a central role in efforts to identify stress-responsive adaptive mechanisms at the PM, as guard cells play a central role in the control of transpiration and water loss under conditions of

drought, heat, salinity, and cold. In a mutant screen for ozone sensitivity, Vahisalu et al. (2008) identified the mutant *slac1-1* that showed constitutively higher stomatal conductance than the wild type. Contemporaneously, Negi et al. (2008) isolated the allelic *cdi3*/*slac1-2* mutant, which is impaired in CO_2-dependent leaf temperature changes. *SLAC1* encodes a membrane protein with ten predicted transmembrane helices and hydrophilic amino- and carboxy-terminals. SLAC1 is also predicted to possess a C_4-dicarboxylate/malic acid transport protein domain, similar to that identified in the TehA and Mae1 proteins from *Escherichia coli* and *Schizosaccharomyces pombe*, respectively (Guzzo and Dubow 2000; Grobler et al. 1995). *SLAC1* promoter GUS fusions showed the gene to be primarily expressed in guard cells, and GFP fusion studies demonstrated that it was localized to the PM (Vahisalu et al. 2008; Negi et al. 2008). Information on the participation of SLAC1 in stomatal control was gained from studies in *slac1-1* plants, demonstrating alterations in stomatal conductance and stomatal closing upon transfer to the dark or upon exposure to low relative air humidity conditions, and a lack of response to large increases in atmospheric CO_2 (Vahisalu et al. 2008). Similar results were also reported for the *slac1-2* mutant (Negi et al. 2008). Moreover, *slac1-1* guard cells were insensitive to ABA and downstream second messengers such as H_2O_2, NO, and oscillating cytoplasmic Ca^{2+}.

Whole cell patch-clamp experiments demonstrated that *slac1-1* guard cells lack the activity of the so-called S-type anion currents, which were clearly activated by μM levels of Ca^{2+} in wild type. From these results, it appears that SLAC1 plays a central role in stomatal closing under adverse environmental conditions (Vahisalu et al. 2008), although it is still not clear if this protein corresponds to the actual anion channel or to a regulatory subunit of the channel complex. In Arabidopsis, there are three other *SLAC1*-like genes (40–50% identity at the amino acid level) that encode proteins localized to the PM, but none of the genes are expressed in guard cells (Negi et al. 2008). However, two of the *SLAC1* homologs, At1g62280 and At5g24030, complemented the CO_2-insensitive phenotype of the *slac1-2* mutant when expressed under the control of the *SLAC1* guard cell-specific promoter. These findings indicate a conserved function despite tissue-specific expression. These studies highlight the importance of PM ion transport processes as direct adaptive mechanisms that mediate plant response to stress.

4 Membrane-Bound Transcription Factor and Response to Abiotic Stress

Another abiotic stress signaling target at the PM could be the recently described membrane-bound transcription factors (MBTF's). Like other transcription factors, MBTFs are proteins that regulate the expression of downstream genes in response pathways and are latent until activation by internal or environmental signals. Signal-induced activation of transcription factors generally leads to a

rapid transcriptional response. Until recently, it was assumed that transcription factors are present as cytosolic pools and that entry to the nucleus is mediated by protein–protein interactions, resulting in their phosphorylation (Leonard and O'Shea 1998) or release from an associated protein (e.g. NF-κB, IκB) (Ghosh et al. 1998). However, MBTFs are membrane-bound proteins that are inactive until undergoing a proteolytic step, which releases them from the resident membrane and allows their entry into the nucleus to modulate gene expression (Kim et al. 2006, 2007a, b; Chen et al. 2008; Yoon et al. 2008). Selective proteolysis of MBTFs to release the trans-acting DNA-binding domain from the membrane anchor adds another level of posttranslational regulation that can respond to abiotic stress at the PM.

As the role of MBTFs in Arabidopsis has been recently been reviewed (Chen et al. 2008), here we only describe the work that has demonstrated the association of stress-related MBTFs to the PM.

Structural analysis of NAC-type transcription factors was used to identify more than 13 members in Arabidopsis and 6 members in rice that possess strong α-helical domains suggestive of MBTF activity. These were designated *NTL*s for *NTM1*-like (Kim et al. 2007a), in reference to the well characterized intracellular membrane-associated *NTM1* MTBF (Kim et al. 2006). Expression of most *NTL*s was detected in leaves, stems, and in roots, with apparently lower expression levels seen in flower and shoot apex tissues (Kim et al. 2006). Eleven *NTL*s were found to be differentially regulated by abiotic stress; *NTL1* and *NTL11* were found to be regulated by heat, and *NTL4* and *NTL7* by cold. *NTL3, NTL6, and NTL8* were strongly upregulated by NaCl, *NTL9* expression increased with osmotic stress, *NTL5* was upregulated by drought and NaCl, and expression of *NTL2* and *NTL3* were influenced by cold, drought, and NaCl. Together, these responses indicate that individual *NTL*s play distinct roles in responses to abiotic stress conditions. However, *NTL* expression in response to hydrogen peroxide was low, suggesting that *NTL*s are not directly associated to responses to ROS (Kim et al. 2007a).

Evidence for the PM localization of NTL8 was demonstrated by GFP fusions to both full length NTL8 or to a truncated version of the protein (Kim et al. 2007a). The full length NTL8 GFP fusion was found to be associated with the PM, while the fusion lacking the NTL8 membrane anchor was detected in the nucleus, in agreement to the NTM1-like nature of NTL8 (Kim et al. 2007b). Overexpression of the truncated form of NTL8 caused a delay in flowering time, associated with a downregulation of specific flowering time genes including FLOWERING LOCUS T (*FT*), FRUITFUL (*FUL*), and CAULIFLOWER (*CAL*) (Kim et al. 2007b). Additionally, *FT* transcript levels were significantly reduced in plants exposed to NaCl, indicating that high salt delayed flowering by repressing *FT* expression via signaling mediated by NTL8. However, *ntl8-1* mutants exhibited only slight differences from wild type in lateral root growth and flowering times (Kim et al. 2007b). Expression of *NTL8* was also shown to be developmentally regulated, with maximal expression observed in germinating seeds (Kim et al. 2007b). A possible role of *NTL8* in germination under salt stress was indicated by an observed decrease in the germination rate of seeds

overexpressing the truncated form of *NTL8*, while germination rate increased in the *ntl8* mutant (Kim et al. 2008).

Another well-studied MBTF of the NTL family, NTL9, is also localized to the PM and appears to be involved in abiotic stress gene regulation (Yoon et al. 2008). Plants overexpressing full length *NTL9* did not show a clear phenotype, which contrasted to the dwarf phenotype with small and curled leaves often associated with abiotic stress that was observed in plants overexpressing *NTL9* lacking the membrane anchor (Yoon et al. 2008). Increased expression of the stress-related genes *SIAH*-Interacting Protein (*SIP*) and Cold-Regulated 15a (*COR15a*) in plants overexpressing the truncated form of *NTL9* further implicated *NTL9* in stress responses (Yoon et al. 2008) as did evidence of posttranscriptional processing of NTL9 and increased abundance of both full length and truncated NTL9 with osmotic stress (Yoon et al. 2008).

5 Other Posttranslational Regulation of PM Proteins

Evidence exists for posttranslational regulation of other plant PM proteins in response to abiotic stress, although the associated upstream signaling is poorly characterized. The turnover of populations of integral PM proteins by trafficking to and from the membrane can be regulated by a variety of environmental stimuli. In the case of the KAT1 inward rectifying K^+ channel, ABA induces the rapid and transient internalization of the channel to an endomembrane compartment from which it can be recycled to the PM upon removal of ABA (Sutter et al. 2007). The soluble *N*-ethylmaleimide-sensitive factor protein attachment protein receptor (SNARE) protein, SYP121, has been proposed to mediate the process (Grefen and Blatt 2009). It has been suggested that this trafficking is a posttranslational adaptive mechanism to allow rapid changes in osmotic solute flux required in response to abiotic stress.

Ubiquitination can also be induced by abiotic stress and alter the turnover and subcellular localization of PM target proteins such as the Arabidopsis aquaporin PIP2;1 (Lee et al. 2009). Overexpression of Arabidopsis RMA1, an ER-localized homolog of a RING membrane anchor E3 ubiquitin ligase, was shown to reduce PIP2;1 protein levels at the PM and inhibited trafficking of PIP2;1 from the ER in protoplasts. In addition, protoplasts from RNA interference (RNAi) plants with reduced expression of *RMA1* showed increased levels of PIP2;1 (Lee et al. 2009). However, increased water permeability was not seen in these protoplasts, and compensatory expression of other *PIP* genes was not investigated. RMA1-mediated ubiquitination was further implicated in abiotic stress responses by the finding that *Capsicum annuum* plants exposed to cold, high salinity, and drought stress (but not ABA) showed rapid induction of transcripts for *Rma1H1*, and overexpression of the *CaRMA1H1* gene in Arabidopsis resulted in plants that exhibited an increased tolerance to drought (Lee et al. 2009).

6 Abiotic Stress and Effects on PM Integrity

Exposure to abiotic stress can result in damage to PM lipids resulting in lesions and membrane leakiness, particularly when osmotic or cold stress factors are involved. Damaged PMs are thought to be repaired by fusion of exocytotic vesicles triggered by a rapid elevation in cytoplasmic Ca^{2+} resulting from entry through the damaged membrane and mediated by the calcium-sensing synaptotagmin protein functioning in parallel with SNARE proteins (Andrews 2005). In a proteomic study of PM proteins from Arabidopsis leaves induced by cold stress, a synaptotagmin protein was identified by its increased abundance (Kawamura and Uemura 2003). Subsequently, it was shown that loss-of-function mutations in *Synaptotagmin1* (*SYT1*) exhibited reduced cellular viability as a consequence of compromised PM integrity and consequent increases in electrolyte leakage and hypersensitivity to NaCl, high osmotic stress, and freezing (Schapire et al. 2008). Further support for the role of this SYT1 in abiotic stress tolerance came from independent studies demonstrating that protoplasts or leaves from *SYT1*-RNA interference (RNAi) plants were freezing-sensitive even in the presence of calcium (Yamazaki et al. 2008).

7 Conclusion

It is evident that plants employ diverse mechanisms, many of which are localized to the PM; to contend with the adverse environmental conditions, they are continuously exposed. The PM not only functions as a protection barrier or interface involved in the maintenance of ionic and metabolite gradients, but it is directly involved in sensing the diversity of external signals, in the transduction of these signals, and in activating mechanisms that help maintain the cellular homeostasis that is disrupted by the imposition of multiple stress conditions.

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The Role of the Plant Plasma Membrane in Microbial Sensing and Innate Immunity

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Abstract Significant progress has recently been made in understanding the molecular mechanisms that determine plant immunity to microbial infection. Several plant plasma membrane pattern recognition receptors recognizing structurally diverse pathogen-derived molecular patterns pathogen-associated molecular patterns (PAMP) have been identified and shown to function in a similar manner as their counterparts mediating microbial pattern recognition and activation of innate immune defenses in animal systems. Receptor-mediated activation of immune response pathways results in the execution of plant defenses that in concert are supposed to halt microbial invasion. In turn, microbial infection strategies have evolved that aim at interfering with plant pattern recognition receptor function and, thus, highlight the importance of PAMP-triggered immunity in general and the important role of the plant plasma membrane as the contact interface between host and potential pathogen in particular.

1 Introduction

The plant immune system consists of two evolutionarily linked branches. The primary plant immune response is referred to as PAMP-triggered immunity (PTI) and is based upon the recognition of invariant structures of microbial surfaces termed pathogen- or microbe-associated molecular patterns (PAMP/MAMP) (Nürnberger et al. 2004; Zipfel and Felix 2005; Chisholm et al. 2006; Jones and Dangl 2006). PAMP-induced immune responses are important for immunity to microbial infection of whole plant species (species or nonhost immunity) and for basal immunity in susceptible host plant cultivars (Zipfel et al. 2004, 2006; Miya et al. 2007).

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Suppression of PTI by microbial effectors (effector-triggered susceptibility, ETS) is mandatory for a pathogen in order to complete its life cycle in a hostile plant environment (Alfano and Collmer 2004; Chisholm et al. 2006; Jones and Dangl 2006). In turn, coevolution between susceptible hosts and virulent pathogens has resulted in the acquisition by individual plant cultivars of resistance (R) proteins that guard microbial effector-mediated perturbations of host cell functions and thus trigger plant immune responses. This type of plant defense is referred to as effector-triggered immunity (ETI) and is synonymous to pathogen race/host plant cultivar-specific plant disease resistance (Chisholm et al. 2006; Jones and Dangl 2006). The following chapter focuses on the particular role of the plant plasma membrane in pathogen sensing and initiation of basal immune response pathways that eventually terminate microbial infections and warrant plant survival.

2 Signals Activating Plant Immunity-Associated Defenses

2.1 Pathogen-Associated Molecular Patterns

Activation of inducible host innate immune defenses depends on the recognition of potential microbial invaders, regardless of their aggressive potential. In 1997, a terminology was proposed to unify the description of innate immunity in animal systems (Medzhitov and Janeway 1997). The authors referred to pathogen-associated molecular patterns (PAMP) as triggers of immune responses in organisms as diverse as human, mice, crustaceans, and insects. Lipopolysaccharides (LPS) derived from Gram-negative bacteria, peptidoglycans from both Gram-positive and Gram-negative bacteria, eubacterial flagellin, unmethylated bacterial DNA fragments, or fungal cell wall-derived glucans, chitins, mannans, and proteins are well-characterized patterns that trigger innate immune responses in numerous vertebrate and nonvertebrate organisms (Ferrandon et al. 2007; Medzhitov 2007). This terminology was enormously informative for molecular plant pathologists, because many of the microbe-associated patterns with immunity-stimulating features were long known as (general) elicitors of cultivar nonspecific defenses in any plants (Nürnberger et al. 2004; Vorwerk et al. 2004; Boller and Felix 2009) (Table 1). Peptidoglycans derived from Gram-positive and Gram-negative bacteria, LPS from Gram-negative bacteria, or the N-terminal 22-amino-acid fragment of eubacterial flagellin (flg22) are potent inducers of defense-associated responses in various plant species (Felix et al. 1999; Newman et al. 2002; Gust et al. 2007; Erbs et al. 2008). This new insight implied a common evolutionary concept of microbial pattern recognition that generally underlies activation of antimicrobial counter-defense in multicellular eukaryotes. In addition, it also suggested a biologically significant role of elicitor recognition in plant immunity that had been predicted for a long time, and that was later experimentally confirmed by showing that plants

Table 1 Selected microbial PAMPs, plant-derived DAMPs, and microbial toxins with proven potential to stimulate plant immunity-associated responses

Signal	Origin	Minimal structural motif (PAMP/DAMP) required for defense activation; mode of action of plant defense activation (Toxins)	Plants mounting immunity-associated responses
Lipopolysaccharide (PAMP)	Gram-negative bacteria (Xanthomonads, Pseudomonads)	Lipid A, lipooligosaccharides	Pepper, tobacco
Peptidoglycan (PAMP)	Gram-positive and Gram-negative bacteria	Muropeptides	*Arabidopsis*, tobacco
Flagellin (PAMP)	Gram-negative bacteria	flg 22 (amino-terminal fragment of flagellin)	Tomato, *Arabidopsis*
Elongation factor (EF-Tu) (PAMP)	Gram-negative bacteria	Elf18 (*N*-acetylated amino-terminal fragment of EF-Tu)	*Arabidopsis* and other *Brassicaceae*
Cold shock protein (PAMP)	Gram-negative and Gram-positive bacteria	RNP-1 motif (amino-terminal fragment of the cold shock protein)	*Solanaceae*
Transglutaminase (PAMP)	Oomycetes (*Phytophthora* spp.)	Pep-13 motif (surface-exposed epitope of the transglutaminase)	Parsley, potato, grapevine, tobacco, *Nicotiana benthamiana*
Lipid-transfer proteins (elicitins) (PAMP)	Oomycetes (*Phytophthora* spp, *Pythium* spp.)	Undefined	Tobacco, turnip
Xylanase (PAMP)	Fungi (Trichoderma spp.)	TKLGE pentapeptide (surface-exposed epitope of the xylanase)	Tobacco, tomato
β-glucans (PAMP)	Fungi (*Pyricularia oryzae*) Oomycetes (*Phytophthora* spp.) Brown algae	Tetraglucosyl glucitol Branched hepta-β-glucoside Linear oligo-β-glucosides	Tobacco, rice, *Fabaceae*
Chitin (PAMP)	All fungi	Chitin oligosaccharides (degree of polymerisation >3)	Tomato, *Arabidopsis*, rice, wheat, barley
Oligouronides (DAMP)	Plant cell wall pectins	Oligomers	Tobacco, *Arabidopsis*
Cellodextrins (DAMP)	Plant cell wall cellulose	Oligomers	Grapevine
Cutin monomers (DAMP)	Plant cuticle	Dodecan-1-ol	Cucumber, tomato, apple
Necrosis-inducing proteins (NLP) (Toxin)	Bacteria (*Bacillus* spp.), fungi (*Fusarium* spp.), oomycetes (*Phytophthora* spp., *Pythium* spp.)	Cytolytic activity	Dicotyledonous plants
Fumonisin B1 (Toxin)	*Fusarium* spp.	Unknown	*Arabidopsis*
Fusicoccin (Toxin)	*Fusicoccum amygdali*	Unknown	Tomato
AAL-Toxin	*Alternaria alternata*	Unknown	*Arabidopsis*
Victorin (Toxin)	*Cochliobolus victoriae*	Unknown	Oat

lacking the ability for PAMP sensing were more susceptible to microbial infection (Zipfel et al. 2004).

PAMPs constitute conserved microbial structures (patterns) that are (1) characteristic of whole classes of pathogens, (2) that are absent from eukaryotic hosts, and (3) that are often indispensable for the microbial lifestyle (Medzhitov and Janeway 1997). For example, Pep-13, a surface-exposed peptide motif present in cell wall transglutaminases (TGase) of various *Phytophthora* species serves as a PAMP for the activation of defense in plants including parsley or potato (Nürnberger et al. 1994; Brunner et al. 2002; Halim et al. 2005). Pep-13 sequences are conserved among *Phytophthora* TGases but are not found in proteins from multicellular eukaryotes. The Pep-13 motif is not only essential for elicitor activity but also important for TGase activity, and knockdown of TGase activity in *Phytophthora infestans* proved to be lethal, suggesting that these enzymes are indispensible for *Phytophthora* biology (Brunner et al. 2002). Similar conclusions were drawn upon investigations on cold-shock-inducible RNA-binding proteins that are found in various Gram-positive bacteria (RNP-1) and that induce defense responses in tobacco (Felix and Boller 2003). It is further conceivable that other microbe-specific patterns with plant immune response-stimulating potential, such as fungal chitin, oomycete-derived glucans, bacterial flagellin, or the bacterial elongation factor, EF-Tu (Kunze et al. 2004), are indispensable for microbial physiology too. Indeed, very recently, it was shown that an N-terminal fragment of bacterial flagellin (flg22) was sufficient not only to trigger well-known PAMP responses in *Arabidopsis* (Felix et al. 1999) but also for the proper functioning of flagellar stability and bacterial motility (Naito et al. 2008). A selection of PAMPs with proven immunity-stimulating activities in plants is presented in Table 1.

During infection, the plant surface may face a blend of different PAMPs that are on display on microbial surfaces (Nürnberger and Lipka 2005; Boller and Felix 2009). For example, the cell walls of many phytopathogenic fungi contain chitins, N-mannosylated glycopeptides and ergosterol, all of which have been reported to trigger plant defense reactions (Basse et al. 1993; Baureithel et al. 1994; Granado et al. 1995). Moreover, various phytopathogenic Gram-negative bacteria harbor plant defense-stimulating LPS and flagellin and produce HrpZ (harpins), which are bacterial proteins that are believed to aid plant infection (He et al. 1993; Felix et al. 1999; Lee et al. 2001). It is, however, currently unclear whether recognition of multiple signals derived from one pathogen may mediate more sensitive perception or, alternatively, if redundant recognition systems may act as independent back-up systems in the same or different tissues.

2.2 Damage-Associated Molecular Patterns

Breakdown products of the plant cell wall were among the first compounds that have been identified to trigger plant immunity-associated defenses (Vorwerk et al. 2004). Plant cell wall-derived oligogalacturonide fragments (fragments of primary

cell wall pectins), cellulose fragments (cellodextrins), or cutin monomers stimulate plant immune responses that are largely indistinguishable from those triggered by microbe-derived PAMPs (Darvill et al. 1994; Fauth et al. 1998; Aziz et al. 2007). Such plant-derived elicitors are likely released by hydrolytic activities from attacking microbes and are thought to be conceptually equivalent to animal tissue-derived "danger" or "alarm" signals. Animal host-derived patterns are produced either upon microbial infection, or as a result of mechanical injury or necrotic cell death (called damage-associated molecular patterns, DAMP), and act as mediators of cell damage that results in the activation of innate immune responses (Seong and Matzinger 2004). Such animal host-derived immunostimulators comprise glucose-starvation proteins, fibronectins, hyaluronan, heat-shock proteins (Hsp), cardiolipin, and β-defensins (Matzinger 2007). Common to all these signals is that they are not released to the blood or lymph system in intact and healthy tissues and, therefore, do not normally get into contact with specialized immune cells patrolling the body for the presence of microbial patterns or determinants of damaged host-self. It is quite conceivable that activation of plant immunity by pectin, cellulose, or cutin fragments constitutes a phenomenon that is conceptually similar to DAMP-induced animal immunity.

2.3 Microbial Toxins as Triggers of Plant Defenses

Phytopathogenic microorganisms produce numerous cytolytic toxins that function as major virulence factors (Van't Slot and Knogge 2002; Glazebrook 2005). An intriguing characteristic of many of these toxins is that they not only cause damage but also trigger plant immunity-associated cellular responses. A list of microbial toxins that trigger plant defenses is given in Table 1. It is most important to note, that in virtually all cases, it is unknown whether toxin-induced plant immune responses is an unavoidable consequence of toxin action or, alternatively, if toxins are also recognized by plants in a PAMP-like fashion.

Necrosis and ethylene-inducing peptide 1-(Nep1) like proteins (NLPs) constitute microbial patterns that are widely found in bacteria, fungi, and oomycetes (Gijzen and Nürnberger 2006; Qutob et al. 2006). In addition to their necrotic activities, several NLPs have been shown to trigger plant innate immunity-associated responses. The recent elucidation of the 3D structure of an oomycete (*Pythium aphanidermatum*)-derived NLP revealed structural similarities with cytolytic toxins produced by marine organisms (Ottmann et al. 2009). Computational modeling of the 3D structure of related proteins from *Pectobacterium carotovorum* and *Phytophthora parasitica* also showed a high extent of fold conservation. As expression of different NLPs in a *P. carotovorum nlp*-deficient strain restored bacterial virulence, it can be assumed that NLPs of prokaryotic and eukaryotic origins are structurally and functionally conserved. NLP mutant protein analyses further revealed that the same structural properties of NLP proteins were required to cause plasma membrane disintegration and cytolysis in dicotyledonous plant cells

and to restore virulence in *nlp*-deficient *P. carotovorum* strains (Ottmann et al. 2009). These results demonstrated that NLPs are cytolytic, virulence-promoting phytotoxins that are derived from an evolutionarily conserved toxin fold with an extraordinarily wide distribution in different kingdoms. We further show that the same fold is also essential for NLP-induced plant defense gene expression, suggesting that NLP-mediated interference with host cell integrity results in cellular distress signaling the activation of plant immune responses. Cellular damage-associated activation of innate defenses in plants is reminiscent of microbial toxin-induced inflammasome activation in vertebrates and, hence, reveals an additional conceptual similarity in eukaryotic innate immunity.

3 Plasma Membrane Pattern Recognition Receptors in Plant Immunity

Forward and reverse genetic approaches have led to the identification of plant plasma membrane receptors that bind microbial patterns, thereby activating the plant surveillance machinery against microbial infection (Nürnberger and Kemmerling 2006; Boller and Felix 2009). The *Arabidopsis* flagellin sensing 2 (FLS2) gene encodes a plasma membrane LRR-receptor kinase (LRR-RK) that recognizes bacterial flagellin through its extracytoplasmic LRR domain (Gomez-Gomez and Boller 2000). FLS2 is the only flagellin receptor in *Arabidopsis* as *fls2* mutants lack flagellin-binding sites and are insensitive to flagellin. Importantly, flagellin-induced immune responses are necessary for the restriction of the growth of virulent *Pseudomonas syringae* pv. *tomato* strain DC3000 (*Pst* DC3000), as fls2 mutants were more susceptible to this pathogen (Zipfel et al. 2004). Thus, bacterial pattern recognition through the PRR FLS2 contributes to plant immunity by halting or limiting microbial growth. This is a most noteworthy finding because it provided unambiguous evidence that PTI is a biologically significant element of the plant immune system.

An N-terminal, acetylated 18-amino-acid fragment (elf18) of *Escherichia coli* elongation factor Tu (EF-Tu) was identified as another PAMP that triggered plant immunity-associated responses in *Arabidopsis* (Kunze et al. 2004). EF-Tu is recognized by the EF-Tu receptor, EFR, and was shown to be a structurally close relative of the LRR-RK, FLS2 (Gomez-Gomez and Boller 2002; Zipfel et al. 2006). As EFR and FLS2 group even into the same clade of LRR-RK genes (LRR XII clade), it can be assumed that other members of this particular clade represent PRRs that sense yet unknown microbial patterns.

A plasma membrane-anchored extracellular LRR-protein (LRR-P) lacking a cytoplasmic signaling domain has been implicated in the recognition of a *Trichoderma viride* xylanase and subsequent activation of immune responses in tomato (Ron and Avni 2004). As the *Arabidopsis* genome harbors 57 LRR-P-encoding sequences (Wang et al. 2008), it is conceivable that these proteins constitute

another class of pattern recognition receptors that are mechanistically similar to animal LRR-P-type PAMP receptors (Nürnberger and Kemmerling 2006). However, whereas LRR-P may be able to facilitate ligand perception, intracellular signal transduction is likely to require additional components.

Fungal chitin perception in rice is mediated through a plasma membrane LysM (lysin motif) receptor protein (LysM-P) that is built of an extracytoplasmic LysM domain linked to a short cytoplasmic domain (Kaku et al. 2006). The LysM domain directly mediates binding of oligomeric chitooligosaccharide fragments (Ito et al. 1997), but it is uncertain whether the cytoplasmic portion of the protein mediates initiation of an intracellular signaling cascade. Alternatively, LysM-P may form complexes with transmembrane proteins carrying cytoplasmic signaling domains. For example, an *Arabidopsis* LysM-RK (CERK1) has recently been implicated in chitin perception (Miya et al. 2007) as *cerk1* mutants lacked chitin sensitivity. It is unclear whether the LysM domain of CERK1 binds chitin physically. Therefore, it remains to be seen whether or not chitin perception and signaling are brought about independently by two types of plant LysM proteins or whether these proteins interact, thereby forming a chitin binding receptor complex.

4 Auxiliary Factors Mediating Pattern Recognition Receptor Function

PRR activation results in ligand-encoded information transfer across the plasma membrane. Subsequent initiation of a host intracellular signaling cascade eventually culminates in the execution of pathogen-nonspecific immune responses that are supposed to halt or slow down microbial ingress. Pharmacological evidence suggested that protein kinase activity is necessary to trigger rapid PAMP-inducible responses in plants, such as influxes of H^+ and Ca^{2+} across the plasma membrane (Felix and Boller 2003; Felix et al. 1991, 1999; Nürnberger et al. 1994), which are considered to be part of early PAMP signal transduction. Point mutations in putative (auto) phosphorylation sites within the protein kinase domain of the flagellin receptor FLS suggested that protein kinase activity is required for the activation of flagellin-inducible plant responses (Robatzek et al. 2006). In addition, overexpression of KAPP, a kinase-associated protein phosphatase that is known to negatively regulate the function of several transmembrane RKs, resulted in flagellin insensitivity and reduced flagellin binding to FLS2 (Gomez-Gomez and Boller 2000). Altogether, phosphorylation of FLS2 (by its own intrinsic PK activity or by another, yet unknown, PK) appears to be an element of flagellin sensing/signaling.

BRI1-associated receptor kinase 1 (BAK1), an LRR-RK that was previously shown to control plant growth by hormone-dependent heterodimerization with the plant brassinosteroid (BR) hormone receptor, BRI1 (an LRR-RK itself) (Wang et al. 2001; Li et al. 2002), has more recently been shown to be important for the functionality of pattern recognition receptors FLS2 and EFR (Chinchilla et al.

2007). BAK1 mutants proved to be (partially) insensitive to both flg22 and elf18. In addition, very rapid flg22-dependent heterodimerization of FLS2 and BAK1 was demonstrated, suggesting that BAK1 acts as a coreceptor serving different LRR-RK (FLS2, EFR, BRI1) in a mechanistically similar way (Chinchilla et al. 2007). In addition to its role as positive regulator of PTI and plant growth, BAK1 appears to fulfill also other functions. *Bak1* mutants also exhibit altered disease-resistance phenotypes to biotrophic and necrotrophic pathogens that are likely the consequence of infection-induced deregulated cell death control (Kemmerling et al. 2007). Thus, BAK1 may act not only as a positive regulator of PTI but also as a negative regulator of plant cell death. Importantly, both plant immunity-associated functions of BAK1 are independent of brassinosteroid activity, because several mutants impaired either in BR sensitivity or biosynthesis were not impaired in flagellin sensitivity or infection-induced runaway cell death (Chinchilla et al. 2007; Kemmerling et al. 2007). Thus, BAK1 has BR-independent, immunity-associated functions in addition to its well-established, BR-dependent role in plant development (Morillo and Tax 2006). Like ERECTA, which was previously implicated in both flower development and plant pathogen resistance (Godiard et al. 2003; Llorente et al. 2005), BAK1 may represent an example of a plant LRR-RK with dual functions in plant development and immunity.

Attenuation and termination of PRR function in animal immunity is achieved mainly by the activities of negative regulators. In *Arabidopsis*, KAPP-mediated inactivation of FLS2 has been suggested as a mechanism to facilitate temporary signaling through PRRs (Gómez-Gómez et al. 2001). In addition, proteasome-dependent, ligand-induced endocytosis of FLS2 has been demonstrated and proposed to be another route to attenuate PRR activity in plant immunity (Robatzek et al. 2006).

5 Suppression of PRR Function: A Major Virulence Strategy of Phytopathogenic Bacteria

Effector-mediated suppression of PTI is a major strategy of virulent pathogens to facilitate infections in susceptible host plants. This was concluded first from experiments that showed that effector secretion-deficient, avirulent mutants of the bacterial strain *Pst* DC3000 (incapable of effector delivery into host cells) triggered a number of plant defenses (callose deposition, defense gene expression), which were suppressed by virulent *Pst* DC3000 (Hauck et al. 2003). Activation of these responses by secretion-deficient bacteria was proposed to be brought about by PAMP perception/PRR activities responses and was later indeed shown to be triggered by various PAMPs (He et al. 2007). *Pst* DC3000 AvrPto and AvrPtoB were the first bacterial effectors that were proven to suppress PTI-associated responses (He et al. 2006). It remained, however, unclear whether these effectors inhibited PTI signaling pathways or interfered directly with PRR function. The

3D-structure elucidation-based identification of AvrPto as a Ser/Thr protein kinase inhibitor (Xing et al. 2007) suggested that soluble protein kinases and/or cytoplasmic PK domains of transmembrane PRRs may be direct targets for bacterial effector activities *in planta*. Importantly, AvrPto has very recently been shown to bind to BAK1 in vivo, thereby inhibiting flg22-induced heterodimerization of FLS2 and BAK1 (Shan et al. 2008). Although AvrPto was also found to target FLS2 when overexpressed *in planta*, moderate accumulation of AvrPto (likely the more realistic scenario during infection) led to preferential binding to BAK1 relative to FLS2 (Shan et al. 2008; Xiang et al. 2008). Because BAK1 is implicated in the function of several PRRs including FLS2 and EFR (Chinchilla et al. 2007), the inactivation of heterodimerization of the PRRs with the cognate coreceptor BAK1 appears to be a powerful strategy for suppression of PTI and subsequent infection of susceptible host plants (Shan et al. 2008). More recently, the ubiquitin ligase activity of AvrPtoB was shown to mediate ubiquitinylation and subsequent destabilization (presumably by degradation via the host 26S proteasome) of FLS2 (Gohre et al. 2008). Thus, multiple microbial effectors target PRR function, thereby underlining the major importance of plant basal defenses for plant immunity, as well as suppression of PTI as a major step towards the establishment of infection. Surprisingly, AvrPtoB also mediated ubiquitination-dependent destabilization of the chitin receptor, CERK1 (Gimenez-Ibanez et al. 2009), suggesting that this protein may not only function in fungal chitin perception but also likely in the perception of bacteria-derived carbohydrate patterns, such as LPS or peptidoglycans.

Additional modes of PTI suppression were suggested for *P. syringae* effectors AvrRpt2 and AvrRpm1 that both inhibit PAMP-induced signaling and compromise host basal immunity through manipulation of a central element of PTI, the plant plasma membrane-attached protein RIN4 (Kim et al. 2005). *Arabidopsis* RIN4 is degraded by the cysteine protease, AvrRpt2 (Century et al. 1997; Mackey et al. 2003; Coaker et al. 2005), whereas AvrRpm1 inactivates RIN4 through phosphorylation by a yet unknown plant protein kinase (Mackey et al. 2002). Importantly, resistant *Arabidopsis* ecotypes that harbor the resistance (R) proteins RPS2 and RPM1 mount AvrRpt2/AvrRpm1 effector-triggered immunity (ETI) that is brought about by R protein-mediated sensing ("guarding") of attempted PTI suppression by these effectors (Mackey et al. 2002, 2003). This is an impressive case for a mechanistic link between PTI and ETI, the two major forms of plant immunity.

Abscisic acid-dependent stomatal closure is observed upon bacterial infection and is due to pattern recognition receptor-mediated perception of bacterial patterns (Melotto et al. 2006). For example, infection of *Arabidopsis* with TTSS-deficient, avirulent *Pst* DC3000 resulted in FLS2-dependent stomatal closure. Importantly, closure of stomata was also observed initially upon infection with the virulent strain *Pst* DC3000, but was reversed at later times of infection, which suggested that virulent pathogens have evolved effectors to reopen stomata that had been closed as a result of PAMP perception. Indeed, stomatal reopening was accounted for by the bacterial toxin, coronatine (Melotto et al. 2006).

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