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Minireview

Plant proton pumps☆

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Abstract **Chemiosmotic circuits of plant cells are driven by proton (H^+) gradients that mediate secondary active transport of compounds across plasma and endosomal membranes. Furthermore, regulation of endosomal acidification is critical for endocytic and secretory pathways. For plants to react to their constantly changing environments and at the same time maintain optimal metabolic conditions, the expression, activity and interplay of the pumps generating these H^+ gradients have to be tightly regulated. In this review, we will highlight results on the regulation, localization and physiological roles of these H^+-pumps, namely the plasma membrane H^+-ATPase, the vacuolar H^+-ATPase and the vacuolar H^+-PPase.**

Keywords: H^+-ATPase; Plasma membrane; Vacuolar; H^+-PPases; Proton pumps

1. Plasma membrane H^+-ATPases: Physiological roles

1.1. Specific PM H^+-ATPases are expressed in cells specialized for transport

Solute flux across the plasma membrane depends to a large extent on the proton motive force available. As such, PM H^+-ATPases are important molecular players that determine and control plant nutrient acquisition and partitioning at the whole plant level (reviewed in Refs. [1–4]). Structure–function relationships of this pump have been reviewed recently [3]. Here we will focus on recent developments that throw new light on the physiological function and regulation of PM H^+-ATPases.

Since the completion of the sequencing of the genome of the model plant *Arabidopsis thaliana* in 2000, the Arabidopsis transcriptome has been characterized in a large number of gene array experiments. An overview of the expression profiles of members of the Arabidopsis PM H^+-ATPase family (*AHA1-11*) [5] can be found in the Genevestigator database (www.genevestigator.org). Two genes, *AHA1* and *AHA2*, are expressed in virtually all tissues and organs. Thus, these genes appear to function as housekeeping genes required for ion homeostasis. Relatively more *AHA1* transcript is found in shoots, whereas *AHA2* is predominantly expressed in roots, especially in root hairs.

AHA3, *AHA4* and *AHA11* also show broad expression throughout the plant, but are not expressed to the same degree. Some degree of specialization is seen as *AHA4* has high expression in root endodermis in accordance with reporter gene analysis studies [6]. Real time RT PCR analysis has confirmed that *AHA1*, *AHA2*, *AHA3* and *AHA11* are the major transcripts found in leaves [7]. Reporter gene analyses show that the *AHA3*promoter is active in leaf phloem companion cells [8].

Expression patterns of *AHA5* (low expression throughout the plant), *AHA6* and *AHA9* (predominantly expressed in anthers), *AHA7*and *AHA8* (almost exclusive expression in pollen) and *AHA10* (highest transcript levels in siliques) suggest that the pumps encoded by these genes have more specialized function. Reporter gene analyses have verified the expression of *AHA9* in anthers [9] and *AHA10* in the endothelium of the developing seed coat [10].

Are all these transcripts translated into proteins? All isoforms, except AHA8, have been detected by mass spectrometry-based proteomics, either in cotyledons, whole seedlings, leaves or stems [11–16]. Four isoforms have been detected by mass spectrometry in most investigations, namely AHA1, AHA2, AHA4 and AHA11 [11,13–16]. These isoforms correspond to the major gene transcripts in seedlings and leaves.

1.2. Single cells may contain more than one isoform of PM H^+-ATPase

Transcriptome analysis and cDNA library screening of *Vicia faba* stomatal guard cells detected expression of at least two plasma membrane H^+-ATPases, but none of these iso-

☆ This review will be divided in three sections. The first section describes the state of plasma membrane H^+-ATPase research and proposes a novel mechanism of H^+-ATPase regulation. The second section reports current vacuolar H^+-ATPase research including its recently identified role in endomembrane protein trafficking. The third section focuses on the H^+-PPase and new evidence that H^+-PPases function in regulating plant growth and development.

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forms are unique to guard cells [17]. Likewise, by fusing endogenous PM H^+-ATPase promoters with the GUS reporter gene and expressing the chimeric genes in transgenic plants, it could be shown that the promoters of two different tobacco PM H^+-ATPases, *NpPMA2* and *NpPMA4*, are active in guard cells [18]. In a recent study, RT-PCR was employed to amplify AHA sequences in isolated Arabidopsis guard cell protoplasts [19]. Surprisingly, all members of the Arabidopsis PM H^+-ATPase gene family (*AHA1-11*) were identified in this particular cell type. The major guard cell transcripts are *AHA1*, *AHA2* and *AHA5*. This confirms the presence of *AHA1* and *AHA2* as house-keeping genes in guard cells and points to *AHA5* as a candidate guard cell specific PM H^+-ATPase. However, as the method employed is very sensitive and not quantitative, it is not clear to what extent each AHA isoform is present in guard cells.

The male gametophyte of the plant is an independent haploid organism that expresses a specific subset of genes [20]. *Nicotiana plumbaginifolia* NpPMA5 H^+-ATPase is expressed in pollen tubes but in many cell types of the sporophyte as well [21]. Four different AHA transcripts have been identified in Arabidopsis pollen grains at different stages of development: *AHA6*, *AHA8*, *AHA9* and *AHA12* [20], the latter being a pseudogene [6]. *AHA12* is expressed at very low levels at the microspore and bicellular states, *AHA6* and *AHA9* peak at the tricellular state, whereas in mature pollen the *AHA8* isogene takes over as the all-dominant transcript. This suggests that during development one *AHA* isogene can take over the role of another.

Lastly, in barley aleurone cells, two isoforms of PM H^+-ATPase have been identified in proteomic analyses [22].

We can conclude that the plasma membrane of individual cells may harbor several PM H^+-ATPases. The reason for this diversity is not known, but might reflect a specific need for ATPases at specific times of development or with specialized functions [23,24].

1.3. Plants with altered PM H^+-ATPase contents

Genetic evidence for the physiological function of PM H^+-ATPase is very scarce, possibly as a result of redundancy among isogenes. PM H^+-ATPase can be overproduced in transgenic plants without changes in plant morphology [25]. Overproduction in tobacco of NpPMA4 resulted in co-suppression of PM H^+-ATPase(s) leading to stunted growth and developmental changes [25].

Knock-out mutagenesis has the potential to generate plants with reduced PM H^+-ATPase. *AHA3* appears to be an essential gene, as a homozygous knockout plant cannot be obtained after crossing heterozygote plants, which are fully viable [26]. A semi-dominant mutant allele of *AHA4*, which in principle could influence other isoforms in the same cell, gives rise to increased salt sensitivity [7], suggesting a role for this pump in controlling ion homoestasis in the endodermis. *AHA10* knockouts produce light-colored seeds with reduced proanthocyanis, the *transparent testa* phenotype, and have altered vacuole morphology [10]. This indicates that AHA10 protein in some way controls vacuolar loading of seed color pigments. Genetic studies involving multiple knockouts and conditional mutants are likely to throw more light on PM H^+-ATPase function in the future.

2. Plasma membrane H^+-ATPases: Post-translational regulation

2.1. There are few examples of regulation of PM H^+-ATPase at the transcriptional level

A large number of external signals result in changes in PM H^+-ATPase activity of plasma membrane vesicles isolated after the treatment. Activating signals include salt [27], and cold acclimation [28]. Inhibiting signals encompass fungal elicitors [29], low temperature [30], and sugar depletion [31].

Although the activity of PM H^+-ATPase is modulated by several physiological signals, there is little evidence that such factors alter PM H^+-ATPase protein abundance and/or gene expression. Unlike a number of genes that encode nutrient transporters and are activated following starvation or supply of specific solutes, PM H^+-ATPase genes show little transcriptional regulation in response to nutrient stresses.

Treatments that result in moderate changes of H^+-ATPase expression include high aluminum [32], iron deficiency [33], high sugar [34] and high salt [27,35]]. An effect of sugars (glucose, fructose and sucrose) on PM H^+-ATPase expression in tomato is specific for two isogenes (*LHA2* and *LHA4*) and is not induced by mannitol, excluding an effect of osmotic stress [34]. Salt and/or osmotic stresses increase the level of PM H^+-ATPase gene expression (*AHA2*in Arabidopsis; [35]) and H^+-ATPase protein content [27].

2.2. Signal transduction pathways leading to PM H^+-ATPase activation

Changes in transcriptional levels are often uncoupled from corresponding changes in protein activities and/or solute fluxes. It is therefore likely that post-translational modifications of plasma membrane H^+-ATPases, rather than the amount of transcript and native protein, determine the activity and downstream developmental attributes of a plant. Regulated exocytosis of secretory vesicles containing already synthesized PM H^+-ATPase is an alternative means to increase PM H^+-ATPase in the plasma membrane and might occur in response to auxin treatment [36].

Phosphorylation and dephosphorylation of proteins is a very common example of a post-translational modification that has the potential to alter protein activity. Short-term post-translational regulation of the PM H^+-ATPase *in planta* targets the C-terminal autoinhibitory domain of the pump (reviewed in Ref. [3]). Thus, promotion or inhibition of pump activity in both cases result from modulating phosphorylation-dependent binding of activating 14-3-3 protein to this domain. The binding of 14-3-3 protein depends on phosphorylation of a Thr residue in the conserved C-terminal sequence motif HYTV (Thr-947 in Arabidopsis AHA2; see [37]). Single particle analysis of the PM H^+-ATPase/14-3-3 protein complex shows that it takes the form of wheel-like structure with six PM H^+-ATPases held together by six 14-3-3 proteins [38,39].

The first phosphorylation site to be identified *in vivo* was the penultimate residue (a Thr corresponding to AHA2 Thr-947) of a spinach PM H^+-ATPase [40]. Subsequently, characterization of phosphorylated peptides derived from Arabidopsis plasma membranes by mass spectrometry has shown that the corresponding Thr residue is phosphorylated *in vivo* in AHA1, AHA2 as well as AHA4 and/or AHA11 (the peptide could have been derived from either isoform) [13,14]. These

studies further show that additional *in vivo* phosphorylation sites exist in the C-terminal domain of AHA1 and AHA2 (Ser-899 and Ser-904) within a conserved sequence motif $GS^{899}YRELS^{904}E$. Protein kinases that phosphorylate PM H^+-ATPase have not been identified. Pump dephosphorylation might involve protein phosphatase 2A (PP2A) as the regulatory subunit of PP2A binds to the C-terminus of PM H^+-ATPase [41].

Stomatal guard cells function as osmotically-driven motors powered by the PM H^+-ATPase and respond to a wide variety of external signals. Blue light reception in guard cells is mediated by phototropins, which initiate a signal transduction pathway that involves an upstream protein phosphatase 1 and a downstream protein kinase that phosphorylates the penultimate C-terminal residue of the PM H^+-ATPase to induce binding of an activating 14-3-3 protein [19,42]. Under drought conditions, closure of the stomatal pore is induced by abscisic acid by a mechanism that involves production of hydrogen peroxide and dephosphorylation of the PM H^+-ATPase [43].

Pulvinar motor cells at the base of leaves function in a similar manner as osmotically driven engines that control leaf movements, which are driven by PM H^+-ATPase. Blue light triggers leaf movements in *Phasaeolus vulgaris* by decreasing turgor pressure in irradiated cells [44]. Like in stomatal guard cells, the response appears to involve phototropins as blue light receptors, but in contrast to guard cells, the result is dephosphorylation of PM H^+-ATPase and a concomitant decrease in PM H^+-ATPase activity.

Toxic levels of aluminum in the soil induces citrate secretion from roots of *Glycine max* to complex aluminum ions by a mechanism involving upregulation of PM H^+-ATPase activity [32]. Increased H^+-ATPase activity is associated with increased threonine-oriented phosphorylation of the pump. Addition of non-metabolizable sugars to roots cause a significant drop in PM H^+-ATPase activity without affecting pump protein levels [31]. The lower pump activity is associated with phosphatase-mediated dephosphorylation of the C-terminal 14-3-3 binding site, which in turn inhibits association of activating 14-3-3 protein with the pump.

2.3. Why is PM H^+-ATPase more effectively regulated at the post-translational level?

Experiments from guard cell protoplasts demonstrate that, although blue light induces only a moderate increase in ATP hydrolytic activity, it promotes a greater activation of proton pumping by PM H^+-ATPases [45]. It appears that native PM H^+-ATPases exist in a partially uncoupled state, and that post-translational activation increases tight coupling between ATP hydrolysis and proton pumping [46–48]. Without this coupling, the native pump is very inefficient and exhibits a coupling ratio between protons pumped per ATP hydrolysed less than 1. Why such futile ATP cycling occurs is not known, but post-translational induction of tight coupling might provide a means for the pump to very rapidly respond to the environment.

What could the mechanism behind uncoupling of the PM H^+-ATPase be? PM H^+-ATPases are P-type ATPases that generate phosphorylated reaction cycle intermediates, (not to be confused with stable regulatory phosphorylation) [3]. K^+ is bound to the proton pump at a site involving Asp-617 in the cytoplasmic domain that is phosphorylated during catalysis [49]. Binding of K^+ to this site induces dephosphorylation of the reaction cycle intermediate by a mechanism involving Glu-184 in the conserved TGES motif of the pump phosphatase domain [49]. Direct dephosphorylation of the reaction cycle intermediate will causes reversion of the pump to an unphosphorylated state without concomitant proton transport. These data identify K^+ as an intrinsic uncoupler of the proton pump. It remains to be tested whether the C-terminal domain of the pump controls the efficiency of this effect.

2.4. PM H^+-ATPases may be regulated by proteins organized in DRMs

The recent identification of PM H^+-ATPase in plasma membrane microdomains resistant to solubilization by mild detergents (detergent resistant microdomains, or DRMs) [16,50] suggests that the plant plasma membrane contains distinct niches that require PM H^+-ATPase functions. All AHAs except AHA3, AHA7 and AHA8 have been detected in DRMs [16]. DRMs isolated from tobacco plasma membranes harbors at least six PM H^+-ATPase isoforms, namely NpPMA2, NpPMA3, NpPMA4, NpPMA5, NpPMA6 and NpPMA9 [50]. The association of PM H^+-ATPase with membrane microdomains provides a straightforward explanation why immunodetection studies consistently show the protein to be present in patches at the plasma membrane (e.g. Ref. [51]). A large number of proteins with known regulatory functions are associated with plant DRMs, including leucine-rich repeat receptor kinases, other protein kinases, hetero-trimeric proteins, GTP-binding proteins and 14-3-3 proteins [16]. This suggests that perception of extracellular signals by receptors in membrane microdomains can result in rapid modulation of the activity of closely associated plasma membrane H^+-ATPases.

3. Vacuolar H^+-ATPase

Among the three proton-pumps found in plant cells, the vacuolar H^+-ATPase (V-ATPase) is the oldest and most complicated enzyme. V-ATPases share a common ancestor with the F-ATPases and the archaebacterial A-ATPases and are distributed throughout all kingdoms of life. Like their congeners, V-ATPases are multisubunit enzymes composed of two subcomplexes: The peripheral V_1 complex consisting of eight subunits (A; B, C, D, E, F, G and H) responsible for ATP hydrolysis, and the membrane-integral V_0 complex comprising up to six subunits (a, c, c′, c″, d and e) responsible for proton translocation (see Fig. 1). Compared to the F-ATPase, the structure of the V-ATPase holocomplex is emerging only slowly. However, structural models based on electron microscopy low-resolution maps of V-ATPases from all eukaryotic kingdoms including plants combined with atomic structures of individual subunits determined by X-ray crystallography show that the general structure of V- and F-ATPases is remarkably similar, suggesting that they might also share a common mechanical design [52]. Indeed, the elegant experiments that visualized rotation of the central stalk in bacterial F-ATPase [53] have been successfully repeated for eukaryotic V-ATPases [54]. Although this leaves little doubt that rotational catalysis is the common mechanism, the rotation kinetics of V1 and F1 are different [55]. Moreover, the fact that

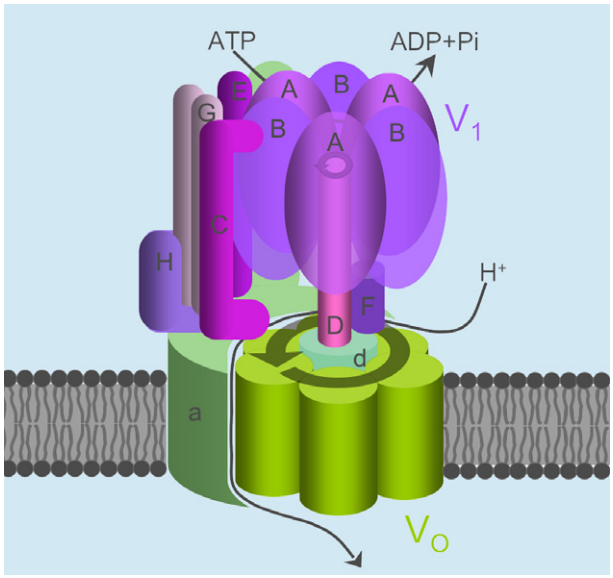

Fig. 1. Schematic model of a plant V-ATPase. The peripheral V_1 complex (purple) and the membrane-integral V_0 complex (green) are joined through a peripheral stalk formed by subunits VHA-a,-C, -E, -G and -H. ATP hydrolysis by the VHA-AB hexamer brings about rotation of the central shaft consisting of subunitsVHA-D and VHA-F, together with the proteolipid-ring made of five copies of VHA-c and one copy of VHA-c″. Rotation of the c-ring promotes H^+-transport across the membrane. Not depicted in this model is VHA-e. Modified after [52].

V-ATPases have additional subunits (C, H, d and e) not found in their bacterial relatives, speaks to the fact that their regulation is likely to be much more complex.

3.1. The even more complex V-ATPase of higher eukaryotes

The *Saccharomyces cerevisiae* V-ATPase has emerged as an important model for V-ATPase structure and function in all eukaryotic cells [56]. However, the fact that in plants, like in all higher eukaryotes, many of the V-ATPase subunits are encoded by gene families, adds an additional layer of complexity that cannot be addressed in the yeast model. In Arabidopsis, the 13 subunits (no subunit c′ is found in plants) are encoded by a total of 27 VHA-genes [57] and if all possible isoform combinations are used we will have to face hundreds of different V-ATPase complexes. We are only beginning to learn how much of this potential is used and what its functional significance is. VHA-A and VHA-c belong to the most highly conserved eukaryotic proteins and it is therefore not surprising that the isoforms found within a species show very little, if any, divergence at the protein level. In the case of the two Arabidopsis genes VHA-c1 and VHA-c3, identical proteins are encoded but whereas VHA-c1 is expressed ubiquitously, VHA-c3 expression is limited to root caps [58]. Similarly, VHA-A1 of tomato is found ubiquitously whereas VHA-A2 is restricted to roots and fruits [59]. In such cases, it seems more likely that the underlying gene duplications were followed by changes in regulatory sequences that allowed evolution of more complex and adaptive expression patterns. However, although the two tomato VHA-A isoforms are 95% identical, in the tissues in which they are co-expressed, they do not occur in the same V-ATPase complexes indicating differences in protein function [59]. Functional differentiation has also been reported for Arabidopsis VHA-B1 and VHA-B3 [60] that go back to a recent duplication in the Arabidopsis genome [57] and are 98% identical. Differences in V-ATPase activity between plant organs or tissues or after changes in the nutritional status have been reported and could either be correlated with changes in the overall structure of the complex or with the presence of individual protein isoforms (reviewed in: [61]). Determining how the Arabidopsis isoforms affect the kinetic and regulatory properties of the holocomplex will, as long as it relies exclusively on measuring enzyme activities in individual tissues or at different time-points, remain a challenging task. However, the use of mutants that lack individual isoforms could greatly facilitate this.

3.2. Mutants to the rescue

Over the past years, T-DNA insertions in a number of VHA-genes have been characterized. Insertions in single-copy genes (*vha-A*, *vha-C*) cause male gametophytic lethality [62] (Liu and Schumacher, unpublished). A *vha-H* mutant has no obvious phenotype [60], however, the T-DNA in *vha-H* is located in an intron leading only to a mild reduction in *VHA-H* mRNA (Wang and Schumacher, unpublished).

For the subunits encoded by more than one gene, the null-mutant phenotypes range from embryo-lethal in the case of *vha-E1* [63] to indistinguishable from wildtype in the case of *vha-B3* (Wang and Schumacher, unpublished). VHA-E1 is a ubiquitous isoform, whereas VHA-E2 is pollen-specific and VHA-E3 expression is restricted to certain tissues [63]. Ubiquitous expression of *VHA-E2* and *VHA-E3* in the *vha-E1* null background would allow addressing the functional divergence of the Arabidopsis VHA-E isoforms. In a similar manner, expression of the tomato VHA-A isoforms in the Arabidopsis *vha-A* mutant would allow to compare their catalytic activities.

3.3. What do we learn from mutant analysis?

At first glance, the lethal loss-of-function phenotypes seem almost trivial as they simply confirm that the V-ATPase is an important enzyme. Although it has been pointed out since the early days of plant V-ATPase research that this pump is found in all compartments of the endomembrane system, it is still frequently perceived as a purely ‘‘vacuolar’’ enzyme. This can in part be explained by the fact that, in the absence of functional evidence, V-ATPases in other compartments could simply be in transit to their final destination at the tonoplast. Therefore, upon closer inspection, the mutant phenotypes have helped to reveal that the activity of the V-ATPase is essential for structure and function of the Golgi apparatus [63,64]. In the light of this result, it seems likely, that the reduced cell expansion observed in plants with reduced VHA expression [58,65] is not solely due to reduced secondary transport of solutes across the tonoplast causing a lack of turgor pressure. A limiting role of the Golgi V-ATPase in cell expansion would also explain why plants that lack the vacuolar H^+/Ca^{2+}-transporter CAX1 do not show a severe growth phenotype [66]. In *cax1* mutants, for reasons that remain to be identified, the activity of the tonoplast V-ATPase is reduced to a similar extent as the total V-ATPase activity in the severely dwarfed *de-etiolated 3* (*det3*) mutant. The *det3* mutation leads to reduced levels of the single-copy encoded subunit VHA-C and therefore affects all V-ATPases [65].

The finding that VHA-a1, one of three Arabidopsis VHA-a isoforms, is preferentially localized in the trans-Golgi network [67] is further proof that plant V-ATPases, like their counterparts in yeast and animals, play important roles in endocytic and secretory trafficking. Indeed, Concanamycin A (ConcA), a specific V-ATPase inhibitor, that has been shown to inhibit secretion and to cause mistargeting of vacuolar proteins in tobacco BY2-cells [68], also blocks trafficking of proteins to the plasma membrane as well as endocytic transport from the TGN to late endosomes in Arabidopsis [67]. Interestingly, *clc-d*, a mutant lacking a TGN-localized member of the Arabidopsis ClC-family of anion transporters (see review by Barbier-Brygoo in this issue), shows hypersensitivity towards ConcA [69]. In animal cells, ClC-mediated anion transport provides the shunt conductance necessary to maintain electrogenic proton transport by V-ATPases [70] and it seems very likely that ClC-d plays a similar role in Arabidopsis.

3.4. Interactions with other proteins

Given the importance that coordinated regulation of all three proton-pumps must have for pH-homeostasis in the cytosol, it is surprising how little we know about common regulatory mechanisms. 14-3-3 proteins, well-known activators of P-type ATPases, would be ideal candidates and have indeed recently been shown to interact specifically with barley VHA-A [71]. This interaction is phosphorylation dependent and in vitro phosphorylation of VHA-A by an unknown tonoplast-associated protein kinase [71] and also by WNK8, a protein kinase identified by its specific interaction with VHA-C [72], has been demonstrated.

3.5. Are V-ATPases more then just ''simple'' proton-pumps?

Last but not least, evidence that V-ATPase subunits can have additional functions independent of proton pumping has added yet another layer of complexity to the biology of V-ATPases. A function for V_0 subunits in membrane fusion was demonstrated for homotypic vacuole fusion in yeast [73] as well as for synaptic vesicle fusion in Drosophila [74]. Mammalian subunit a4 has been shown to act as a pH-sensor that is able to communicate luminal pH to cytosolic proteins [75]. Recent results from Arabidopsis are perhaps even more surprising: VHA-B1 has been identified in nuclear complexes with hexokinase and the phenotype of a*vha-B1* mutant indicates that this subunit is involved in glucose signaling [60]. If this holds true, it will be extremely interesting to see if other V-ATPase subunits also lead double-lives.

4. H^+-PPases: A conserved family of PPi-driven H^+-pumps

4.1. PPi is a byproduct of biosynthetic processes characteristic of actively growing cells

H^+-pyrophosphatases (H^+-PPase; EC 3.6.1.1) are highly hydrophobic (14–17 α-helical transmembrane domains) single-subunit proteins of about 80 kDa believed to function as homodimers in the generation of proton gradients across endomembranes using the energy of the phosphoanhydride bond of pyrophosphate (PPi) molecules [76–78]. They are found mainly in higher plants, some protozoa, and several species of eubacteria and archeubacteria [79,80]. Prototypical plant H^+-PPases have overall amino-acid sequence identities of 85% or greater, and they have been localized to vacuolar, Golgi, and plasma membranes [76,79,81–84]. Plants have two phylogenetically distinct types of H^+-PPases: type I that depend on cytosolic K^+ for their activity and are moderately sensitive to inhibition by Ca^{2+}, and type II which are K^+-insensitive but extremely Ca^{2+}-sensitive. In an elegant study combining phylogenetic analyses and site-directed mutagenesis, Belogurov and Lahti demonstrated that the replacement of a conserved alanine residue at position 460 by a lysine converted the K^+-dependent H^+-PPase from *C. hydrogenoformans* into K^+-independent form. This transition appeared to be due to a simple substitution of K^+ with the NH^{3+} group of the lysine residue [85]. Both types of H^+-PPase require Mg^{2+} as a cofactor [77,79,83,86]. What these varying metal requirements and sensitivities mean at the physiological level remains obscure and requires further investigation.

4.2. Bacterial H^+-PPases aid to decipher molecular details of plant H^+-PPases

The high degree of homology (86–91% at the amino acid level) observed between vacuolar H^+-PPases from higher plants and bacteria allows for meaningful structure–function experiments to be performed in more amenable systems [77,79]. Using cysteine-scanning analysis of cysteine substitution mutations of the type II H^+-PPase (ScPP) from *Streptomyces coelicolor* expressed in *E. coli*, Mimura et al. determined the topological arrangement of conserved motifs and transmembrane domains in ScPP [80]. Based on these results, they propose a topological model (see Fig. 2) applicable to all H^+-PPases. In this model, the catalytic region that contains Mg-PPi binding, PPi hydrolysis and energy conversion motifs is formed by five cytoplasmic loops (*e*, *i*,*k*, *m* and *o*), three of which are conserved among all H^+-PPases [80]. Furthermore, this model concurs with earlier models derived from immunochemical analysis and computer algorithms predicting that the C-terminal segment of plant H^+-PPases is exposed to the cytoplasm [76,77,79,80,87]. However, Van et al. have presented an alternative topological model for plant H^+-PPases using the TopPred II algorithm that predicts the C-terminal segment exposed to the vacuolar lumen [88,89].

Site-directed thiol cross-linking and selective oxidation experiments with the H^+-PPase of ScPP suggest that the higher order oligomeric state of this enzyme consists of at least two or three sets of dimers [90] and that two opposing cysteine residues ($Cys^{253}$and Cys^{621}) are required for reversible enzyme inactivation by oxidation [91]. As all known type II, but not type I, H^+-PPases share cysteine residues corresponding to the Cys^{253} and Cys^{621} of ScPP, the authors speculate that redox control of enzyme activity could be unique to type II H^+-PPases [91]. However, as Cys^{621} of ScPP is conserved among type I H^+-PPases, the authors further speculate that intermolecular disulfite cross-linking might be a redox mechanism for the regulation of type I H^+-PPases [91]. These findings suggest that plant H^+-PPases may be regulated in a manner similar to what is seen in the barley V-ATPase which has been reported to be inhibited by H_2O_2 and reactivated by reduced glutathione *in vitro* [92]. As H_2O_2 generated by plasma membrane NADPH oxidases can function as a signaling molecule in stress and growth responses (reviewed in [93,94]). It is quite possible that redox control of these H^+-gradient generators may regulate multiple downstream processes.

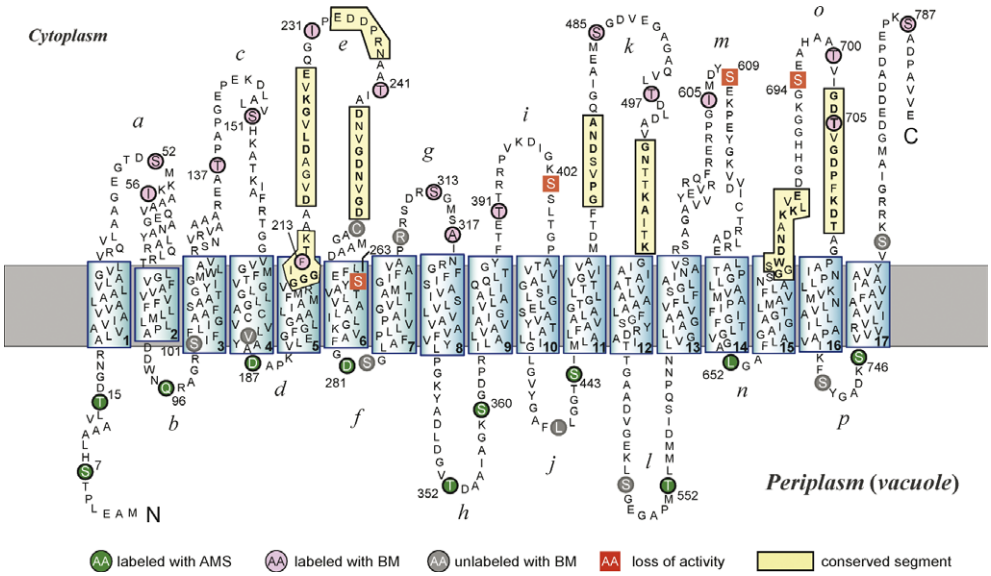

Fig. 2. Model of the membrane topology of *Streptomyces coelicolor* H^+-PPase. The transmembrane domains (1–17) are boxed and numbered. Conserved motifs, such as DVGADLVGKVE, are marked with yellow boxes. The residues replaced with cysteines in the cysteine-less H^+-PPase mutant for cysteine-scanning mutagenesis are marked with circles. Residues that are accessible from the periplasm are indicated by green circles and those that are not accessible are indicated by light purple circles. Residues that are in a hydrophobic environment are indicated by white letters in gray circles. Residues that cause inactivation of the enzyme by substitution with cysteine are indicated by red squares. Plant vacuolar H^+-PPases lack the 17th transmembrane domain and the C-terminal tail. This is a modified version of Figure 8 reported by Mimura et al. [80].

Other interactions may also regulate H^+-PPase activity. A recent sequence analysis of two vacuolar H^+-PPases from *Vitis vinifera* identified a putative 14-3-3 interaction motif (RQFNTIP) that is conserved among several type I H^+-PPases [95]. However, experimental evidence of 14-3-3 interactions with H^+-PPases remains to be demonstrated.

4.3. Heterologous expression of plant H^+-PPases in yeast facilitates the identification of structural determinants of function

Using site-directed mutagenesis on the mung bean (*Vigna radiata*) H^+-PPase, Hsiao et al. generated a series of mutants in which conserved histidine residues were singly replaced by alanines [96]. The enzymatic and proton pumping activities were tested in the budding yeast *Saccharomyces cerevisiae*. Only the mutation of H716 significantly decreased the enzymatic activity, proton transport, and the coupling ratio of the mung bean H^+-PPase [96]. Interestingly, H716 is proximal to the DX_3DX_3D acidic domain in the cytosolic loop *o* (see Fig. 2) [80,96]. The transmembrane domain 5 (TM5) is highly conserved among plant H^+-PPases and has a conspicuous lower degree of hydrophobicity that suggest it could be involved in proton translocation [89]. Alanine scanning mutagenesis approach was used to identify critical amino acid residues along TM5 domain of mung bean H^+-PPase [89]. The important reduction in apparent coupling ratios (50% of controls) of mutants in the GYG motif localized at the middle of the TM5 suggested that this motif might be directly or indirectly involved in proton translocation [89]. A site directed mutagenesis study with the type I H^+-PPase AVP1 from *Arabidopsis thaliana* expressed in yeast revealed that neither E427 nor K461 (associated with TMDs 9 and 10, respectively) play a direct role in H^+ translocation. The authors suggested that interactions between these two residues have a structural impact favoring H^+ translocation [97].

Plant H^+-PPases have relative conserved amino acid sequences at the C-terminal region that presumably locates in the vicinity of the cytoplasmic loops of the catalytic region [79,80,98]. Biochemical characterization of C-terminal deletion mutants of mung bean H^+-PPase expressed in yeast revealed that the removal of the last 5 amino acid residues (ΔC5) resulted in a dramatic inhibition of PPi hydrolysis, H^+ translocation and coupling efficiency. However, a mutant lacking the last 10 amino acids (ΔC10) significantly restored the enzyme's activity. The authors speculate that in the ΔC5 mutant the C-terminal moiety could be introduced into potential hydrophobic surroundings, and that in the ΔC10 mutant this possibility does not stand because of the nature of the remaining amino acids [98]. PPi hydrolysis and proton pumping activities were lost in mutants with larger deletions (ΔC14, ΔC20, and ΔC25) [98]. Interestingly, ΔC10 mutants showed a higher K^+ binding constant (0.23 mM^{-1}) than that of the wild type enzyme (0.13 mM^{-1}), suggesting that the C-terminus of plant H^+-PPases is not only required to sustain the enzymatic activity but that it may be involved in K^+-dependent regulation [98].

5. Role of plant H^+-PPases in growth and development

5.1. Characterization of avp1-1 Arabidopsis null mutant revealed a previously unrecognized role for H^+-PPases in auxin transport

A potential role for plant H^+-PPases in growth and development was hinted by a study of pear fruit development, showing that both protein levels and activity of a type I H^+-PPase were enhanced in young fruit at the cell division stage [99]. In keeping with these early observations, Li et al. reported that the overexpression of the type I H^+-PPase AVP1 in Arabidopsis resulted in increased cell division at the onset of organ

formation, hyperplasia, and increased auxin transport. Furthermore, *avp1-1* null mutants displayed severely disrupted root and shoot development and reduced auxin transport (Fig. 3). Changes in the expression of AVP1 affected the abundance and activity of the plasma membrane (PM) H^+-ATPase that correlate with apoplastic pH alterations and auxin transport efficiencies [81]. Therefore, it has been hypothesized that in addition to its established role in the maintenance of vacuolar pH, the type I H^+-PPase of *Arabidopsis thaliana* facilitates the trafficking of the P-type H^+-ATPase to the PM [81]. Support for this hypothesis comes from early immunogold electron microscopy observations that confirmed the presence of the H^+-PPase at the PM, trans Golgi network, multivesicular bodies and tonoplast of cauliflower inflorescence cells [84]. Furthermore, double labeling experiments (H^+-PPase after PM H^+-ATPase) of PM from *Ricinus communis*sieve elements resulted in reduced staining of the H^+-PPase. This observation is consistent with the localization of both H^+-pumps in close proximity at the PM [100]. PM localized H^+-PPases are unlikely to function directly in apoplastic acidification [101], but their role in endosomal acidification is thermodynamically feasible and their presence at the PM could be explained as a dynamic one. Of significance, H^+-PPase immunogold studies with *Ricinus communis* seedlings have shown strong PM staining at phloem tissues of cotyledons and roots, whereas in the mesophyll and cortical cells the staining was mainly vacuolar [82]. Interestingly, tissues in which H^+-PPase has been shown to localize at the PM [82,84,100] are also tissues involved in polar auxin transport [102]. Zandonadi et al. reported that exogenous indoleacetic (IAA) and humic acids (HA) induced lateral root development through a concerted activation of PM and tonoplast H^+-pumps from maize roots [103]. The H^+-PPase showed sensitivity to a broader range of IAA than the other two pumps [103]. Consistent with the model presented by Li et al. these studies suggest a more upstream role for H^+-PPases in mediating the cascade of events leading to the activation of the other H^+-pumps [81]. Further research is needed to gain an insight into the *in vivo* localization of plant H^+-PPases and their possible role in mediating auxin fluxes that regulate organogenesis. It is important to emphasize that

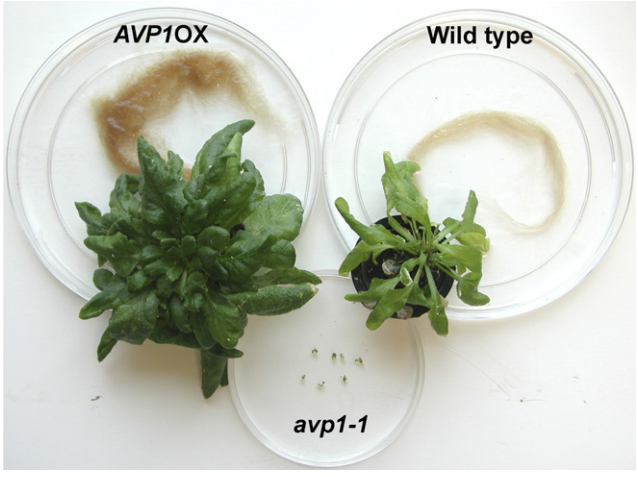

Fig. 3. Phenotypes associated with gain- and loss-of-function mutants of Arabidopsis type I H^+-PPase AVP1. AVP1 overexpressing line *AVP1*OX and Col-0 wild type hydroponically grown for 40 days. Loss-of-function *avp1-1* 20-day-old seedlings grown in solid media.

the severity of the developmental phenotypes associated with *avp1-1* in the Col-0 background is not obvious in other Arabidopsis ecotypes (Yang and Gaxiola, unpublished). This discrepancy suggests that the mechanism by which AVP1 mediates mobilization of other H^+-pumps may be multi-factorial.

5.2. Environmental cues and growth regulators involved in the regulation of expression and/or enzymatic activities of plant H^+-PPases

In a different physiological scenario, stimulation of the H^+-pumping activity of the vacuolar ATPase of *Kalanchoe blossfeldiana* by activation of the H^+-PPase has been documented during high Crassulacean acid metabolisms CAM [104]. Co-precipitation of both pumps argues for a close localization *in vivo* [104]. Transcriptional regulation of two barley H^+-PPase genes (*HVP1* and *HVP10*), a gene coding for the catalytic subunit of the vacuolar H^+-ATPase (*HvVHA-A*) and a gene coding for a Na^+/H^+ antiporter (*HvNHX1*) was monitored in response to additions of exogenous abscisic acid (ABA), auxin and gibberellin (GA). While all the above genes were induced upon ABA treatment, GA had no effect. Auxin induced the expression of the *HVP1*and the *HvNHX1* genes but not of the *HVP10* nor the *HvVHA-A*genes suggesting a hormone specific *modus operantis* for the regulation of each of the H^+-PPases and vacuolar H^+-ATPase [105]. It is puzzling that the expression and activity of antiporter and both H^+-pumps are enhanced upon treatments with these two antagonistic growth regulators. The physiological meaning of these results needs further investigation. A report by Zhang et al. describes a connection between nitric oxide signal transduction and enhanced salt tolerance in maize via the stimulation of H^+-PPase and H^+-ATPase activities [106]. It is not clear if the enhanced H^+-pumping activities are due to changes in abundance or regulation of function. A study with suspension-cultured cells of *Catharanthus roseus* revealed that H^+-pumping activities of vacuolar H^+-PPase and H^+-ATPase were activated under Pi limiting conditions. Interestingly, Western blot analysis revealed no change in protein abundance [107]. Similar results were obtained with rye plants grown under conditions where the mineral nutrients were diluted 1000 fold. Here again the changes on both H^+-pumps were qualitative [108]. An intriguing finding relates to the restricted expression of the maize *Vpp1* gene encoding for a H^+-PPase to the aleurone layer [109]. Its expression identifies it as an aleurone cell fate developmental marker, but its physiological role remains obscure and requires further investigation.

6. Overexpression of H^+-PPases as a potential biotechnological approach

6.1. Enhanced expression of H^+-PPases improves plant growth in the presence of saline and water deficit stress conditions

Overexpression of the type I H^+-PPase AVP1 in Arabidopsis resulted in plants with enhanced salt tolerance and drought resistance. The salt tolerant phenotype of these plants was explained by an increased uptake of Na^+ into their vacuoles. The drought related phenotype was originally attributed to an enhanced vacuolar osmoregulatory capacity [110]. However, further characterization of these AVP1-overexpressing plants revealed a dramatic enhancement of their root development

(see Fig. 3), with obvious implications for their ability to withstand drought [81]. In keeping with these observations, a commercial cultivar of tomato (*Lycopersicon esculentum*) engineered to overexpress the Arabidopsis type I H^+-PPase displayed enhanced recovery capacity when challenged with soil water deficit stress conditions. Furthermore, this phenotype resulted from the development of more robust root systems [111]. Improved salt tolerance has been achieved in tabacco plants engineered with either a H^+-PPase of the bacterium *Rhodospirillum rubrum* [112] or a H^+-PPase from the halophyte *Thellungiella halophila* [113]. In the latter report, a comparison between the performance of the halophyte H^+-PPase and the Arabidopsis AVP1 pump showed no difference [113]. The salt tolerant phenotype of rice plants expressing a Na^+/H^+ exchanger of *Suaeda salsa* was compared to plants engineered to co-express this exchanger with the Arabidopsis AVP1 H^+-PPase. Co-expressing plants outperformed controls and single Na^+/H^+ transgenics [114]. Guo et al. reported that the expression in Arabidopsis of a H^+-PPase from the halophyte *Suaeda salsa* confers salt and drought tolerance [115]. Similarly, the overexpression of the wheat H^+-PPase TVP1 improved Arabidopsis salt- and drought-tolerance [116]. Unfortunately, none of these recent reports analyze the root systems of the engineered plants. Only Zhao et al. make mention of root systems, reporting that the rice plants co-expressing the Na^+/H^+ exchanger and the H^+-PPase have an enhanced rhizosphere acidification capacity [114]. It is tempting to speculate that the increased root proliferation and apoplast/rhizosphere acidification capacities evident in Arabidopsis, tomato and rice plants engineered to overexpress the type I H^+-PPase AVP1 [81,111,114] could be instrumental in producing plants that exhibit increased resilience to mineral deficiencies.

Although there has been a significant advance in our understanding of plant H^+-PPases, many unanswered questions remain. Among the most critical are the following:

- Is the localization (i.e., tonoplast, endosomes, plasma membrane) of plant H^+-PPases tissue dependent varying with developmental and/or environmental cues?
- What is the meaning of H^+-PPases co-precipitating and/or co-localizing with either vacuolar or PM H^+-ATPases?
- Why do plants have K^+-dependent and K^+-independent H^+-PPases? Is there a physiological meaning?
- Does the number of isoforms of H^+-PPases present in a given plant genome (i.e., Arabidopsis 2 isoforms, rice 5 isoforms) relate to their growth habits?

7. Concluding remarks

Analysis of Arabidopsis mutant phenotypes has revealed that, like their counterparts in yeast and animals, V-ATPases play important roles in endocytic and secretory trafficking. Further, manipulation of the type I H^+-PPase AVP1 contributes to the regulation of apoplastic pH and to auxin transport, likely by mediating the trafficking of PM H^+-ATPase and associated proteins. Regulated exocytosis has been suggested to increase PM H^+-ATPase protein in the plasma membrane in response to auxin, but the involvement of other H^+ pumps in this process remains to be investigated. It is plausible to speculate a scenario where the trafficking of proteins destined to vacuoles, endosomes and plasma membrane is energized by the regulated activity of one or more H^+-pumps. For example, increased H^+-PPase-driven vesicular trafficking may be favored in actively dividing tissues where both ATP consumption and PP_i generation are high. The unexpected link between a PM H^+-ATPase (AHA10) and vacuolar accumulation of secondary metabolites suggests that PM H^+-ATPases control energization of the vacuolar membrane either directly or indirectly, e.g. via control of vesicle trafficking. The dynamic plasticity of all these H^+-pumps, as well as their fundamental role in maintenance of cytosolic pH-homeostasis, may require a set of shared regulatory mechanisms. 14-3-3 proteins, well-known activators of PM H^+-ATPases, would be ideal candidates and have recently been shown to interact specifically with the V-type H^+-ATPase. Furthermore, a putative 14-3-3 interaction motif has been identified in H^+-PPases.

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