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Review

The plant plasma membrane H^+-ATPase: structure, function and regulation

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Abstract

The proton-pumping ATPase (H^+-ATPase) of the plant plasma membrane generates the proton motive force across the plasma membrane that is necessary to activate most of the ion and metabolite transport. In recent years, important progress has been made concerning the identification and organization of H^+-ATPase genes, their expression, and also the kinetics and regulation of individual H^+-ATPase isoforms. At the gene level, it is now clear that H^+-ATPase is encoded by a family of approximately 10 genes. Expression, monitored by in situ techniques, has revealed a specific distribution pattern for each gene; however, this seems to differ between species. In the near future, we can expect regulatory aspects of gene expression to be elucidated. Already the expression of individual plant H^+-ATPases in yeast has shown them to have distinct enzymatic properties. It has also allowed regulatory aspects of this enzyme to be studied through random and site-directed mutagenesis, notably its carboxy-terminal region. Studies performed with both plant and yeast material have converged towards deciphering the way phosphorylation and binding of regulatory 14-3-3 proteins intervene in the modification of H^+-ATPase activity. The production of high quantities of individual functional H^+-ATPases in yeast constitutes an important step towards crystallization studies to derive structural information. Understanding the specific roles of H^+-ATPase isoforms in whole plant physiology is another challenge that has been approached recently through the phenotypic analysis of the first transgenic plants in which the expression of single H^+-ATPases has been up- or down-regulated. In conclusion, the progress made recently concerning the H^+-ATPase family, at both the gene and protein level, has come to a point where we can now expect a more integrated investigation of the expression, function and regulation of individual H^+-ATPases in the whole plant context.

Keywords: Plant; Plasma membrane; Transport; H^+-ATPase; Regulation

1. Introduction

P-type ATPases form a large family of membrane proteins which couple ATP hydrolysis to the active transport of cations or other compounds such as phospholipids across cell membranes [1,2]. During the catalytic cycle, an aspartyl phosphate intermediate is formed, hence their 'P-type name' [3].

The plasma membrane H^+-ATPase in plants and fungi couples ATP hydrolysis to proton transport. This creates the pH and potential difference across the plasma membrane required by secondary transporters whose activity is directly dependent upon the proton motive force (Fig. 1).

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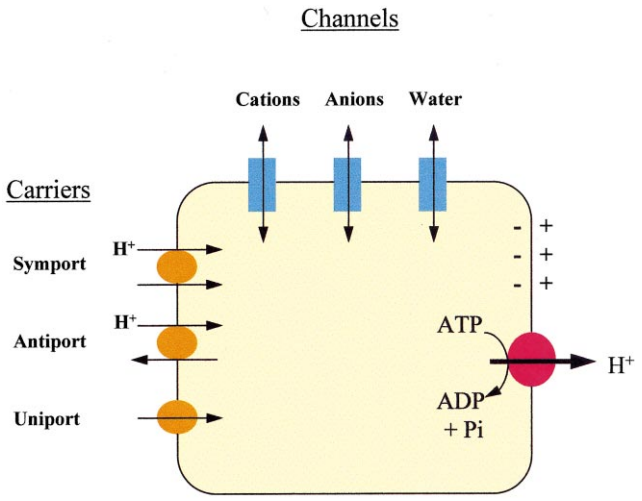

Fig. 1. Primary and secondary transports across the plasma membrane. The electrochemical gradient created by H^+-ATPase is used by secondary transporters (channels and carriers) to move ions and organic compounds across the plasma membrane. Water transport through aquaporins may not respond directly to the proton electrochemical gradient, but to the osmotic potential and, thus, the solute movement.

In plants, the plasma membrane H^+-ATPase also participates in other functions essential for normal plant growth such as salt tolerance, intracellular pH regulation and cellular expansion [4–7]. In this review, we will begin with a brief description of the physiological roles of the plant plasma membrane H^+-ATPase. We will then cover its structural and enzymatic properties and finally discuss its regulation at gene and protein levels.

2. Physiological roles

2.1. Secondary transport

The major role of plasma membrane H^+-ATPase is to activate secondary transport. For instance, mineral absorption from the soil can occur against concentration gradients, and, in this case, relies upon the energy provided by the electrochemical gradient created across the plasma membrane (Fig. 1). Many transporters usually work in symport or antiport with a proton ([8]; see contributions by Blumwald, Forde and Schachtman, this issue).

The H^+-ATPase-dependent electrochemical gradient also confers the energy necessary for the transport of organic compounds. For instance, many sugar/proton and amino acid/proton symports have been identified (reviewed in [9,10]; see contribution by Bush, Lemoine and Sauer, this issue). A well characterized example is the sucrose transporter involved in sugar loading from the apoplasm of the source tissues into the phloem vessels.

2.2. Salinity tolerance

In some respects, salinity tolerance relies on secondary transporters. Salt is toxic to plant cells. In order to avoid its accumulation in the cytosol, plants have developed various mechanisms involving secondary transport. A response to the accumulation of toxic ions in the cytosol is their compartmentalization within the vacuole, while another response is their extrusion out of the cell (reviewed in [11–13]). In each case, a Na^+/H^+ antiport seems to be involved and thus activation of this process by a vacuolar and plasma membrane primary proton transporter is expected. During salt stress, H^+-ATPase mRNA does, in fact, accumulate in roots and expanded leaves of *Atriplex nummularia*, as well as in tobacco cultured cells [11,14–16]. Stimulation at the enzyme level was also observed in tobacco cells [17] and in the salt marsh plant *Spartina* [18].

2.3. Stomata aperture and osmoregulation

The opening and closing of stomata are conducted by the swelling and shrinking of guard cells, resulting from massive ion and water fluxes through specific channels [19,20]. Different signals such as CO_2, humidity, light, fungal toxins and hormones can regulate stomata opening. These signals affect H^+-ATPase as well as K^+ and anion channels and lead to water uptake, turgor increase and cell swelling, eventually leading to stomata opening due to the unusual wall structure of guard cells. The role of the H^+-ATPase in these mechanisms is to provide the electrochemical potential required for driving ion movement, but also to control the opening/closing of voltage-gated channels. This explains the high expression of H^+-ATPase in guard cells [21]. Similarly, the H^+-ATPase activity may also be involved in other turgor-related phenomena such as leaf movements (reviewed in [22]).

2.4. Intracellular pH regulation

Intracellular pH remains constant during plant growth (reviewed in [23]), but little information is known about how this is regulated. It is not clear how the H^+-ATPase might be involved in this phenomenon. The dependence of the H^+-ATPase according to the pH was clearly demonstrated by in vitro assays and varies according to the isoform [24] or the activation state of the H^+-ATPase (discussed below). While the internal pH is maintained at pH 7.5 [23], the optimum pH of the H^+-ATPase is slightly below 7.0. Acidification of the cytosol might thus activate the H^+-ATPase and enhance the extrusion of protons. This should therefore contribute to alkalinization of the cytosol. However, it has also been shown in *Medicago sativa* root hairs that changes in H^+-ATPase do not affect cytoplasmic pH [25]. Since other controls regulate H^+-ATPase and many transport and metabolic pathways involve H^+, it is difficult, given our present knowledge, to have a clear idea of what regulates what.

2.5. Cellular expansion

Acidification of the external medium caused by activation of the plasma membrane H^+-ATPase initiates cellular expansion (reviewed in [26,27]). This mechanism, known as the acid growth theory, could be associated with auxin, a hormone assumed to activate the H^+-ATPase by an as yet unknown mechanism. Acidification of the apoplasm, followed by cell elongation, is also observed with the fungal toxin fusicoccin [28], known to activate plant H^+-ATPases [29]. According to the theory, apoplastic acidification leads to the wall-loosening process [30,31] and to hyperpolarization of the plasma membrane inducing K^+ uptake [32]. This uptake promotes osmotic changes allowing water influx by plasma membrane aquaporins, favoring cell elongation [33].

2.6. Conclusion

From this brief survey of the physiological roles of the plasma membrane H^+-ATPase, it seems clear that this enzyme acts like an one-man-band, expected to adapt to the many different situations encountered by all cell types during plant life. This may involve multiple regulatory systems to control this enzyme and also raises the question of the specific roles possibly played by the several members of the H^+-ATPase gene family (discussed below).

3. H^+-ATPase structure

P-type ATPases form heterosubunit complexes such as the Na^+,K^+-ATPase and H^+,K^+-ATPase, which are composed of the catalytic α-subunit and a glycosylated β-subunit, or comprise a single subunit such as the sarcoplasmic and plasma membrane Ca^{2+}-ATPases and plasma membrane H^+-ATPases in plants and fungi. Nevertheless, they all share a catalytic subunit of approximately 100 kDa. The functional similarity between plant and fungal H^+-ATPases is evident from the capability of the former to partly replace the latter (see below). It is, therefore, relevant to discuss some of the properties of the fungal H^+-ATPases which have been under investigation for a longer period. The molecular nature of the plasma membrane H^+-ATPase was evident after purification of a 100 kDa polypeptide from the fungi *Schizosaccharomyces pombe*, *Saccharomyces cerevisiae* and *Neurospora crassa*; it was capable of forming a β-aspartyl phosphate intermediate and was endowed with an ATP-dependent proton-pumping activity after reconstitution into liposomes (reviewed in [34]). It should be noted that two small proteolipids, *PMP1* and *PMP2*, were suggested as regulating the *S. cerevisiae* plasma membrane H^+-ATPase but the regulation mechanism was not identified, and a physical association between the H^+-ATPase and proteolipids was not demonstrated [35].

Based on hydropathy analysis and experimental data locating the amino- and carboxy-terminal domains at the cytoplasmic face of the plasma membrane, a 10-transmembrane model is often suggested for the plasma membrane H^+-ATPase (Fig. 2) although eight- or 12-transmembrane models have also been proposed (reviewed in [2,4,5,34,36]). The controversy surrounding the number of transmembrane spans has recently been bolstered by the three-dimensional map of the plasma membrane H^+-ATPase from *N. crassa* determined at 8 Å resolution by electron crystallography of two-dimensional crystals [37]. The data resulted in a map with

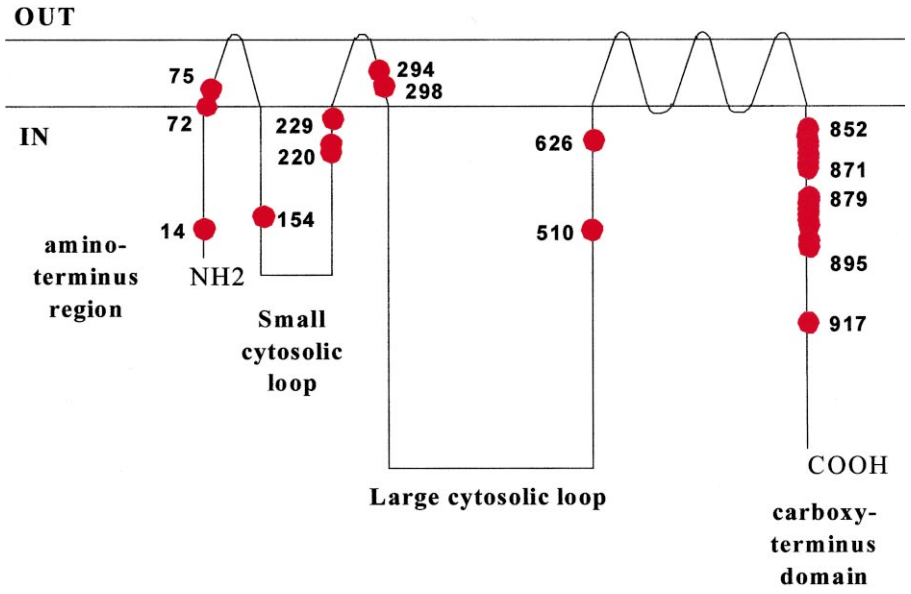

Fig. 2. Predicted topology of the plant plasma membrane H^+-ATPase. Although still a prediction, this topology represents the most widely proposed model for plant plasma membrane H^+-ATPases. Numbering refers to the *N. plumbaginifolia* PMA2 residues adjacent to the membrane spans, as predicted by the DNAStar program. Activating mutations of the *N. plumbaginifolia* PMA2 isoform are represented by dots. Position of the mutated residues is indicated (data from [106,107]).

10 transmembrane helices and four cytoplasmic domains.

Information about transmembrane domains involved in cation transport has come mainly from studies on the sarcoplasmic reticulum Ca^{2+}-ATPase and the Na^+,K^+-ATPase, where the transmembrane spans 4, 5 and 6 would seem to be implicated in cation transport (reviewed in [38,39]). The mechanism for proton transport by the H^+-ATPase has not yet been determined, in spite of large scale mutagenesis throughout the predicted transmembrane regions of the yeast plasma membrane H^+-ATPase [40–42].

The specific function of the amino-terminal region of the H^+-ATPase is not yet known. The small cytoplasmic loop of Na^+,K^+-ATPase and Ca^{2+}-ATPase was suggested as being implicated in conformational changes during the catalytic cycle [43]. Certain mutants in the small loop of the yeast H^+-ATPase were shown to be defective in the coupling between proton transport and ATP hydrolysis [44,45].

The large cytoplasmic loop contains the aspartate residue that is phosphorylated during the catalytic cycle [46] and the ATP binding domain identified by sequence similarity with other P-type ATPases and by labeling with fluorescein-5′-isothiocyanate [47].

The H^+-ATPase C-terminal region has a regulatory function in yeast [48] and in plants [49,50], acting as an auto-inhibitory domain (see below).

The quaternary structure of yeast and plant plasma membrane H^+-ATPases is still unresolved. After detergent solubilization, it was suggested that the *S. pombe* H^+-ATPase formed an oligomer with 8–10 monomers [51] while the *N. crassa* H^+-ATPase formed stable hexamers [37,52]. Nevertheless, solubilization could alter the quaternary structure as suggested in [53] for the plant H^+-ATPase. These authors conducted a radiation inactivation analysis on the red beet plasma membrane H^+-ATPase under three forms: plasma membrane vesicles, detergent-solubilized enzyme preparations or reconstituted liposomes. The results indicated that the plasma membrane-associated and reconstituted H^+-ATPases formed a dimer, while the solubilized enzyme was present in a monomeric form. Similar experiments were performed on the plasma membrane-bound *N. crassa* H^+-ATPase, yielding a target size of 230 kDa, close to the expected value for a dimeric form [54]. Finally, it was shown that monomers of the *N. crassa*

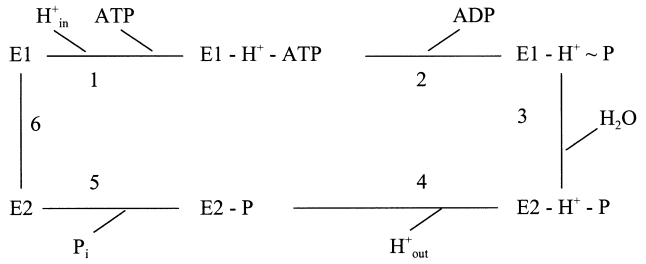

Fig. 3. Reaction cycle of the plasma membrane H^+-ATPase. This reaction cycle (see the text for the description of the various steps) was originally proposed for the Ca^{2+}-ATPase (reviewed in [38]) and has been extended to H^+-ATPase (discussed in [34]).

H^+-ATPase, reconstituted in proteoliposomes, were able to catalyze efficient ATP hydrolysis-driven proton translocation [55]. In conclusion, although the minimal functional unit for proton transport is the 100 kDa catalytic monomer, the quaternary structure of the H^+-ATPase in plasma membranes, in situ, is still an open question.

4. Catalytic cycle

The catalytic cycle proposed for the plasma membrane H^+-ATPase is based mainly on the current models for the mammalian Na^+,K^+-ATPase and Ca^{2+}-ATPases. For the latter, the general scheme (Fig. 3) includes two distinct conformational states of the enzyme, termed E1 and E2. There is evidence that these two conformers have different affinities for the translocated cations. Similarly, they differ in reactivity at the nucleotide binding site, which can be phosphorylated by the γ-phosphate of ATP in the E1 form or by free Pi in the E2 form (reviewed in [38]). Yeast and plant plasma membrane H^+-ATPases have also been shown to form a phosphorylated intermediate [46,56–58].

The reaction cycle of the plasma membrane H^+-ATPase can be formulated as shown in Fig. 3. ATP and proton bind to the E1 form of the enzyme (step 1). The order of binding is not known, although Blanpain et al. [59] have proposed that the change in conformation from E2 to E1 is facilitated by the fixation of the proton, followed by fixation of ATP. A high energy phosphorylated intermediate is formed and ADP is released (step 2). A conformational change then occurs, shifting the enzyme from the E1 to the E2 conformation (step 3), followed by the release of the proton to the exterior (step 4). Finally, Pi is released and the enzyme returns to E2 (step 5). The transition step between E2 and E1 (step 6) has been suggested to be the rate limiting step [60]. Vanadate, a potent inhibitor of P-type ATPases, is thought to mimic Pi and block the enzyme in its E2 form.

5. Enzymatic properties

The substrate for the plasma membrane H^+-ATPase is MgATP. In plants, the K_m for MgATP varies between 0.3 and 1.4 mM and the optimum pH is around 6.6 [21,61,62]. The specific ATPase activity in purified plasma membranes is usually in the range of 1–2 μmol Pi/min/mg. The existence of several genes encoding the plasma membrane H^+-ATPase and the simultaneous expression of several of these in the same organs (see below) has made it difficult to perform a precise biochemical study of a single isoform in plant tissues. This problem was overcome by the heterologous expression of plant H^+-ATPase genes in yeast.

Three H^+-ATPase isoforms of *Arabidopsis thaliana* (AHA1, AHA2 and AHA3) were expressed in the yeast *S. cerevisiae* [63–65]. In this system, the chromosomal yeast gene *PMA1*, encoding the yeast plasma membrane H^+-ATPase, was placed under the control of the inducible *GAL1* promoter, while the plant H^+-ATPase gene, carried by a plasmid, was placed under the control of the constitutive yeast *PMA1* promoter. The shift from galactose to glucose arrested yeast *PMA1* expression and led to the exclusive expression of the plant gene. The yeast cells transformed with the AHA2 isoform showed a very weak capacity for growth on glucose medium while yeast cells transformed with AHA1 and AHA3 isoforms showed no capacity at all for growth on glucose. Nevertheless, the plant enzymes were found to be expressed in a biochemically functional form in yeast internal membranes, and could thus be isolated and their enzymatic properties studied. When compared with AHA3, both AHA1 and AHA2 had apparently higher turn-over rates for ATP hydrolysis, exhibited 10-fold lower apparent K_m values for ATP and 3-fold higher sensitivities to vanadate. In addi-

tion, AHA2 seemed to be activated more than either AHA1 or AHA3 by lysophosphatidylcholine (LPC), a phospholipid known to activate plant plasma membrane H^+-ATPases [65] (see below).

Another strategy was developed in our laboratory for the expression of two *Nicotiana plumbaginifolia* isoforms (PMA2 and PMA4) [24,66]. These two isoforms were chosen because they are highly expressed in tobacco and represent the two major H^+-ATPase subfamilies found in plants. To prepare the recipient strains, the two yeast H^+-ATPase genes, *PMA1* and *PMA2*, were inactivated by gene disruption. Yeast was kept alive on galactose medium by the presence of the *PMA1* gene, placed on a *URA3* centromeric plasmid under the control of the *GAL1* promoter. The plant gene under the yeast *PMA1* promoter was then introduced. A shift from galactose to glucose medium allowed exclusive expression of the plant H^+-ATPase gene; at the same time, the *PMA1*-carrying plasmid was removed by counter-selection on a *URA3* suicidal medium [66]. This technique eliminated the risk of recombination between the yeast and plant H^+-ATPase genes and prevented residual expression of yeast H^+-ATPase. The *N. plumbaginifolia* isoforms were functionally expressed in internal and in plasma membranes and, in contrast with the *A. thaliana* isoforms tested up to now, allowed yeast growth even in the absence of the yeast H^+-ATPase genes. Biochemical comparison of the *N. plumbaginifolia* PMA2 and PMA4 isoforms revealed enzymatic differences which could be related to distinct functional properties since yeast growth at acidic pH was dependent on the isoform. Indeed, while yeast expressing PMA2 was not able to sustain growth at a pH lower than 5.0, yeast expressing PMA4 grew at pH as low as 4.0. This was correlated to a higher acidification of the external medium and an increase in the ATPase activity of PMA4 compared to PMA2 [24].

Inevitably, the finding of different enzymatic and physiological properties of plant H^+-ATPases expressed in yeast raises the question of their actual roles in plants. With regard to the enzymatic properties, tools to isolate a specific isoform from plant tissues will need to be developed. At the physiological level, transgenic approaches aimed at increasing or decreasing expression of specific H^+-ATPases have been developed (see below).

6. Regulation

Given their multiple physiological roles, and the high ATP consumption by plasma membrane H^+-ATPases, these enzymes are expected to be tightly regulated. As a matter of fact, much data have been obtained which mark the regulatory properties at transcription, translation and enzymatic levels.

6.1. Transcriptional regulation

6.1.1. Multigenic family

The plant plasma membrane H^+-ATPase is encoded by a multigene family. Ten genes have been identified in *A. thaliana*, five of which have been characterized to various levels [67–71]. Nine genes have been identified in *N. plumbaginifolia* and eight of them have been studied in more detail [72–76]. In tomato, seven genes were identified and two of them have been characterized [77–79]. Five genes have been found so far in *Vicia faba* [80,81]. Two genes were characterized in *Oryza sativa* [82,83], *Solanum tuberosum* [84] and *Zea mays* [85,86]. Finally, one gene was identified in *Phaseolus vulgaris* [87] and *Zostera marina* [88].

All of the full-length plant H^+-ATPase cDNA clones isolated so far can be grouped into two subfamilies (Table 1) that diverged before the emergence of monocot and dicot species [74]. These two subfamilies probably represent the most expressed genes in plant species. Other genes selected as genomic clones represent other subfamilies whose expression seems to be more restricted [69,71,76].

6.1.2. Tissue-specific expression

Given the diversity of biological functions supported by H^+-ATPases and the multiplicity of factors affecting their activity, the question arises as to whether certain isoforms are specialized to work under specific environmental conditions or in specific cell types, tissues or organs. Several approaches have been used to study the differential expression of isoforms, including immunodetection, Northern blot analysis, in situ hybridization and reporter gene techniques.

Immunological studies suggested that H^+-ATPase accumulates in particular plant tissues or in cell types such as the root cap, root hairs and epidermis, guard

cells and the transfer and stellar cells. In some cases, asymmetric distribution within the cell has been observed [89–94]. These studies pointed to cell types in which H^+-ATPase expression was high, but they could not distinguish between various isoforms. The situation was different with in situ detection of tagged *Arabidopsis* AHA3 introduced in transgenic plants, which allowed specific detection of this one isoform in companion cells [95].

Northern blot analysis of various organs showed a differential expression pattern for different H^+-ATPase genes in several species such as *N. plumbaginifolia* [73,74], *Arabidopsis* [69,71], tomato [78] and *V. faba* [81].

Finally, reporter gene (β-glucuronidase) and in situ hybridization techniques enabled the analysis of single gene expression at the cell type level. In *A. thaliana*, the various isoforms seemed to be individually expressed in specific tissues, as revealed by the β-glucuronidase method: *aha2* in root hairs [5], *aha3* in phloem [96] and *aha10* in developing seeds [69]. By contrast, in *N. plumbaginifolia*, *pma4* was highly expressed in several cell types, including companion cells, root epidermis and hairs, as well as guard cells, indicating that the same isoform can activate mineral nutrition, sugar loading into the phloem and stomata aperture. Another observation was that up to three H^+-ATPase genes might be expressed in the same cell type at the same developmental stage, suggesting that isoforms with distinct kinetics might co-exist in the same cell [75]. Other genes have a very restricted pattern of expression, such as *pma6* which, at the vegetative stage, is mainly expressed in the short trichomes (leaf hairs), and is presumably involved in secretion of secondary metabolites [76]. The maize MHA2 gene was found to be expressed, like the *N. plumbaginifolia pma4*, in various cell types [86].

It will be interesting to analyze how the H^+-

Table 1
H^+-ATPase genes of the plasma membrane of higher plants characterized so far

Subfamily	Species	Genes	References
I	*Lycopersicon esculentum*	*lha1*	[77]
	N. plumbaginifolia	*pma1*	[73]
		pma2	[72]
		pma3	[73]
	O. sativa	*osa1*	[82]
		osa2	[83]
	S. tuberosum	*pha1*	[84]
	V. faba	*vha4*	Rassau, D. and Hedrich, R., personal communication
	Z. mays	*mha1*	[85]
II	*A. thaliana*	*aha1*	[67]
		aha2	[68]
		aha3	[70]
	L. esculentum	*lha4*	[79]
	N. plumbaginifolia	*pma4*	[74]
	P. vulgaris	*bha1*	[87]
	S. tuberosum	*pha2*	[84]
	V. faba	*vha1*	[80]
		vha3	Rassau, D. and Hedrich, R., personal communication
		vha5	Rassau, D. and Hedrich, R., personal communication
	Z. mays	*mha2*	[86]
	Z. marina	*zha1*	[88]
Others	*A. thaliana*	*aha9*	[71]
		aha10	[69]
	N. plumbaginifolia	*pma5*	Arango, M., unpublished data
		pma6	[76]
		pma7	[76]
		pma8	[76]

ATPase gene family has evolved in the plant kingdom. This will require us to obtain the expression profile of the whole gene family for at least two species. However, the current picture seems to show that two gene subfamilies encode the most highly expressed H^+-ATPases in plant species, while variations in gene organization and expression are clearly apparent within these two subfamilies.

6.1.3. Environmental regulation

In addition to tissue-specific expression, the plant H^+-ATPases are differentially expressed according to environmental factors. Studies performed on different plant species often used Northern blot analysis to illustrate changes in mRNA levels after modification of external conditions. It was shown that H^+-ATPase gene expression in specific tissues was enhanced by NaCl or salt stress [14,15,88,97], low hydritation potential [98], darkness [84], hormones such as auxin [86] and metabolizable sugars [79]. Several *N. plumbaginifolia pma* genes, monitored by the β-glucuronidase reporter, were shown to be activated by salt stress in roots (*pma2*, *pma4*, *pma5* and *pma6*) or mechanical stress in the cortical parenchyme of the young nodes (*pma6*) [76]. This experimental approach has the advantage of examining one gene at a time and allowing a direct analysis of the sequences involved in transcription regulation.

6.2. Translational regulation

The 5′-untranslated region (leader) of the transcript for most plant H^+-ATPase genes is longer than in most other plant transcripts and contains a short upstream open reading frame (uORF) of 5–13 codons, suggesting translational control. Studies performed on the *pma1* and *pma3* leaders from *N. plumbaginifolia* revealed that the long leader has an activating effect in mRNA translation whereas the uORF acts more like an inhibitor. It was found that ribosomes were able to initiate translation of the small upstream reading frame and, thereafter, to reinitiate at the H^+-ATPase reading frame, thus contrasting with the Kosak model [99,100]. Although the physiological meaning and precise regulatory mechanism at the translational level are not yet resolved, it seems clear that a regulation step occurs at this stage, possibly by modulating reinitiation.

6.3. Enzymatic regulation

6.3.1. Regulatory role of the H^+-ATPase carboxy-terminal region

In addition to transcriptional and translational regulatory mechanisms, modulation might also occur at the enzyme level. There is now convincing evidence that the C-terminus of the H^+-ATPase acts as an auto-inhibitory domain. The initial data came from studying the effect of glucose on the yeast H^+-ATPase. When yeast cells were incubated in glucose, prior to homogenization and plasma membrane preparation, the K_m for ATP decreased, the V_{max} increased, the pH optimum shifted to more alkaline values and vanadate sensitivity increased [101]. These effects could be mimicked by deletion of the 11 carboxy-terminal amino acids of the H^+-ATPase [102]. Thus, the carboxy-terminal region of the proton pump contains a negative regulatory domain, the function of which is switched off by glucose metabolism. In addition, phosphorylation of the yeast PMA1 was shown to be dependent upon the presence of glucose, suggesting a possible involvement of protein kinases in ATPase regulation [103,104].

The C-terminal region of the plant H^+-ATPase is longer than and structurally unrelated to that of the yeast H^+-ATPase. However, it is also involved in regulation. The first data resulted from biochemical approaches which showed that trypsin treatment of plant plasma membranes resulted in H^+-ATPase activation concomitant with cleavage of a C-terminal fragment [49,50]. Genetic approaches confirmed these data, since deletion of the last 92 residues of the *A. thaliana* AHA2 isoform expressed in yeast allowed yeast growth after replacement of the endogenous yeast H^+-ATPase genes while the full-length AHA2 enzyme did not [64]. With both trypsin treatment and genetic deletion, ATPase activity increased, its apparent K_m decreased, its pH optimum shifted to more alkaline values (from 6.5 to 7.0) and stimulation by LPC was reduced [49,50,64,105]. Subsequently, a more precise study was performed by progressive deletions from the C-terminal end of AHA2 [105]. The results showed that deletion of the last 38 residues reduced the K_m and shifted the pH optimum but did not modify the V_{max}, nor the complementation capacity of AHA2. However, deletion of 51 (or more) residues increased molecular ATPase activity,

and complementation of yeast growth was obtained after deletion of 61 (or more) residues [105].

It is not necessary to delete a large part of the C-terminal domain to fully activate the plant H^+-ATPase since this could be obtained by point mutations of specific residues of this region. Indeed, 19 residues of the C-terminus of the *N. plumbaginifolia* PMA2 isoform [106,107] and one residue of the *A. thaliana* AHA1 isoform [108] were found to activate the enzyme and to improve yeast growth when expressed in yeast. These mutants showed similar kinetic behavior as truncated H^+-ATPases and, in addition, an improved coupling ratio between proton-pumping and ATP hydrolysis [106,108]. The analysis of the mutation distribution within the carboxy-terminal region of PMA2 revealed that two close regions (A852–K871 and R879–V895) within the first half of the C-terminal domain concentrated the large majority of the point mutations, while the second half did not [107] (Fig. 2). Recently, a systematic alanine scanning mutagenesis was performed on the last 87 residues of the *A. thaliana* AHA2 isoform [109]. Among five mutations that conferred growth at pH 4.0, three were at positions also mutated in the *N. plumbaginifolia* PMA2. An additive set of 19 mutations was scattered throughout the region analyzed, but the improvement of H^+-ATPase activity and yeast growth they conferred was more limited.

6.3.2. Other regulatory regions

The carboxy-terminus is not the only region to regulate the enzyme, since point mutations of PMA2 activating the ATPase were discovered in seven other regions: in the amino-terminal region, the first and fourth transmembrane spans and the small and large cytoplasmic loops [106,107] (Fig. 2). Partial tryptic digestion showed that the C-terminal of internal PMA2 mutants had a conformational change making the carboxy-terminal region more accessible to trypsin [107]. It was thus surprising that a mutation in the amino-terminal region had the same effect as the deletion of the C-terminal region. This implies that regulatory signaling leading to modulation of ATPase and proton-pumping activities are not necessarily restricted to the C-terminal region but could also affect other domains of the enzyme. Nevertheless, these modifications should eventually result in the displacement of the inhibitory domain from its interaction site.

6.3.3. Fusicoccin and regulatory 14-3-3 proteins

Fusicoccin is a toxic compound, produced by the fungus *Fusicoccum amygdali*, which provokes loss of water, cell elongation, germination and rhizogenesis [110–112]. In vivo treatment with fusicoccin induces hyperpolarization of the plasma membrane and acidification of the external medium [112], suggesting a possible effect on the plasma membrane H^+-ATPase. In several studies, the H^+-ATPase isolated from in vivo treated plants was shown to be in an activated state, characterized by a higher V_{max}, a reduced K_m and an alkaline shift in the pH optimum [113–115]. These features were similar to those of the plant H^+-ATPase deleted of its C-terminal region, suggesting that fusicoccin somehow modifies the conformation of the C-terminus [114–117]. Clues to the fusicoccin signaling machinery have been obtained with the discovery that a 30 kDa doublet was present in purified fusicoccin receptor preparations [114,118,119]. The proteins of this doublet were sequenced and identified as members of the 14-3-3 protein family [120–122]. The molecular mechanism which leads to the fusicoccin-induced C-terminus displacement is still undetermined and at least two models have been proposed. One favors regulation through direct interaction between the fusicoccin receptor and the H^+-ATPase, whereas the other envisages the occurrence of a transduction chain.

14-3-3 Proteins are highly conserved proteins that are ubiquitously expressed in eukaryotic organisms including yeast, plants and animals (reviewed in [123,124]). These proteins have been shown to regulate many cellular targets in these organisms. 14-3-3 Proteins are acidic proteins and are known to form dimers [125]. Each monomer contains a domain that could bind an α-helix of a substrate protein, potentially dimerizing substrates or binding different substrates into close proximity [125–127]. Binding of 14-3-3 proteins to the target protein has been shown to involve a phosphorylated motif in the target [128,129].

Recent data indicated that 14-3-3 proteins interact directly with the plant plasma membrane H^+-ATPase and induce the displacement of the C-terminal domain. In vivo treatment of plant tissues with fusi-

coccin, which produced a stimulation of H^+-ATPase activity, concomitantly brought a marked increase in plasma membrane-associated 14-3-3 proteins [122,130]. In addition, the amount of fusicoccin bound to plasma membrane appeared to parallel H^+-ATPase stimulation [131]. Recently, it has been shown that 14-3-3 proteins bind directly to the H^+-ATPase C-terminal region and that fusicoccin stabilizes the H^+-ATPase/14-3-3 complex formed [132–134]. The H^+-ATPase/14-3-3 complex thus represents an activated state of the enzyme which can be stabilized by fusicoccin binding in vivo [132,133,135] or in vitro [134,136]. Moreover, heterologous expression of the *N. plumbaginifolia* PMA2 H^+-ATPase isoform in yeast generated a high level of fusicoccin binding site in plasma membranes, reflecting the formation of the PMA2/14-3-3 complex in yeast plasma membranes [137].

Providing PMA2 with a 6-His-tag at its N-terminus allowed us easily to purify this H^+-ATPase from yeast membranes and, surprisingly, it was shown that part of the purified PMA2 H^+-ATPase isoform co-purified with the two yeast 14-3-3 proteins without any in vivo or in vitro fusicoccin treatment, illustrating that, for this isoform, fusicoccin treatment was not necessary for 14-3-3 binding [138]. This could explain why the wild-type *N. plumbaginifolia* H^+-ATPase isoforms allowed yeast growth when they replaced the yeast H^+-ATPase genes. This hypothesis could be supported by the observation that, when the PMA2 isoform was tagged at the very end of the C-terminal region, 14-3-3 proteins no longer copurified with the PMA2 H^+-ATPase, suggesting that the tag placed at the C-terminus prevented 14-3-3 binding. However, in this case, ATPase activity was reduced and yeast growth was prevented [138]. This suggests a relationship between binding of 14-3-3 proteins on the H^+-ATPase and yeast growth capacity for cells expressing plant H^+-ATPase genes.

6.3.4. Other compounds affecting H^+-ATPase activity

Lysophosphatidylcholine (LPC) has been shown to activate plant H^+-ATPases by increasing the V_{max}, decreasing the apparent K_m and displacing the pH optimum [50,139,140]. Interestingly, deletion of the C-terminal region or treatment with fusicoccin produced an H^+-ATPase that was no longer stimulated by LPC [105,114], suggesting that LPC activates H^+-ATPase by displacing the auto-inhibitory C-terminal domain from its interaction site. The binding site for LPC on the plant H^+-ATPase has not yet been identified. It was shown that LPC slowed down proteolytic cleavage of the C-terminal region by trypsin [107] while competition experiments with the fungal toxin beticolin-1 (which inhibits the wild-type and truncated H^+-ATPase in similar ways) suggested that LPC binds to a site outside the C-terminal region [141]. Considering a possible regulatory role for LPC in plant H^+-ATPase activity, it is interesting to note that phospholipase A_2 activity has been detected in oat root plasma membranes [139] and in cytosolic fractions of leaves from *V. faba* [142]. Phospholipase A_2 catalyzes the cleavage of phosphatidylcholine into LPC and free fatty acids, indicating a possible regulatory function for this enzyme in H^+-ATPase activity (reviewed in [143]).

Other compounds have been reported as modulators of plant H^+-ATPase activity but the physiological role and the molecular interaction of these compounds and the H^+-ATPase are still poorly understood. The beticolin-1 toxin produced by *Cercospora beticola* [144,145] and the phytotoxic lipodepsipeptides produced by the plant pathogen *Pseudomonas fuscovaginae* [146] inhibited H^+-ATPase activity. Cholesterol and stigmasterol were found to stimulate the H^+-ATPase while other sterols behaved as inhibitors [147].

Finally, cytosolic Ca^{2+} was found to regulate H^+-ATPase activity, since H^+-ATPase in guard cells was reversibly inhibited by Ca^{2+} at physiological concentrations (K_i around 0.3 μM) [148]. This regulation does not probably have a direct effect on the enzyme, since Ca^{2+} did not modify the activity of plant H^+-ATPase purified from yeast (Morsomme, P., unpublished data). Since Ca^{2+} is known to bind 14-3-3 proteins [149,150], its effect on H^+-ATPase might be mediated through the 14-3-3 complex. The regulation by Ca^{2+} might as well occur through a kinase signaling pathway (see below).

6.3.5. Regulatory phosphorylations

There is also evidence that plant H^+-ATPase is regulated by a phosphorylation/dephosphorylation mechanism. In 1988, Schaller and Sussman [151] showed that the oat root plasma membrane H^+-ATPase was phosphorylated at serine and threonine

residues in a Ca^{2+}-dependent manner. Complementary experiments performed on red beet storage tissue revealed that syringomycin stimulated in vitro plasma membrane H^+-ATPase phosphorylation on serine and threonine residues [152].

The next step was then to determine the possible influence of H^+-ATPase phosphorylation on its activity. Thus, it was shown that purified plasma membranes from tomato cells, exposed to a specific elicitor of *Cladosporium fulvum*, displayed an increase in plasma membrane H^+-ATPase activity, concomitant with dephosphorylation of the enzyme by a membrane-bound phosphatase [153]. This phosphatase was suggested as being activated by G proteins, themselves activated by the receptor to the fungal elicitor [154]. Once the fungal elicitor was removed, H^+-ATPase activation was reduced by a mechanism involving rephosphorylation of the enzyme by two kinases. The first, possibly a protein kinase C (PKC), was suggested to rephosphorylate H^+-ATPase very rapidly (within 1 h), while the second, a Ca^{2+}/calmodulin-dependent protein kinase, would act later and depend on the prior activation by the PKC-like kinase [155]. Recently, it was shown in tobacco cells that in vitro dephosphorylation of H^+-ATPase also led to increased activity [156].

Ca^{2+}-dependent phosphorylation of beet root H^+-ATPase was recently correlated to a decrease in ATPase and proton-pumping activities of the H^+-ATPase in reconstituted proteoliposomes [157]. These data could explain the inhibition of the ATPase activity previously observed in the presence of a μM concentration of calcium [148]. These phosphorylation data thus suggest that phosphorylation of the H^+-ATPase inhibits its activity.

The situation became more complex when other studies proposed that H^+-ATPase phosphorylation led to its activation. In this case, however, phosphorylation was related to a fusicoccin activation effect [158,159]. As mentioned previously, 14-3-3 proteins bind to a consensus motif containing a phosphorylated residue [128]. As 14-3-3 proteins bind to the C-terminal region of plant H^+-ATPases, it was therefore expected to find a phosphorylated residue within this domain, although no consensus motif was present within this region. It was effectively shown recently that the penultimate residue (threonine 948) of the H^+-ATPase purified from spinach leaf tissues is phosphorylated [159]. The authors suggested that threonine 948 participates in 14-3-3 binding, since its phosphorylation was protected by fusicoccin-induced 14-3-3 binding [159]. Using the yeast expression system, we recently observed that the penultimate threonine residue of PMA2, when part of a 14-3-3 complex (in this case, in the absence of fusicoccin), was phosphorylated [138].

It thus seems that phosphorylation can, on the one hand, activate H^+-ATPase by phosphorylating the C-terminal domain and possibly inducing 14-3-3 binding and, on the other hand, inactivate H^+-ATPase at a yet unknown site.

6.3.6. Model of the regulation of plant H^+-ATPases: from yeast to plant

Heterologous expression of plant H^+-ATPases in yeast has led to the identification of unexpected regulatory mutants and has revealed a new pathway for studying the role of 14-3-3 proteins in H^+-ATPase regulation, while other experiments on both yeast and plant material have indicated the importance of regulatory phosphorylation. Is it possible to integrate the results obtained from these various approaches? In the light of the data and observations mentioned above, we would propose that the plant H^+-ATPase exists under three different forms (Fig. 4). The inhibited form corresponds to the plant enzyme phosphorylated elsewhere, other than in the C-terminal region and is characterized by its very weak activity. Dephosphorylation at this site converts the enzyme into a state of weak activity. Phosphorylation at the penultimate threonine and 14-3-3 binding raises the H^+-ATPase to a state of high activity. This state is also attained by the H^+-ATPase deleted from its C-terminal domain or stimulated by LPC. Within a cell, the H^+-ATPase might simultaneously exist under several forms. For instance, in the yeast expressing the wild-type tobacco PMA2, this H^+-ATPase exists in both the weak and high activity states since part of the enzyme is engaged in a complex with 14-3-3 proteins. The point mutants localized in various regions of PMA2 and characterized by a higher ATPase activity and improved yeast growth have an enzyme whose C-terminal domain is more amenable to phosphorylation and 14-3-3 binding. Therefore, the equilibrium between the weak and high activity forms is displaced towards

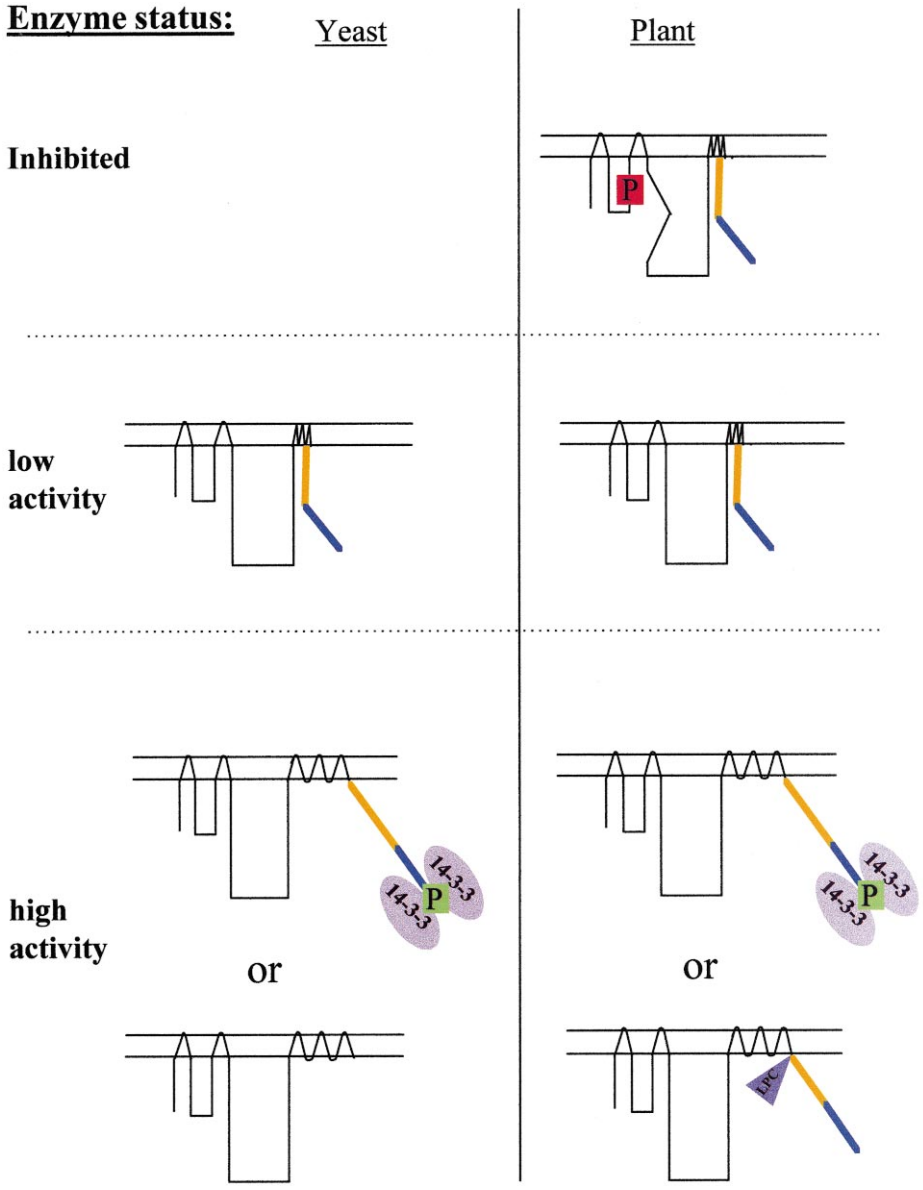

Fig. 4. Regulatory model for the plant plasma membrane H^+-ATPase. The figure displays the various forms proposed for plant plasma membrane H^+-ATPase expressed in a yeast or plant background. The various states are explained in the text. The thick bar represents the C-terminal region divided into a domain (the first half, colored in orange) that binds to and inhibits the enzyme and the other domain (the second half, colored in blue) that binds to a 14-3-3 dimer (depicted by the pair of ovals). The green square represents the phosphorylated penultimate threonine residue. The red square represents the phosphorylated residue that has not yet been localized, but the existence of which has been supported by experiments performed on plants. The triangle represents LPC.

the latter. The three-conformation model is still hypothetical. We could also consider that the 'inhibiting' phosphorylation occurs in the H^+-ATPase/14-3-3 complex, resulting in the removal of 14-3-3 dimer. In this case, the 'inhibited' and 'low activated' states would be equivalent. Moreover, this model does not take into account the possibility that regulation implies H^+-ATPase dimerization. Indeed, the plant H^+-ATPase can exist at least on the monomeric and dimeric form (see above). We can thus not exclude that these states have different properties and that, for instance, 14-3-3 proteins play a role in the dimerization process.

The activating mutants obtained with PMA2 and

AHA2 carry another message. If we only consider the 19 PMA2-activating mutations that are localized within the carboxy-terminal domain (Fig. 2) and the five AHA2 mutations that allowed yeast growth at pH 4.0, we notice that all but two are positioned in the first half of this domain (orange-colored in Fig. 4). This probably represents the segment interacting with the rest of the enzyme and conferring partial inhibition. The second half of the enzyme (blue-colored in Fig. 4), which comprises the phosphorylated threonine at the penultimate position, interacts with 14-3-3 proteins. We may hypothesize that this association weakens the binding of the first half to the rest of the enzyme and hence releases the inhibitory effect. An important question remains to identify how 14-3-3 proteins are associated with the H^+-ATPase C-terminus in the 14-3-3 clamp. Different possibilities might exist: (i) the 14-3-3 dimer binds two H^+-ATPases linked together by their C-terminal region. In this case, the site of interaction of the H^+-ATPase with the 14-3-3 dimer would be the extreme end of the C-terminus and this association would lead to the H^+-ATPase dimerization. (ii) The 14-3-3 dimer binds, on one hand, the H^+-ATPase C-terminus and, on the other hand, another region of the H^+-ATPase. (iii) Finally, we cannot exclude that the second partner could be another protein interacting with the H^+-ATPase.

7. Physiology of transgenic plants

To understand the physiological implications of H^+-ATPase activity and regulation, experiments must be carried out on intact plants. The first step in this direction was recently reported by Young et al. [160] who expressed in *A. thaliana* the phloem-specific AHA3 H^+-ATPase isoform with a modified C-terminal region. Transgenic plants were characterized by improved growth at low pH during seedling development.

In our laboratory, we obtained transgenic tobacco plants that co-suppressed expression of PMA4, while expression of the isoforms belonging to the first subfamily (*pma1*, *pma2* and *pma3*) was unaffected. The development of co-suppressed plants was stunted and retarded. The soluble sugars accumulated in the leaves, while the sucrose translocation through the leaf main vein was concomitantly reduced [161]. These data showed the importance of the PMA4 H^+-ATPase in sugar translocation in the phloem vessels, between source and sink tissues.

8. Conclusions

The complex regulation of the plant H^+-ATPase at the transcriptional, translational and post-translational levels illustrates the very precise control exerted on this key enzyme. Several questions still need to be addressed including (i) the relationship between the expression of the different isoforms and their physiological roles in distinct cell types during plant development and (ii) the integration of regulatory mechanisms into plant transport physiology. While yeast is very useful for comparing the enzymatic properties of different isoforms and for approaching some aspects of post-translational regulation, reverse genetics should now be applied in plants by modifying the expression level of the ATPase or by expressing regulatory mutants. (iii) Finally, at the enzyme level, the structure has still to be determined and will probably be the limiting step for approaching the mechanism of proton transport. Resolving the three-dimensional structure of P-type ATPases is thus an important challenge for the future.

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