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Plasma Membrane H$^+$-ATPase Regulation in the Center of Plant Physiology

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ABSTRACT

The plasma membrane (PM) H$^+$-ATPase is an important ion pump in the plant cell membrane. By extruding protons from the cell and generating a membrane potential, this pump energizes the PM, which is a prerequisite for growth. Modification of the autoinhibitory terminal domains activates PM H$^+$-ATPase activity, and on this basis it has been hypothesized that these regulatory termini are targets for physiological factors that activate or inhibit proton pumping. In this review, we focus on the posttranslational regulation of the PM H$^+$-ATPase and place regulation of the pump in an evolutionary and physiological context. The emerging picture is that multiple signals regulating plant growth interfere with the posttranslational regulation of the PM H$^+$-ATPase.

Key words: blue light, pathogens, hormonal regulation, protein phosphorylation/dephosphorylation, proton pump, stomata

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INTRODUCTION

The plasma membrane (PM) H$^+$-ATPase is an electrogenic pump that exports cellular protons. In addition to generating a transmembrane chemical gradient of H$^+$ (ΔpH; acidic on the outside), it also establishes an electrical gradient (the membrane potential; negative on the inside). As almost all other transport proteins in the plant PM are energized by this combined electrochemical gradient of protons, it has been a long-standing hypothesis in plant physiology that the PM H$^+$-ATPase is a powerhouse for plant growth (reviewed in Palmgren, 2001; Arango et al., 2003; Sondergaard et al., 2004; Duby and Boutry, 2009). However, although the biochemistry of the pump is known in great detail and its structure has been determined (reviewed in Morth et al., 2011), strong evidence for its role in plant growth has remained sparse until recently, when strong links between posttranslational regulation of PM H$^+$-ATPase and essential physiological functions of plants have emerged. The aim of this review is to provide an overview of this exciting and rapidly developing field.

Physiological evidence for the notion that PM H$^+$-ATPase is a driver of growth was mainly based on studies using pump inhibitors, such as vanadate, and on high expression of PM H$^+$-ATPase in cells specialized in solute transport such as stomatal guard cells, phloem companion cells, root epidermis, xylem parenchyma, and transfer cells (Parets-Soler et al., 1990; reviewed in Sondergaard et al., 2004). However, none of the inhibitors used in physiological studies are specific for the PM H$^+$-ATPase, and correlation between the amount of protein and specific functions only hints at a mechanistic link, but does not prove it. Until recently, genetic evidence for the physiological role of PM H$^+$-ATPase has also been sparse, for three main reasons. First, PM H$^+$-ATPase expression levels are surprisingly constant in response to a plethora of environmental stimuli, including nutrient limitation and surpluses (Maathuis et al., 2003). Second, each plant has multiple PM H$^+$-ATPase genes (11 in *Arabidopsis*) and each cell type can express several different isoforms. For example, wild-type *Arabidopsis* plants carrying knockouts of either of the two major PM H$^+$-ATPase genes, *Autoinhibited H$^+$-ATPase isoform 1* and *2* (*AHA1* and *AHA2*, respectively) (Harper et al., 1989; Pardo and Serrano, 1989; Baxter et al., 2003), grow normally under standard conditions, whereas the double knockout is embryo lethal (Haruta et al., 2010). Although these findings confirm that PM H$^+$-ATPase is essential for plant growth, its specific physiological roles are unclear, and often only become apparent under stress conditions that reduce the transmembrane electrical gradient and/or external proton

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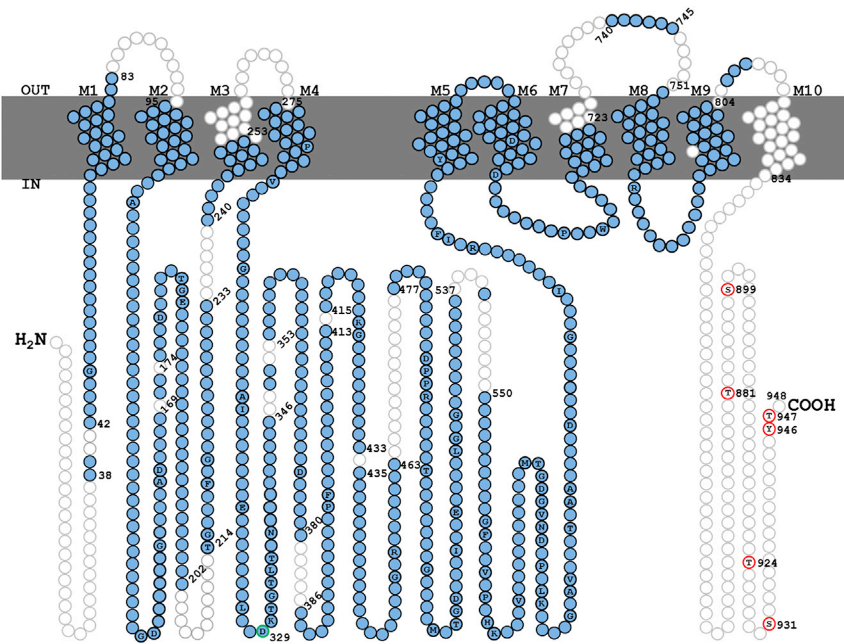

Figure 1. The Terminal Domains Are the Most Variable in Distantly Related PM H⁺-ATPases from Embryophyta, Chlorophyta, and Rhodophyta.

A 2D model of *Arabidopsis thaliana* Autoinhibited PM H⁺-ATPase 2 (AHA2). The polypeptide is integrated into the membrane by 10 transmembrane segments (M1–M10) and has most of its remaining mass, including the N- and C-terminal domains, exposed on the cytosolic side of the membrane. A total of 135 predicted PM H⁺-ATPase sequences from green algae (18), red algae (2), and plants (115) were aligned using the Multiple Sequence Comparison by Log-Expectation (MUSCLE) program (Edgar, 2004). Light-blue circles represent conserved regions in which inserts or deletions were never observed. Fully conserved amino acid residues are indicated with letters inside circles. White circles indicate regions where distantly related sequences do not align. The two termini (the latter including M10) represent regions not conserved between red algae, green algae, and higher plants. Red circles mark *in vivo* phosphorylated residues of AHA2 (Rudashevskaya et al., 2012) discussed in the text. Green circle marks Asp-329, located in the P domain, which is phosphorylated during each catalytic cycle. The numbering of residues refers to the AHA2 sequence. Accession numbers of the sequences aligned are listed in Supplemental Table 1.

chemical gradient (Haruta et al., 2010; Haruta and Sussman, 2012). Third, even ectopic overexpression of unmodified PM H⁺-ATPase typically has no effect on plant growth (Gévaudant et al., 2007). A plausible explanation for this is that the gene product of PH H⁺-ATPase is autoinhibited and that activation requires posttranslational modification (Palmgren, 1991). However, when constitutively activated mutant forms of PM H⁺-ATPase are expressed throughout the plant, developmental problems arise that likely result from the expression of active ATPase in cells where it is not normally expressed (Gévaudant et al., 2007).

Strong genetic evidence for specific physiological roles of PM H⁺-ATPases has recently emerged, most strikingly in stomatal biology. In a screen for *Arabidopsis* mutants with constitutively opened stomata, a mutant *open stomata2* (*ost2*) line was found to carry a single point mutation in *AHA1* that rendered it constitutively active (Merlot et al., 2007). Thus, expression of an activated form of this specific PM H⁺-ATPase isoform from its natural promoter produces a specific effect in stomatal guard cells that causes them to be constitutively swollen. Although it has long since been hypothesized that the PM H⁺-ATPase energizes the opening of stomatal apertures (Zeiger, 1983), this landmark study of 2007 provided the first genetic evidence that this is indeed the case. Furthermore, this work highlighted the physiological importance of the C-terminal regulatory domain. Similarly, guard cell-directed overexpression of *AHA2* in wild-type plants causes stomatal opening, but only when the plants are irradiated with blue light, which induces posttranslational activation of the recombinant pump (Wang et al., 2014b). These findings reinforce the paradigm that PM H⁺-ATPase-related phenotypes are first apparent when the gene product occurs in an activated form and in specific cell types.

ALTERNATION BETWEEN TWO ACTIVITY STATES OF THE PM H⁺-ATPASE IS REGULATED AT THE POSTTRANSLATIONAL LEVEL

Biochemical and structural studies of mutant fungal and plant PM H⁺-ATPases have shed light on the molecular mechanisms underlying proton pumping and pump regulation (Morth et al., 2011). The following picture has emerged: The pump is folded from a single polypeptide and is thus structurally simpler than multi-subunit rotary H⁺-ATPases. The model pump AHA2 comprises 948 amino acid residues (Figure 1). The polypeptide folds into 10 mainly α-helical hydrophobic segments that are integrated as a unit into the membrane. The catalytic activity is assigned to the major cytoplasmic part of the pump formed by the A, N, and P domains (Figure 1; Portillo and Serrano, 1988; Pedersen et al., 2007),where, in addition, the two hydrophilic terminal domains play regulatory roles (Figure 1). During the catalytic cycle the enzyme forms a phosphorylated intermediate in the P domain, not to be confused with any of the posttranslational phosphorylations discussed in the present review. The C-terminal domain of approximately 100 residues inhibits pump function possibly by interfering with the catalytic domains (Palmgren et al., 1991). The N-terminal 10 amino acid residues appear to assist the C-terminal domain in this function (Ekberg et al., 2010). Depending on the status of the terminal domains, PM H⁺-ATPases from both yeast and plants exist in two activity states: an autoinhibited state, where ATP hydrolysis is only loosely coupled to H⁺ transport, and an upregulated state with tight coupling between ATP hydrolysis and H⁺ pumping (Figure 1; Venema and Palmgren, 1995; Baunsgaard et al., 1996; Pedersen et al., 2015). Uncoupling is illustrated by the fact that the autoinhibited state of the yeast

Residue/ motif[a]	Function	Protein kinase	Reference	Rhodophyta	Chlorophyta	Bryophyta		Embryophyta	
						I	II	AHA1 group AHA4 group AHA5 group	AHA10 group
Glu-10	Mutation leads to pump activation	?	Ekberg et al., 2010	+	–	(+)[b]	(+)[b]	(+)[c]	(+)[b]
N-Terminal E/DXXXXLL	Putative vacuolar targeting signal	–	Wang et al., 2014c	–	–	–	–	–	(+)[d]
Thr/Ser-881	Phosphorylation leads to pump activation	PSY1R	Fuglsang et al., 2014	–	–	(+)[c]	+	+	(+)[c]
Ser-899	Phosphorylation leads to pump inhibition	FER	Haruta et al., 2014	–	–	–	–	(+)[c]	–
Thr-924	Assists in binding of 14-3-3 protein	?	Fuglsang et al., 2003	–	–	+	+	+	(+)[c]
Ser-931	Phosphorylation inhibits binding of 14-3-3 protein	PKS5	Fuglsang et al., 2007; Duby et al., 2009	–	–	(+)[e]	+	+	+
Tyr-946	Assists in binding of 14-3-3 protein	?	Fuglsang et al.,1999	–	–	+	+	+	(+)[f]
Thr-947	Phosphorylation allows for binding of 14-3-3 protein	?	Fuglsang et al., 1999, 2003	–	–	+	+	+	+

Table 1. N- and C-Terminal Residues Considered Important for the Regulation and Targeting of PM H+-ATPases.
[a]*Arabidopsis* AHA2 nomenclature.
[b]Appears inconsistently.
[c]Except for *Oryza sativa Japonica* (OSA1b; CAD29313).
[d]Except for *Beta vulgaris* 10 (Betvu10, XP_010685192).
[e]Except for *Physcomitrella patens* HA6 (PpHA6; Pp1s302_18V6.1).
[f]Tyr can also occur at this position.
[?]The protein kinase is not known.

PM H+-ATPase, Pma1p, requires eight-fold more ATP than the upregulated state to pump the same number of protons (Pedersen et al., 2015).

Regulation by Lipids

The monoacyl signaling lipid lysophosphatidylcholine was the first factor found to activate the plant PM H+-ATPase (Palmgren and Sommarin, 1989). *In vitro*, lysophospholipids directly relieve the autoinhibitory effect of both the N- and C-terminal domains (Wielandt et al., 2015). Although lysophospholipids are the most potent activators of the PM H+-ATPase identified to date, the physiological relevance of lysophospholipids in PM H+-ATPase regulation is uncertain. Lysophospholipids can be generated externally during plant–microbe interactions (Wi et al., 2014), and an active uptake system for lysophospholipids was recently identified (Poulsen et al., 2015). This could be an avenue for PM H+-ATPase activation, but more research is needed to clarify the *in vivo* relevance of lysophospholipids in plants.

Regulation by Phosphorylation

It is now evident that phosphorylation of the PM H+-ATPase plays a major role in its regulation. Multiple phosphorylation sites are present *in planta* (Rudashevskaya et al., 2012; those with characterized roles are illustrated in Table 1 and Figure 2).

The first discovered and most studied phosphorylation event is that of the penultimate residue, which in plants is threonine (Thr-948 in AHA1; Thr-947 in AHA2; Figure 1). Phosphorylation of this residue creates a binding site for 14-3-3 proteins, which stabilize the upregulated state of the pump in which C-terminal autoinhibition is released (Jahn et al., 1997; Olsson et al., 1998; Fuglsang et al., 1999; Svennelid et al., 1999; Maudoux et al., 2000). In eukaryotic cells, 14-3-3 proteins are abundant cytoplasmic proteins that bind tightly to other proteins and, as a result, stabilize conformations that can represent, e.g., active or inactive states (Ferl, 2004; Chevalier et al., 2009). For a 14-3-3 protein to bind, its target sequence needs to be phosphorylated, and the C-terminal sequence His/Ser-Tyr-pThr-Val-COOH (pThr denotes phosphorylated Thr) represents a 14-3-3 protein motif only found in PM H+-ATPases.

The protein kinase that phosphorylates the penultimate residue has not yet been identified. Phosphorylation of another residue further upstream in the C-terminal domain (Thr-881 in AHA2; Figure 1) activates the pump by a mechanism that does not affect binding of 14-3-3 protein as demonstrated both for Thr-881 in *Arabidopsis* (Niittylä et al., 2007; Fuglsang et al., 2014) and the corresponding residue Thr-889 in tobacco PMA2 (Piette et al., 2011). By contrast, two *in vivo* phosphorylation events (on Thr-924 or Ser-931; Figure 1) have been found to inactivate the PM H+-ATPase (Fuglsang et al., 2007; Duby

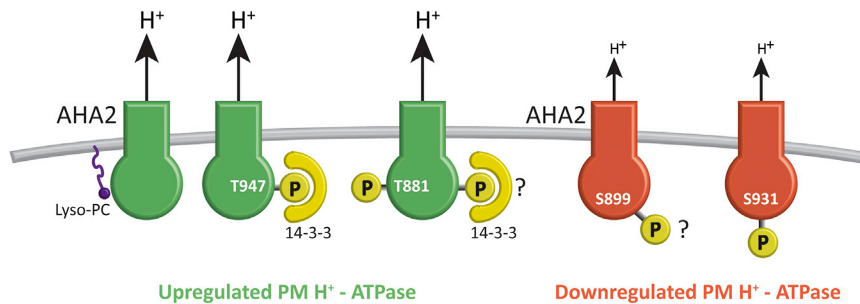

Figure 2. Posttranslational Regulation Allows the PM H+-ATPase to Alternate between Two Regulatory States.
In the downregulated state (right, pumps marked in red) the pump can still hydrolyze ATP, but the coupling efficiency between energy consumption and transport of H^+ is low. In the upregulated state (left, pumps marked in green) the coupling efficiency and H^+ transport rates are high. Terminal regulatory domains control transition between the two states. Phosphorylation (P) of the C-terminal penultimate threonine residue (Thr-947 in the *Arabidopsis* pump AHA2) creates a binding site for activating 14-3-3 protein. Also, binding of the signaling lipid lysophosphatidylcholine (Lyso-PC) and phosphorylation at Thr-881 activate the PM H^+-ATPase. Lyso-PC activation is independent of phosphorylation and 14-3-3 protein. It is not known whether phosphorylation at Thr-881 in the C-terminal domain interferes with or is dependent on binding of 14-3-3 protein (as indicated by the question mark). Phosphorylation at Ser-899 or Ser-931, both in the C-terminal domain, is associated with pump inactivation. Phosphorylation at Ser-931 blocks the binding of 14-3-3 protein, but it is not known whether the same holds true for phosphorylation at Ser-899. Only well-characterized regulatory events are shown.

et al., 2009). Phosphorylation of Ser-931 and Thr-924 (corresponding to Ser-938 and Thr-931 in PMA2 from *Nicotiana tabacum*) interferes with binding of 14-3-3 proteins regardless of whether Thr-947 is phosphorylated (Fuglsang et al., 2007; Duby et al., 2009). Thus, independent of phosphorylation of Thr-947, 14-3-3 proteins cannot bind if Ser-931 is phosphorylated. Most likely, phosphorylation of Ser-931 produces a steric hindrance that interferes with 14-3-3 binding. Whether phosphorylation of Ser-899 has a related effect on binding of 14-3-3 protein is unknown.

Mechanism of Fusicoccin Action

The toxin fusicoccin secreted by the fungi *Fusicoccum amygdali* has been used intensively in the study of PM H^+-ATPase activation (Marrè, 1979). Fusicoccin activates the PM H^+-ATPase by promoting 14-3-3 protein binding to it in an essentially irreversible manner (Olsson et al., 1998; Svennelid et al., 1999). The fusicoccin receptor is only formed when the C-terminal end of PM H^+-ATPase interacts with 14-3-3 protein (Baunsgaard et al., 1998; Svennelid et al., 1999). This simultaneous binding of fusicoccin and 14-3-3 protein to the PM H^+-ATPase has been described at the structural level (Würtele et al., 2003; Ottmann et al., 2007). Thus, the fusicoccin molecule elegantly fills a cavity in the interaction surface between PM H^+-ATPase and 14-3-3 proteins to tightly bridge the two partners, and it was initially believed that phosphorylation of the penultimate residue was a prerequisite for binding of 14-3-3 protein to the PM H^+-ATPase. However, fusicoccin stabilizes the complex between 14-3-3-protein and peptides representing the PM H^+-ATPase C terminus also in the absence of phosphorylation (Fuglsang et al., 2003). Application of fusicoccin to plants has been reported to increase phosphorylation levels of the penultimate Thr. This has been interpreted as a sign of fusicoccin-mediated protein kinase activation, but is more likely the result of bound 14-3-3 protein protecting the pThr from phosphate removal by protein phosphatases.

Autoinhibitory Regulatory Terminal Domains of PM H+-ATPase Appeared Early in Plant Evolution

When aligning PM H^+-ATPase sequences from multiple plant genomes and their closest relatives, we found that a penultimate threonine residue is present and strictly conserved in land plants (Bryophyta and Embryophyta) (Table 1). It was previously reported that a number of moss sequences deposited in sequence databases do not contain this residue (Pedersen et al., 2012; Okumura et al., 2012a, 2012b); however, these sequences were only draft versions of predicted open reading frames. Further, when examining the genomic sequence of *Physcomitrella patens* isoform 7 (PpHA7; Pp1s404_34V6.1), which has been reported to lack this residue (Okumura et al., 2012a; Pedersen et al., 2012), we observed a sequence just downstream of the predicted open reading frame that encodes the conserved motif Tyr-Thr-Val-STOP, including the penultimate threonine. PM H^+-ATPase-related sequences in green algae (Chlorophyta) and red algae (Rhodophyta), the sister group of Viridiplantae, typically have extended C-terminal regions of around 100 amino acid residues. However, whereas their core catalytic region is conserved with that of land plant PM H^+-ATPases (Figure 1), they show a very low degree of sequence conservation in their terminal domains and lack a penultimate threonine residue. Such a residue is also absent from fungal PM H^+-ATPase sequences. A penultimate threonine is essential for 14-3-3 binding even in mosses (Okumura et al., 2012a, 2012b), and the omnipresence of this residue in mosses but complete absence in green algae suggests that 14-3-3 protein-mediated regulation of PM H^+-ATPases occurred in the first land plants concomitantly with their divergence from green algae. Strikingly, Ser-931, phosphorylation of which inhibits 14-3-3 protein action, evolved at the same time (Table 1).

Regulatory Evolution of PM H+-ATPase

A phylogenetic tree based on PM H^+-ATPase sequences in which the regulatory terminal regions have been deleted is shown in Figure 3. A striking feature of the tree is that sequence variation in the catalytic core is relatively large within and between fungi, red algae, and green algae (as evidenced by the extended branch lengths), but extremely restricted within and between groups of land plants (Figure 3). Furthermore, two clades of moss sequences involving the same species are evident: one with a very short connecting branch (Bryophyta I) is situated within the Angiosperms clade (light blue in top of Figure 3) and has conserved regulatory

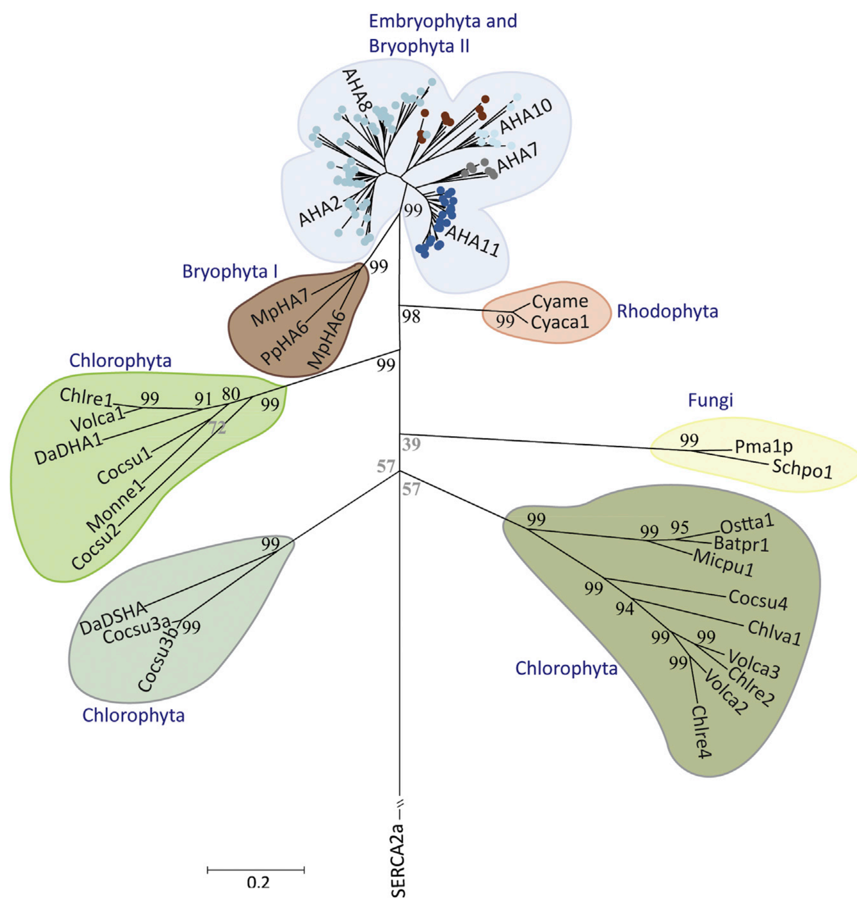

Figure 3. Evolutionary Relationship between Classes of PM H+-ATPases in Plants, Algae, and Fungi.
PM H+-ATPases of land plants cluster in a dense group reflecting limited phylogenetic evolution within land plants compared with algae pumps. Previously characterized or predicted PM H+-ATPase sequences from Embryophyta (represented here by Bryophyta, Lycopodiophyta, Angiospermae, Chlorophyta, Rhodophyta, and fungi; 137 in total) were aligned with SERCA2a (*Oryctolagus cuniculus)* as an outgroup using the MUSCLE program. Subsequently, all positions where gaps were evident in one or more sequences were eliminated (marked with white circles in Figure 1; gaps in the SERCA2a sequence were not accounted for when eliminating sequence positions). The final dataset of core sequences included 654 amino acids, but not the N-terminal and C-terminal domains or transmembrane segment M10. The evolutionary history was inferred using the maximum likelihood method based on the JTT matrix-based model (Jones et al., 1992). The tree with the highest log likelihood (−39 616.5206) is shown. Following a bootstrap analysis with 1000 replicate trees, the percentage of trees in which the associated taxa clustered together is shown next to the branches. Bootstrap values below 75% are marked in gray and represent the positions of nodes that are uncertain. Evolutionary analyses were conducted in MEGA6 (Tamura et al., 2013). Abbreviations are as follows. Angiospermae, dicotyledones: AHA, *Arabidopsis thaliana*. Lycopodiophyta (clubmosses; primitive vascular plants): SmAHA, *Selaginella moellendorffii*; Bryophyta (mosses): PpHA, *Physcomitrella patens*; Marpo, *Marchantia polymorpha*; Chlorophyta (green algae): Chlva, *Chlorella variabilis*; Chlre, *Chlamydomonas reinhardtii*; Cocsu, *Coccomyxa subellipsoidea* C-169; Volca, *Volvox carteri* f. *nagariensis*. Rhodophyta (red algae): Cyaca, *Cyanidium caldarium*; Cyame, *Cyanidioschyzon merolae* strain 10D. *Fungi:* Pma1p, *Saccharomyces cerevisiae*; Schpo1, *Schizosaccharomyces pombe*; SERCA2a, P2-type (non-P3A) ATPase from *Oryctolagus cuniculus*. Database accession numbers are given in Supplemental Table 1. Scale bar represents 0.2 amino acid substitutions per site.

residues (Figure 4), whereas the other (Bryophyta II) with a long branch is outside this clade and does not have similar regulatory residues (Figure 3). Altogether, it appears that restriction in sequence variation occurs concomitantly with the evolution of key residues in the terminal regulatory domains (compare Figure 4 with Table 1), whereas residues essential for the core pump mechanism are conserved in all branches of the tree (Figure 1). The implications of this observation are unclear, but it seems that the delicate regulatory apparatus within the single PM H+-ATPase polypeptide of land plants has evolved to a stage where even minor changes in structure affect pump function and regulation.

A Vacuolar Subgroup Lacks Conserved Phosphosites

The phosphorylated residue Thr-881 is present in all land plant PM H+-ATPase sequences except for a clade of sequences that cluster with AHA10 (Table 1; Figure 4; Baxter et al., 2005). The same is true for Thr-924 (Table 1), which plays a role in interaction with 14-3-3 protein (Fuglsang et al., 2003) and is phosphorylated *in vivo* (Rudashevskaya et al., 2012), although the effect of phosphorylation is not known. AHA10 and related proteins are distinct from PM H+-ATPases in that they are targeted to the tonoplast (Verweij et al., 2008; Appelhagen et al., 2015), where they have specialized functions (Baxter et al., 2005; Verweij et al., 2008; Aprile et al., 2011; Appelhagen et al., 2015). In AHA10-related sequences, we detected an N-terminal dileucine motif preceded further upstream by one or more acidic residues (Table 1; the sequence **EDLDKPLL** in AHA10), which for other membrane proteins has been demonstrated to be a vacuolar membrane targeting signal (Wang et al., 2014c). The presence of this motif supports the notion that these pumps are exclusively localized to the vacuolar membrane. The inconsistent occurrence of Thr-881 and Thr-924 in the AHA10 clade and the fact that no AHA10-related sequences have been identified in Bryophyta suggest that AHA10-related proteins represent a more advanced stage of plant evolution, where 14-3-3 protein-mediated regulation has been either substantially modified or lost. In support of this notion, an N-terminal residue (Glu-10), which has been found to interfere with autoinhibition (Ekberg et al., 2010), is strictly conserved in Embryophyta except within the AHA10 clade where it only occurs sporadically (Table 1).

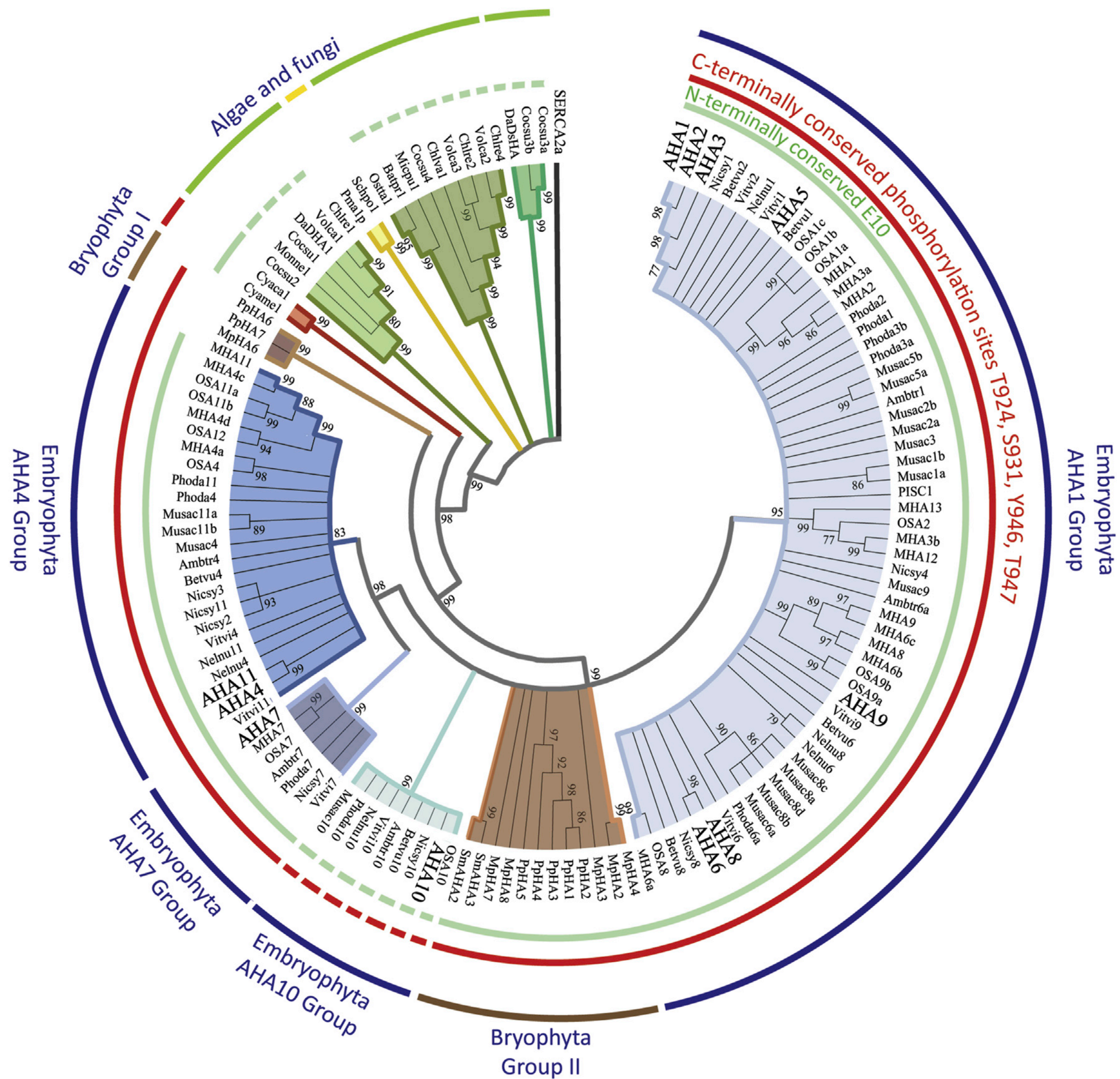

Figure 4. Evolution of the Core Structure in the Plant PM H⁺-ATPase Family and Emergence of Key Regulatory Residues.
A bootstrap consensus tree inferred from 1000 replicates and derived from that shown in Figure 3 was taken to represent the evolutionary history of the taxa analyzed. Branches corresponding to partitions reproduced in less than 75% of bootstrap replicates were collapsed. Concentric rings indicate specific features of the sequences. On the outer ring, PM H⁺-ATPases can be divided into subgroups based on phylogeny. The first group (the *AHA1 group*) contains pumps that cluster together with *Arabidopsis* AHA1, 2, 3, 5, 6, 8, and 9 isoforms. A second less well defined group contains only bryophyte (primitive land plant) pumps. A third group contains pumps with high similarity to the vacuolar-localized AHA10 isoform (AHA10 group). A fourth group contains pumps similar to AHA7 (AHA7 group). A fifth group contains pumps that cluster with AHA 4 and 11 isoforms (AHA4 group). Finally, a group contains a subset of ancient bryophyte pumps with high identity to pumps of green algae. Middle and inner rings indicate the occurrence of key regulatory residues in the C- (red ring) and N-terminal (green ring) domains of PM H⁺-ATPases. Abbreviations are as follows. Angiospermae (flowering plants), monocotyledons: Phoda, *Phoenix dactylifera* (date palm); Musac, *Musa acuminata* subsp. *malaccensis* (banana); Zeama, *Zea mays* (maize). Orysa, *Oryza sativa* var. *japonica* (rice). Angiospermae, dicotyledones: AHA, *Arabidopsis thaliana*; Vitvi, *Vitis vinifera* (wine grape); Betvu, *Beta vulgaris* subsp. *vulgaris* (sugar beet); Nicsy, *Nicotiana sylvestris* (wood tobacco); Nelnu, *Nelumbo nucifera* (sacred lotus). Amborellales (ancestral flowering plants): Ambtr, *Amborella trichophoda*; Gymnosperms: Picsi, *Picea sitchensis* (sitka spruce). Other Abbreviations of species names are given in the legend of Figure 3. Database accession numbers are given in Supplemental Table 1.

Recently, new plant mutants and tools to detect dynamic phosphorylation events at high cellular resolution have provided a wealth of information regarding the physiological role of plasma membrane H⁺-ATPase regulation. Large-scale phosphoproteomic studies have greatly enhanced our understanding of physiological factors controlling PM H⁺-ATPase phosphorylation (Benschop et al., 2007; Niittylä et al., 2007; Nühse et al., 2007; Monneuse et al., 2011). Furthermore, antibodies that recognize the phosphorylated C-terminal end of PM H⁺-ATPases have been raised (Bobik et al., 2010a; Hayashi et al., 2011) and can be used for immunohistochemical detection of pumps phosphorylated at this residue (Hayashi et al., 2011). Exciting discoveries that have resulted from these advances are reviewed below.

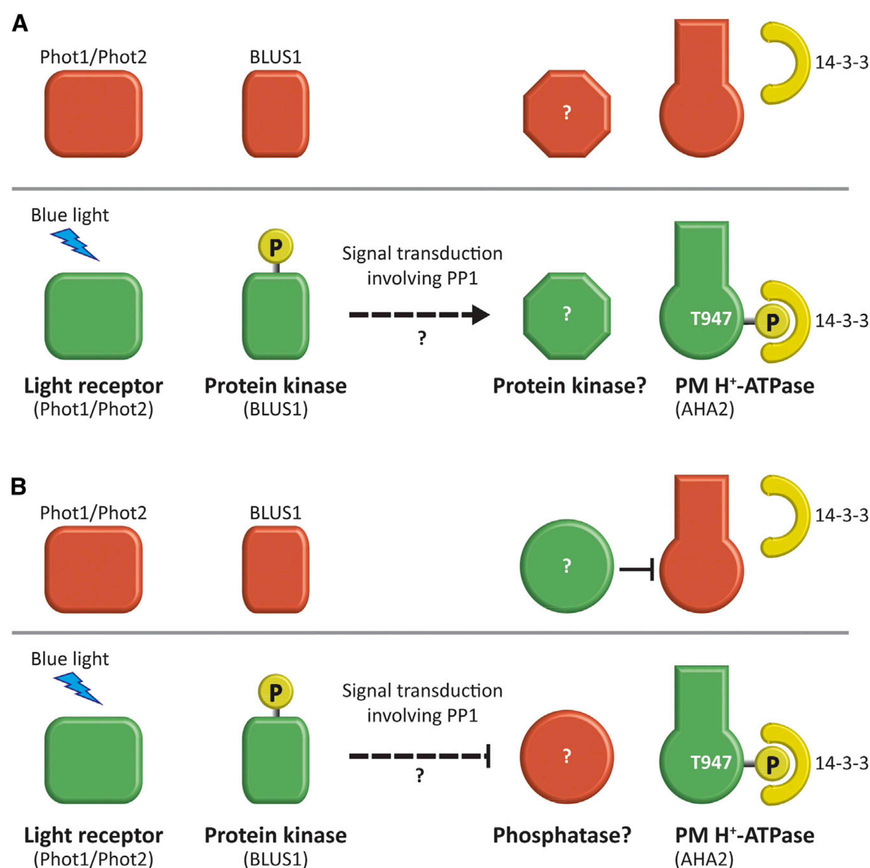

Figure 5. Regulation of PM H^+-ATPase by Blue Light.
Blue light is perceived by phototropins, which are blue light-activated protein kinases with the protein kinase BLUS1 as substrate. Two models for further signaling events leading to activation of the PM H^+- ATPase are shown. Green figures indicate an upregulated protein and red figures an downregulated protein.
(A) According to the current model, activated BLUS1 indirectly activates the hitherto unidentified protein kinase (as indicated by the question mark) that phosphorylates Thr-947, which creates a binding site for activating 14-3-3 protein.
(B) According to the model proposed here, which is analogous to auxin activation of the pump (Spartz et al., 2014), BLUS1 inactivates a protein phosphatase, which has phosphoThr-947 as its substrate. This increases steady-state levels of phosphorylated PM H^+-ATPase, which can interact with activating 14-3-3 protein. The model assumes that the protein kinase phosphorylating Thr-947 is continuously active.

MULTIPLE GROWTH REGULATORY FACTORS TARGET THE C-TERMINAL REGULATORY DOMAIN AND HAVE OPPOSING EFFECTS ON PM H^+-ATPASE ACTIVITY

It has recently become apparent that PM H^+-ATPase activity is regulated by virtually all factors known to regulate plant growth, including multiple environmental signals, such as blue light, peptide hormones, and classical plant hormones.

Regulation by Blue Light

Blue light was the first physiological factor found to induce phosphorylation of the penultimate residue of PM H^+-ATPases in guard cells, and triggers the binding of 14-3-3 protein and activation of the PM H^+-ATPase (Figure 5; Kinoshita and Shimazaki, 1999). The initial receptors of blue light are phototropins (PHOT1 and PHOT2), which are protein kinases (Kinoshita et al., 2001). Following activation by blue light, phototropins phosphorylate another protein kinase, BLUE LIGHT SIGNALING 1 (BLUS1) (Figure 5; Takemiya et al., 2013). The substrate of BLUS1 is uncertain. The penultimate Thr-947 of the PM H^+-ATPase is unlikely to be a substrate for BLUS1 as another protein in blue light signaling, type 1 protein phosphatase (PP1) (Takemiya et al., 2006), seems to act downstream of BLUS1. Thus, an inhibitor of PP1 suppresses PM H^+-ATPase phosphorylation, but has no effects on BLUS1 phosphorylation (Takemiya et al., 2013). These data are in accordance with a model proposed by Kinoshita and Hayashi (2011) in which the target of blue light-induced signaling is thought to be an unidentified protein kinase that phosphorylates Thr-947 in the PM H^+-ATPase. According to this model (depicted in Figure 5A), the protein kinase and the PM H^+-ATPase are both inactive in the absence of blue light, but active in its presence. An alternative model also merits consideration. As discussed below, this model (depicted in Figure 5B) predicts that PM H^+-ATPase Thr-947 is phosphorylated by a constitutively active protein kinase. Instead of activating a protein kinase, pump activation proceeds by inhibition of a protein phosphatase that removes the Thr-947 phospho group.

Cell-Surface Receptors for Peptides Are Directly Linked to the Regulation of PM H^+-ATPase

The cell membrane harbors hundreds of different receptor kinases that receive environmental signals at the receptor domain on the extracellular side of the membrane and convert these signals into cellular responses via an intracellular protein kinase domain. PM H^+-ATPase interacts with multiple such receptor kinases (Caesar et al., 2011; Fuglsang et al., 2014; Pertl-Obermeyer et al., 2014; Ladwig et al., 2015). Binding of growth-promoting brassinosteroids to the brassinosteroid receptor BRI1 induces membrane hyperpolarization (Caesar et al., 2011). While the mechanism is not clear, membrane hyperpolarization requires the protein kinase function of BRI1 and, furthermore, BRI1 interacts directly with AHA1. It is therefore possible that the mechanism involves phosphorylation of AHA1. However, regulation does not involve the penultimate threonine, as a mutant of AHA1 devoid of this residue also responds to brassinosteroids (Witthöft et al., 2011).

Recent evidence demonstrates that a stimulated receptor kinase can indeed activate the PM H^+-ATPase directly, without the need

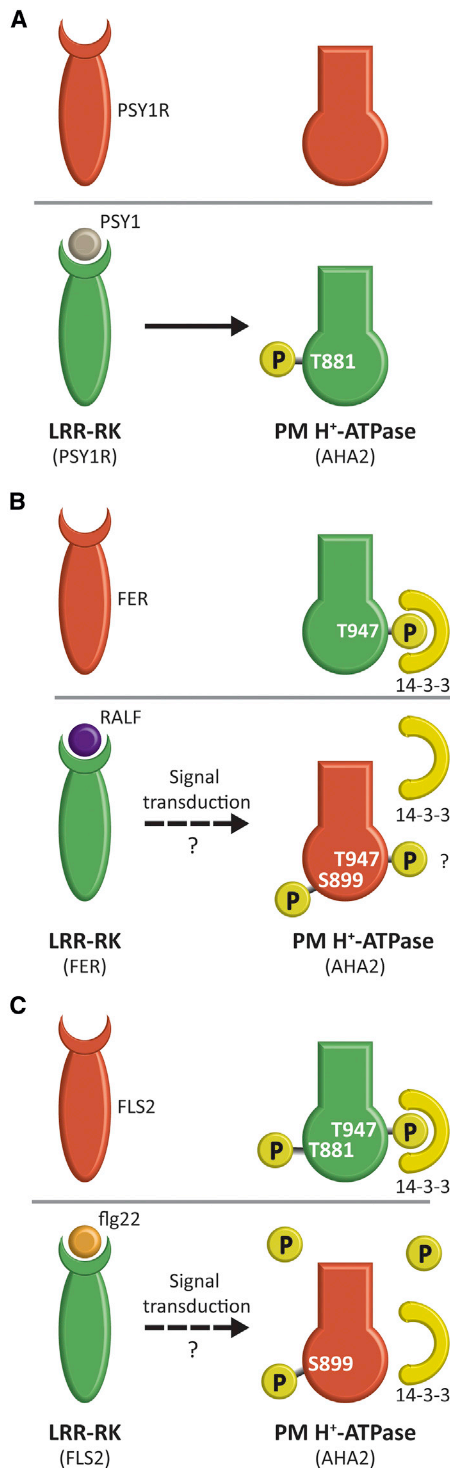

Figure 6. Regulation of PM H$^+$-ATPase by Receptor Kinases at the Cell Surface.

Green figures indicate an upregulated protein and red figures an down-regulated protein.

(A) PSY1R is activated by extracellular binding of the peptide hormone PSY1. Activated PSY1R directly phosphorylates the PM H$^+$-ATPase at Thr-881, which causes pump activation. Although phosphorylation by PSY1R does not affect Thr-947 phosphorylation levels, it is not known whether phosphorylation of Thr-881 activates the pump independently of 14-3-3 protein binding (as indicated by the question mark).

(B) Binding of the peptide hormone RALF to the receptor kinase FERONIA (FER) results in phosphorylation of the PM H$^+$-ATPase at Ser-899, which causes pump inactivation. Phosphorylation is not direct and is likely mediated by other protein kinase(s).

(C) Binding of flg22, the recognized part of bacterial flagellin, to the receptor kinase FLS2 modifies the phosphorylation levels at three PM H$^+$-ATPase sites, with each event leading to pump inactivation.

for other signaling components (Figure 6A; Fuglsang et al., 2014). The PSY1 receptor, PSY1R a Leucine-Rich-Repeat Receptor Kinase (LRR-RK), is a surface receptor for a growth-promoting peptide, PSY1, which is secreted by cells and perceived by (other) cells that are sensitive to the signal. PSY1R interacts directly with the C-terminal domain of AHA2. Within minutes after applying PSY1, PSY1R phosphorylates AHA2 on Thr-881 and concomitantly activates H$^+$ pumping (Fuglsang et al., 2014).

Following binding of the peptide hormone RALF to the FERONIA (FER) receptor kinase, the Ser-899 residue of the PM H$^+$-ATPase is phosphorylated, which results in pump inactivation (Figure 6B; Haruta et al., 2014), The protein kinase that phosphorylates Ser-899 appears to differ from FER. In apparent contradiction, when tested in a yeast complementation assay, a phosphomimetic mutant of Ser-899 (aha2Ser899Asp) appears to be more active than a mutant that cannot be phosphorylated at this site (aha2-Ser899Ala) (Rudashevskaya et al., 2012). However, both mutants do not complement as well as the wild-type AHA2, which might be due to the presence of endogenous protein kinases in the heterologous expression system employed, which modulate the activity of the introduced pump (Rudashevskaya et al., 2012). Exposure of plants to the flagellin fragment flg22, which is perceived by the receptor kinase FLAGELLIN-SENSING 2 (FLS2), likewise leads to phosphorylation of Ser-899 of the PM H-ATPase (Figure 6C; Nühse et al., 2007), but, as FER is also phosphorylated in response to flg22 treatment, the mechanism of flg22-induced phosphorylation of Ser-899 may indirectly involve FER.

Auxin

The classical growth hormone, auxin indole-3-acetic acid (IAA), has long since been proposed to activate the PM H$^+$-ATPase (the acid growth theory; Rayle and Cleland, 1992). In *Arabidopsis* cell cultures, phosphorylation of the penultimate threonine residue of AHA1 and AHA2 increases in the presence of IAA (Chen et al., 2010). Hypocotyls respond to IAA application with increased levels of PM H$^+$-ATPase phosphorylation at the penultimate threonine concomitant with stimulation of PM H$^+$-ATPase activity (Takahashi et al., 2012). A well-studied pathway of auxin action involves the TIR1/AFB auxin receptors that control degradation of transcription factors responsible for auxin-induced alterations in gene transcription (Salehin et al., 2015). However, as the effect of IAA on PM H$^+$-ATPases is evident in a *tir1-1 afb2-3* double mutant and in the presence of a TIR1/AFB inhibitor, auxin-mediated regulation of PM H$^+$-ATPase activity appears to involve receptor(s) other than TIR1/ARF and may occur at the posttranslational rather than at the transcriptional level. This is supported by the fact that IAA does not affect PM H$^+$-ATPase protein levels (Takahashi et al., 2012). IAA has also been shown to stimulate the production of lysophospholipids by activating

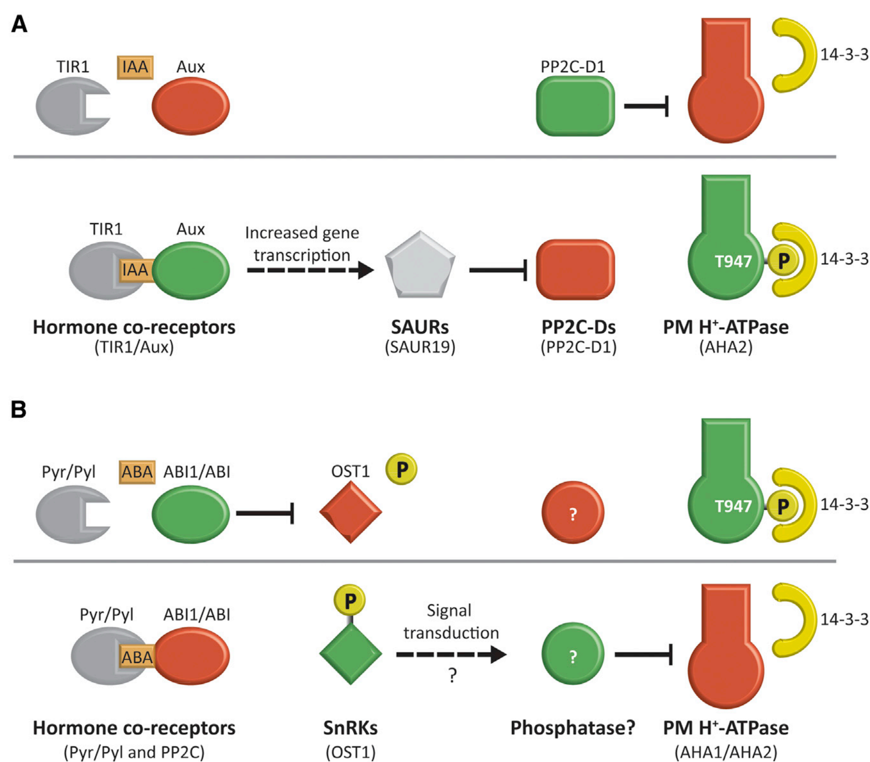

Figure 7. Regulation of PM H$^+$-ATPase by Two Major Plant Hormones that Have Opposing Effects on Plant Growth. Green figures indicate an upregulated protein and red figures an downregulated protein.

(A) Regulation by the growth-promoting hormone auxin. The natural auxin, indoleacetic acid (IAA), serves as molecular glue that brings TIR1 and AUX together in one complex. This initiates a series of events that relieves repression of transcription at multiple genes including *SAUR19*. The product of this gene inhibits PP2D-C, a protein phosphatase (unknown as indicated by the question mark) that removes the phosphate groups at Thr-947 of the PM$^+$-ATPase, thereby increasing the steady-state levels of phosphorylated PM H$^+$-ATPase. The model assumes that the protein kinase that phosphorylates Thr-947 is continuously active.

(B) Regulation by the growth-retarding hormone abscisic acid (ABA). ABA serves as molecular glue that combines a soluble Pyr/Pyl receptor protein (multiple isoforms exist in the cell) and a protein phosphatase (ABI1 or ABI2) into one complex. In the complex, ABI1/ABI2 is inactive and can no longer dephosphorylate and thereby inactivate the OST1 protein kinase. It is unknown how the active OST1 protein kinase causes PM H$^+$-ATPase dephosphorylation and inactivation. According to the model proposed here, which is analogous to auxin activation of the pump, OST1 action directly or indirectly causes activation of a protein phosphatase (unknown as indicated by the question mark), which has phosphoThr-947 as its substrate. This decreases the steady-state levels of phosphorylated PM H$^+$-ATPase, which can no longer interact with activating 14-3-3 protein.

phospholipase A_2 (Lee et al., 2003), which could provide a link to posttranslational activation of PM H$^+$-ATPase.

Recently, light has been cast on the IAA-mediated regulation of PM H$^+$-ATPases, which supports a new paradigm for the posttranslational regulation of these pumps (Spartz et al., 2014). This model is based on the findings that the SAUR19-24 subfamily of auxin-induced SMALL AUXIN UP-RNA (SAUR) genes promotes cell expansion in a PM H$^+$-ATPase-dependent manner (Figure 7A; Spartz et al., 2012, 2014) and, notably, that the presence of SAUR19 correlates with increased levels of PM H$^+$-ATPase phosphorylation at the C-terminal domain (Spartz et al., 2014). SAUR19 does not appear to regulate the PM H$^+$-ATPase directly, but interacts with and inhibits protein phosphatase type 2C proteins of the PP2C-D subfamily (Spartz et al., 2014). These findings are explained in a model in which IAA activates the transcription of SAUR proteins which, at the posttranslational level, inhibit PP2C-D phosphatases that specifically target the PM H$^+$-ATPase (Spartz et al., 2014). Thus, in the presence of SAUR proteins, the penultimate threonine of H$^+$-ATPase remains phosphorylated and the PM H$^+$-ATPase is active (Figure 2). In support of a role of protein phosphatases in the direct regulation of PM H$^+$-ATPases, it has been demonstrated that the regulatory subunit A of phosphatase 2A (PP2A-A) (Fuglsang et al., 2006) and a phosphatase type 2C (Hayashi et al., 2010) interacts with the C-terminal domain of the PM H$^+$-ATPase.

The finding that a specific phosphatase indeed influences phosphoThr-947 levels (Spartz et al., 2014) prompts us to consider whether dephosphorylation is controlled by a general mechanism that regulates the phosphorylation status of this residue. During optimal growth conditions, approximately one-third of the population of different PM H$^+$-ATPase pumps are phosphorylated at the residue corresponding to Thr-947 (Bobik et al., 2010b). Constant high phosphorylation levels of the penultimate residue, even in the absence of growth-promoting or inhibiting signals indicates that the activated state of the pump is required for basal growth, and suggests that Thr-947 is phosphorylated by a constitutively active system.

Abscisic Acid

Abscisic acid (ABA) strongly inhibits plant growth. Physiological concentrations of ABA (submicromolar) reduce phosphorylation at the penultimate threonine residue of the PM H$^+$-ATPase in both suspension cell cultures (Chen et al., 2010) and guard cells (Hayashi et al., 2011). As this effect of ABA is lost in mutants with defects in upstream ABA signaling proteins phosphatases 2C (ABI1 and ABI2) and protein kinase (OST1/SnRK2.6) (Hayashi et al., 2011), ABA-mediated downregulation of Thr-947 phosphorylation is dependent on early events in the ABA signal transduction pathway. How ABA exerts its effect on the PM H$^+$-ATPase is not known. In principle, ABA could inhibit any element in the blue light-induced signal transduction pathway and thereby lead to PM H$^+$-ATPase phosphorylation or, alternatively, it may promote dephosphorylation of the phosphoprotein directly via a protein phosphatase (Figure 7B).

In hypocotyls, the application of ABA also reduces Thr-947 phosphorylation in an ABI1-dependent manner (Hayashi et al., 2014).

In roots, ABA inhibits PM H+-ATPase activity, and this effect is lost in a mutant lacking six ABA receptors (a sextuple *pyr1 pyl1 pyl2 pyl4 pyl5 pyl8* knockout), an overexpressor of the ABA-inhibited protein phosphatase 2C (*HAB1*), and a double mutant of ABA-activated protein kinases (*snrk2.2/snRK2.3*) (Planes et al., 2015). The ABA-activated protein kinase SnRK2.2 phosphorylates the C-terminal domain of AHA2 *in vitro*, but the specific phosphorylation site is unknown (Planes et al., 2015). However, in roots, ABA was not found to interfere with Thr-947 phosphorylation of the PM H+-ATPase.

Other Classical Plant Hormones

Gibberellic acid promotes Thr-947 phosphorylation in PM H+-ATPases, whereas cytokinin (kinetin) decreases phosphorylation at this residue (Chen et al., 2010). Depending on the experimental conditions, methyl jasmonate, as well as its mimic coronatine, a toxin secreted by strains of *Pseudomonas syringae*, may induce opening or closure of stomata (Yan et al., 2015; Zhou et al., 2015) and root hair formation (Zhu et al., 2015) via a mechanism that alters the regulation of the PM H+-ATPase. However, this mechanism is not clear and could be indirect.

PM H+-ATPASE REGULATION IS TARGETED BY MICROORGANISMS ATTEMPTING TO INVADE THE PLANT

The waxy cuticle of plant epidermal cells is an effective barrier for microorganisms attempting to invade plants, but when open the stomatal pore provides a convenient entry point for many pathogens (Melotto et al., 2006). A classic example of a pathogen that takes control of the PM H+-ATPase in guard cells to enforce opening of the stomatal pore is the fungus *Fusicoccum amygdali* (discussed above).

In general, following an encounter with a pathogen the first reaction of plants is to close their stomatal pores. The bacterial pathogen *P. syringae* pv *tomato* (*Pst*), which infects both tomato (*Solanum lycopersicum*) and *Arabidopsis thaliana* (Xin and He, 2013), is studied as a model organism in plant–pathogen interactions, and recently, exciting links between *Pst* infection and PM H+-ATPases were identified (Liu et al., 2009, 2011; Chung et al., 2014; Lee et al., 2015; Zhou et al., 2015). Mutant *Arabidopsis* lines with deregulated hyperactive AHA1 (*ost2-1D* and *ost2-2D*) have constitutively open stomata and, when sprayed with virulent *Pst* DC3000, these plants are much more susceptible to infection than are wild-type plants (Liu et al., 2009). However, when mutant plants are injected with the bacterium or sprayed with non-motile mutant bacteria, their susceptibility does not differ from that of wild-type plants. This suggests that the mobile bacteria enter the plant via the open stomata. Importantly, in mutant *ost2* lines expressing hyperactive AHA1, stomata fail to close in response to virulent *Pst* DC3000 (Liu et al., 2009). This supports the notion that inactivation of the PM H+-ATPase is part of the closing process.

What mechanism underlies pathogen-induced stomatal closure? Within minutes of *in vivo* elicitation with flg22, a fragment of bacterial flagellin, the phosphorylation status of AHA1 and AHA2 changes at three sites in the autoinhibitory C-terminal domain (Benschop et al., 2007; Nühse et al., 2007). Thus, phosphorylation at Thr-947 (the penultimate residue) and Thr-881 decreases, whereas phosphorylation at Ser-899 increases. Phosphorylation of Thr-947 is required for 14-3-3 protein binding (see above), whereas phosphorylation of Thr-881 activates the PM H+-ATPase (Fuglsang et al., 2014). Therefore, dephosphorylation at each of these two sites is expected to inactivate the pump. *In planta* phosphorylation of Ser-899 coincides with apoplastic alkalinization (Haruta et al., 2014), which is consistent with the assumption that phosphorylation at this site leads to pump inactivation. In summary, sensing of a flagellin fragment alters the phosphorylation levels of PM H+-ATPases at three sites, with each event independently leading to pump inhibition. This strongly suggests that effective inhibition of PM H+-ATPase contributes to stomatal closure following pathogen perception.

In plant genotypes sensitive to *Pst*, the pathogen takes control of the opening apparatus and, after a few hours, the stomata reopen. How does *Pst* bring about stomatal reopening? A pathogen target in this process appears to be the plant protein RPM1-INTERACTING PROTEIN 4 (RIN4). When RIN4 is overexpressed in *Arabidopsis*, the plants are much more susceptible to infection and, conversely, plants lacking RIN4 are more resistant (Kim et al., 2005). *rin4* knockout lines do not reopen their stomata following exposure to virulent *Pst* DC3000 (Liu et al., 2009).

The following lines of evidence indicate that RIN4 activates the PM H+-ATPase: (1) RIN4 interacts with the C-terminal regulatory domain of the PM H+-ATPases AHA1 and AHA2; and (2) when RIN4 is overexpressed, proton pumping by PM H+-ATPase increases (Liu et al., 2009). How RIN4 activates the PM H+-ATPase is not clear, and it is not known whether this process involves phosphorylation and/or 14-3-3 protein binding. It has been suggested that phosphorylated RIN4 associates with a different set of target proteins, possibly bringing one or more kinases in proximity to the C terminus of the AHA protein, leading to pump activation (Liu et al., 2011).

A simple model for stomatal reopening by pathogens that has emerged recently is based on the following observations: The ability of RIN4 to interact directly with the PM H+-ATPase increases when it is phosphorylated at three different positions (Lee et al., 2015) and, as demonstrated by phosphomimetic mutants of RIN4, a stronger activation is seen when all three residues are mutated to aspartate residues. The invading pathogen utilizes this feature by injecting a protein that triggers specific phosphorylation of RIN4 at Thr-166 (Chung et al., 2014). In the case of *Pst* DC3000, such a protein is AvrB (Chung et al., 2014; Zhou et al., 2015). The result is increased PM H+-ATPase activity and stomatal opening.

POSTTRANSLATIONAL REGULATION OF PM H+-ATPASES REGULATE IONIC TRANSPORT ACROSS THE PM

In *Arabidopsis*, AHA2 is the major root PM H+-ATPase (Harper et al., 1989), and the roots of *aha2* mutants exhibit a reduced ability to acidify their surroundings (Haruta et al., 2010). Although the growth of *aha2* plants is reduced in stress

conditions that reduce the plasma membrane proton motive force, these plants are more resistant to toxic cations, such as lithium and cesium, than are wild-type plants (Haruta et al., 2010; Haruta and Sussman, 2012). The absence of AHA2 is predicted to result in a lower membrane potential and, thus, a smaller driving force for the uptake of positively charged toxins; therefore, the acquired resistance to toxic cations in the absence of AHA2 supports the notion that PM H^+-ATPases powers nutrient uptake.

Among the abiotic stresses, salinity is a serious threat to plant growth. Unlike animals, plants do not have a primary active transport system that exports Na^+, and the only molecular system for Na^+ extrusion is the Na^+/H^+-antiporter SOS1, which is energized by the electrochemical gradient generated by the PM H^+-ATPase. As noted above, an overexpressed mutant form of PM H^+-ATPase that is constitutively active confers increased salt tolerance (Gévaudant et al., 2007). Likewise, overexpression of wild-type PM H^+-ATPase increases the fitness of plants under salt-stress conditions (Wang et al., 2013). Conflicting reports have appeared regarding changes in PM H^+-ATPase protein levels during salt stress in plants (Monneuse et al., 2011; Zhang et al., 2012), but recent evidence suggests that rapid posttranslational activation of the PM H^+-ATPase contributes to salt tolerance in halophytic species (Bose et al., 2015). Indeed, salt stress induces phosphorylation and 14-3-3 protein binding to the pump (Janicka-Russak et al., 2013).

In alkaline soil, the response to salt stress is different. If the growth medium of a plant is alkaline, the plant (neutral cytoplasmic pH) depends on the membrane potential to drive nutrient uptake and on an active plasma membrane H^+-ATPase. Accordingly, plant tolerance to alkaline pH increases in the absence of Salt Overly Sensitive 2-Like Protein Kinase5 (PKS5 kinase, also known as CIPK11 and SnRK3.22), which phosphorylates Ser-931 to inhibit PM H^+-ATPase activity (Fuglsang et al., 2007), and decreases in the absence of chaperone J3, which interacts with and represses PKS5 kinase activity (Yang et al., 2010). An even stronger phenotype is observed when plants are exposed to salt stress under alkaline conditions. In plants where the PM H^+-ATPase is deactivated due to increased PKS5 activity (e.g. in *pks5*-3, *pks5*-4, or *j3* mutant backgrounds), the sensitivity to salinity under alkaline conditions is markedly increased. The opposite is observed in plants with decreased PKS5 activity (e.g. in the *pks5-1* mutant background), which are more tolerant to salt at alkaline pH (Yang et al., 2010). A possible explanation for this is that under alkaline-stress conditions an inside negative potential supports Na^+ uptake, and the SOS1 antiporter lacks the proton motive force needed to extrude Na^+.

To mobilize phosphate bound to soil particles, plants secrete organic acids such as citrate. Citrate also chelates toxic Al^{3+} in the soil, and citrate exudation is therefore an important mechanism for achieving tolerance to aluminum. In several plant species, upregulation of this process correlates tightly with increased PM H^+-ATPase activity, phosphorylation of Thr-947, and increased binding of 14-3-3 protein (Shen et al., 2005; Tomasi et al., 2009; Chen et al., 2015). Thus, by creating an outside positive membrane potential, PM H^+-ATPase appears to contribute to the charge balance during secretion of the organic acid.

Many vascular plants form mycorrhizal symbioses with fungi to improve the uptake of nutrients, particularly phosphate. In arbuscular mycorrhiza, where the fungus forms an invagination directly into root cells, specific PM H^+-ATPase isoforms (such as HA1/MtAHA1 of *Medicago truncatula*) are required at the interface between the plant PM and the fungal hypha to facilitate import of phosphate into the plant cell (Krajinski et al., 2014; Wang et al., 2014a; Hubberten et al., 2015). Legumes engage in a symbiotic relationship with nitrogen-fixing bacteria in root nodules. In *M. truncatula*, a specific PM H^+-ATPase isoform (MtAHA5) expressed in roots is phosphorylated at multiple sites in the C-terminal domain within 1 h of treatment with secreted bacterial nodulation (Nod) factors (Nguyen et al., 2015). Two of the phosphorylated residues correspond to Thr-881 and Thr-947 in AHA2, which strongly suggests that Nod factors induce post-translational activation of the PM H^+-ATPase (Nguyen et al., 2015).

CONCLUDING REMARKS

Following the identification of an autoinhibitory domain in plant PM H^+-ATPases, it was suggested that multiple environmental stimuli that regulate plant growth target this domain (Palmgren, 1991). This hypothesis has been strongly supported in recent years and, in particular, important roles for PM H^+-ATPase regulation in guard cell and root physiology have emerged. Only a few of the protein kinases responsible for phosphorylating PM H^+-ATPase phosphosites have been identified; notably, the protein kinase targeting the penultimate Thr residue is still not identified. Investigations of the physiological role of protein phosphatases and phospholipids in PM H^+-ATPase regulation are also still in their infancy. Future studies should aim to elucidate all elements of the signal transduction pathways by which growth-controlling factors regulate PM H^+-ATPases, and establish how these pathways are integrated into the physiology of the plant.

SUPPLEMENTAL INFORMATION

Supplemental Information is available at *Molecular Plant Online*.

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