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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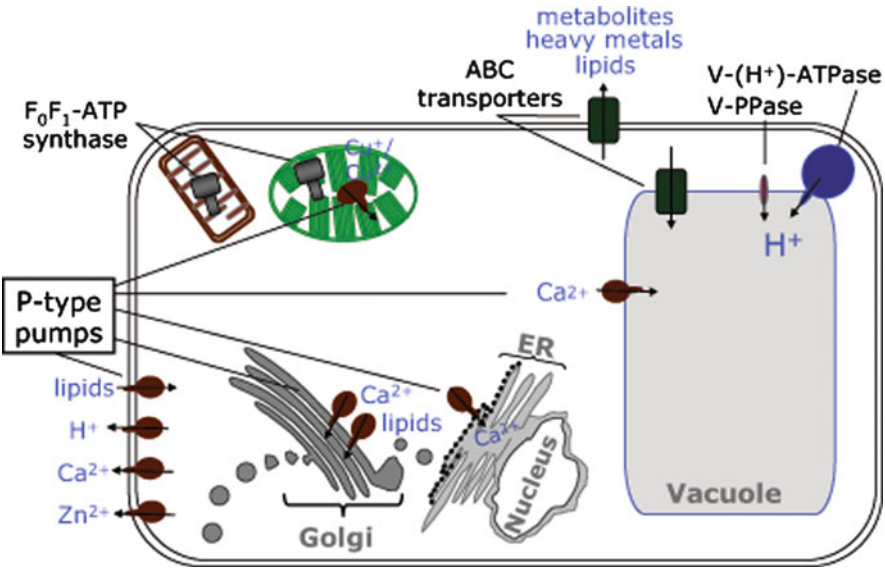

Fig. 1 Main pumps in plant cells. Mitochondria and chloroplasts contain the F_OF_1-synthase involved in ATP synthesis during respiration and photosynthesis, respectively. In the vacuole, a vacuolar H^+-ATPase and a vacuolar pyrophosphatase (V-PPase) are in charge of generating H^+-gradients required 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

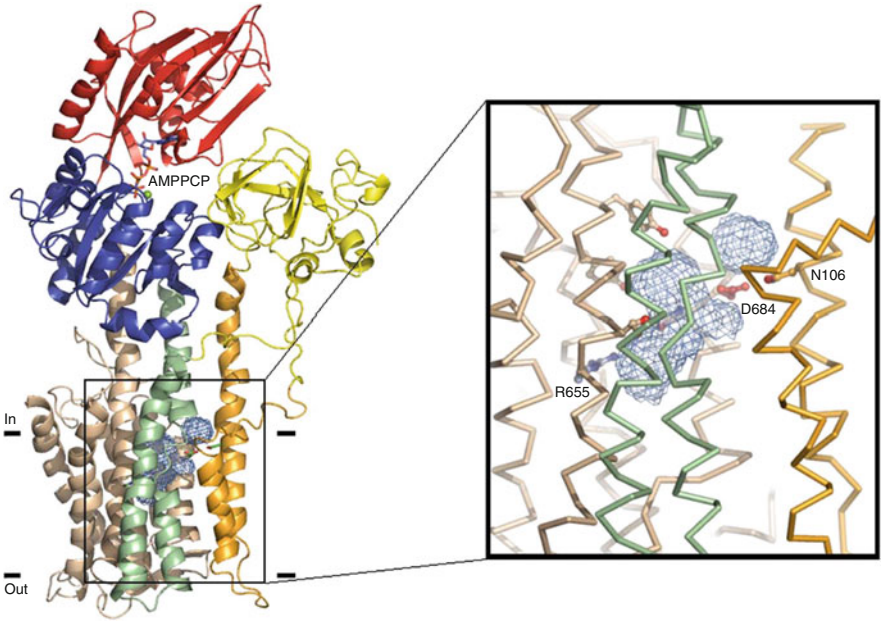

Fig. 2 Structure of a plant plasma membrane H^+-ATPase at 3.4 Å resolution (Pedersen et al. 2007). The structure represents an active form of the AHA2 proton pump, without its autoinhibitory C-terminus, in complex with Mg-AMPPCP, a nonhydrolyzable ATP homolog. Ten transmembrane helices (*orange*, *green*, 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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