Plant Physiol. (1995) 108: 1–6

Update on H^+-ATPase

The Plasma Membrane H^+-ATPase[1]

A Highly Regulated Enzyme with Multiple Physiological Functions

Baudouin Michelet and Marc Boutry*

Unité de Biochimie Physiologique, Université Catholique de Louvain, Place Croix du Sud 2–20, B-1348 Louvain-la-Neuve, Belgium

The proton-pump ATPase (H^+-ATPase) of the plant plasma membrane acts as a primary transporter by pumping protons out of the cell, thereby creating pH and electrical potential differences across the plasmalemma (Fig. 1). Transport of many solutes (ions, metabolites, etc.) into and out of the cell involves secondary transporters whose ability to function is directly dependent on the proton-motive force created by the H^+-ATPase.

Depending on the electrical charge of the solute to be transported, the direction of its transport, and its concentration on either side of the membrane, it is possible to predict from Figure 1 the type of transport protein required. For instance, the uptake of a cation is energetically favorable because of the positive external electrical potential, and therefore requires only a diffusion facilitator, such as a channel protein or a uniport. Conversely, to be energetically favorable, the uptake of an anion must be accompanied by the uptake of one or more protons in a symport system. In addition to activating secondary transport, the H^+-ATPase promotes more specialized physiological functions.

THE PLASMA MEMBRANE H^+-ATPASE IS INVOLVED IN MANY PHYSIOLOGICAL FUNCTIONS

Activation of Secondary Transport

In plants the uptake of mineral nutrients from the soil occurs against a concentration gradient. Minerals enter the root via transport proteins located in the plasma membrane, they are loaded into the xylem sap, and then they leave the xylem to enter the plant symplast via the same type of transport proteins. Those transport proteins that have been characterized at the molecular level in various laboratories are shown in Figure 1.

Depending on the K^+ concentration in the soil, K^+ import occurs via two different transport systems. The first is a low-affinity transport system cloned and characterized as a K^+ channel, which allows K^+ to enter the cell (Schachtman et al., 1992; Sentenac et al., 1992). This channel operates at high external K^+ concentrations (typically >1 mM). The second is a high-affinity K^+ transport system, cloned and characterized as a $1H^+/1K^+$ symport (Schachtman and Schroeder, 1994). It thus energizes K^+ transport by using up two positive charges per K^+ transported and by decreasing the proton chemical gradient. Theoretically, it permits plants to grow in soil in which the K^+ concentration is as low as 27 nM while maintaining the intracellular physiological K^+ concentration at about 100 mM (Schachtman and Schroeder, 1994), i.e. creating a K^+ concentration ratio of 4 million across the plasmalemma! A recently cloned high-affinity NH_4^+ transport system (Ninnemann et al., 1994) is not included in Figure 1, because its mode of action is not yet known.

Anion uptake is opposed by the negative internal membrane potential, and to be energetically favorable it must be accompanied by proton uptake. Two transport systems with different affinities for NO_3^- have been characterized in plant roots. A low-affinity system, induced by culture in high-NO_3^- conditions, has been cloned from *Arabidopsis thaliana* (Tsay et al., 1993). This carrier is a H^+/NO_3^- symport with a transport stoichiometry of two or more H^+ per NO_3^-. Plant plasma membranes contain many other ion transport systems. Some have been identified electrophysiologically but have not yet been cloned and functionally characterized by heterologous expression.

Carbon and nitrogen are reduced and assimilated in some organs and then distributed throughout the plant, mainly as Suc and amino acids, respectively. The loading of these organic solutes into the conducting vessels is energetically unfavorable when it takes place against a concentration gradient. Here, again, the proton-motive force created by the H^+-ATPase provides the energy required for these transports. Sugar/H^+ and amino acids/H^+ symports have been cloned by heterologous expression in yeast (Riesmeier et al., 1992; Hsu et al., 1993; Kwart et al., 1993; Sauer and Stolz, 1994).

[1] The experimental work on ATPase carried out in this laboratory was supported by grants from the Belgian Services Fédéraux des Affaires Scientifiques, Techniques et Culturelles, the Belgian Fund for Scientific Research, and the European Community's BIOTECH program.

* Corresponding author; e-mail boutry@fysa.ucl.ac.be; fax 32–10–473872.

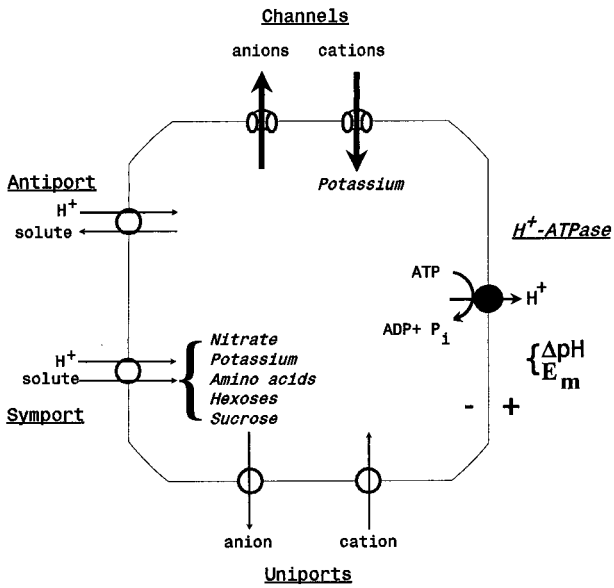

Figure 1. Theoretical scheme of secondary tranporters energized by the plasma membrane H^+-ATPase and identification of all corresponding cloned transport proteins. The H^+-ATPase acts as a primary transporter by creating a proton-motive force comprising two components, a pH difference (ΔpH) and a potential difference (E_m), which can provide the energy for solute transport via secondary transporters. Secondary transporters consist of channels and carriers. Channels permit the very rapid diffusion of ions across the plasma membrane; in some cases their aperture is regulated by the membrane potential. Carriers permit a much slower flow of solutes and operate either as uniport (a single solute is transported) or as symport and antiport (when two solutes are transported in the same or opposite directions, respectively). All the cloned carriers are proton symports, which use the proton-motive force to activate the transport of another solute. Those transport proteins whose genes have been cloned are shown in italics (see text for references).

Cell Turgor and Related Functions

Osmotically stressed cultured plant cells respond by activating H^+-ATPase-mediated H^+ efflux (Curti et al., 1993), which in turn allows the uptake of more K^+ followed by osmotic adaptation. This illustrates how the H^+-ATPase can be involved in various turgor-related phenomena such as plant and cell growth, organ and stomata movement, and salinity tolerance.

Salinity imposes two stresses on the cell: one is the loss of turgor due to the hypertonicity of the extracellular medium, and the other is a direct effect of toxic ions on metabolism. The H^+-ATPase has been shown to be involved in salinity tolerance (Niu et al., 1993), but clearly it is not the sole factor involved in these processes. Indeed, the vacuole and its own membrane proteins play a major role, for instance, by depleting the cytoplasm of Na^+ and Cl^-. Moreover, salt-tolerant species may limit the influx of Na^+ (Spickett et al., 1993).

The H^+-ATPase also influences cell growth. Auxin activates the proton pump, resulting in loosening of the cell wall, either by breaking acid-labile bonds or by activating lytic enzymes within the wall (Rayle and Cleland, 1992). As a consequence, the wall yields under the cell turgor pressure. The H^+-ATPase is again required to compensate for the loss of turgor pressure accompanying cell enlargement. Another important protein in this process is aquaporin, which is found in the vacuolar membrane and in the plasma membrane (Chrispeels and Maurel, 1994). This channel protein permits rapid water flow in response to osmotic pressure, thus allowing its conversion into turgor pressure.

Cell turgor changes also promote organ movement and the modification of stomatal aperture. The pulvinal motor cells induce organ movement by swelling and shrinking during phenomena such as solar tracking by the plant to optimize orientation of photosynthetic leaves, or by the swift and spectacular reactions to touch found in specific plant species, such as the sensitive plant (*Mimosa pudica*). Swelling and shrinking result from massive water and ion fluxes through specific channels, processes in which the H^+-ATPase may be more or less directly involved.

The mechanisms regulating the swelling and shrinking of guard cells and leading to the opening and closure of stomata are better understood (reviewed by Kearns and Assmann, 1993). The guard cells are sensitive to CO_2 concentration, humidity, light, and hormones. These signals affect the H^+-ATPase as well as K^+ channels and anion channels. Activation of the H^+-ATPase leads to plasma membrane hyperpolarization and the subsequent opening of inward K^+ channels. K^+ influx may be accompanied by Cl^- influx and leads to water uptake, turgor increase, and cell swelling. Upon swelling, the unusual wall structure of the guard cells causes stomatal opening.

Intracellular pH Regulation

Metabolism of nitrate, import of solutes via H^+ symports, anaerobiosis, temperature changes, and light-to-dark transitions are all examples of pH-perturbing factors in plants. Under most conditions, the cytoplasmic H^+ concentration is maintained almost constant. The intracellular pH is mainly regulated by the H^+-ATPase and by intracellular H^+ utilization, especially in malate metabolism (reviewed by Kurkdjian and Guern, 1989). The H^+-ATPase has a pH optimum of 6.6, i.e. well below the physiological pH of the plant cell cytoplasm (usually around 7.2–7.5). Thus, whenever protons start accumulating in the cytoplasm, the activity of the H^+-ATPase increases, resulting in the expulsion of the excess H^+ from the cell. Cytoplasm alkalinization, which may result from increased ATPase pumping activity, can trigger important cellular events in response to hormonal and developmental signals (see Blatt and Armstrong, 1993, and review by Kurkdjian and Guern, 1989). Consequently, the H^+-ATPase might act as an intermediate in certain signal transduction pathways rather than simply being the final target.

H^+-ATPase Biochemistry

The plasma membrane H^+-ATPase is called a P-type ATPase because it undergoes phosphorylation during its catalytic cycle. The enzymatic cycle and kinetics of the plant H^+-ATPase are reviewed by Briskin and Hanson

(1992) and Serrano (1989). As shown in Figure 2, the H^+-ATPase, a protein with a molecular mass of about 100 kD, is composed of a single polypeptide that is predicted to be anchored in the plasma membrane by 10 membrane-spanning regions (Wach et al., 1992). It transports one proton per molecule of ATP hydrolyzed and has a pH optimum of about 6.6 and a K_m for MgATP of 0.3 to 1.4 mM. Potentials of about −150 mV and pH differences of two units are routinely observed across plant plasma membranes. Its specific activity in purified plasma membranes is usually of the order of 1 to 2 μmol P_i min^{-1} mg^{-1} protein, and it is inhibited by vanadate, dicyclohexylcarbodiimide, diethylstylbestrol, and erythrosin B, but not by NaN_3 or oligomycin (two inhibitors of the mitochondrial and chloroplastic ATPases), nor by nitrate (an inhibitor of the vacuolar membrane ATPase) or molybdate (an inhibitor of nonspecific phosphatases).

The variations in certain of the biochemical parameters cited above for whole-organ plasma membrane preparations can now be explained by the existence of several H^+-ATPase isoforms with different properties. For example, the variability in the K_m for MgATP is explained by biochemical studies on the different isozymes from *A. thaliana* expressed in the yeast *Saccharomyces cerevisiae* (Palmgren and Christensen, 1994); in this heterologous system, two (AHA1 and AHA2) of the three isoforms studied have a K_m of 0.15 mM, and the third (AHA3) has a K_m of 1.5 mM.

THE H^+-ATPASE IS REGULATED AT MANY DIFFERENT LEVELS

H^+-ATPase activity in the plant seems to be regulated by an extraordinary variety of mechanisms. This is probably justified by the numerous pivotal roles of this enzyme in plant physiology. In most cases, however, the level at which regulation occurs has not yet been clearly defined. Moreover, until recently it was not clear if a single H^+-ATPase was responsible for the whole range of roles and regulatory functions ascribed to this enzyme. Molecular genetic studies have shown that there are several genes coding for H^+-ATPases (Sussman, 1994). The existence of a gene family (at least 7 genes in tomato [Ewing and Bennett, 1994] and 10 in *A. thaliana* [Harper et al., 1994]) is certainly the major factor that permits the fine regulation of the H^+-ATPase in different cells and tissues. The differences in the observed kinetic properties of different H^+-ATPase isoforms may also confer an advantage, but until now it has not been possible to relate these differences to the plant physiology. H^+-ATPase gene duplications seem to have occurred early in angiosperm speciation (Moriau et al., 1993; Ewing and Bennett, 1994) and possibly correspond to specialization of H^+-ATPase lineages. Comparison of the mode of expression of corresponding genes in distant species may lead to a better understanding of this phenomenon. The molecular analysis of H^+-ATPase genes and gene products has already shed light on several regulatory events.

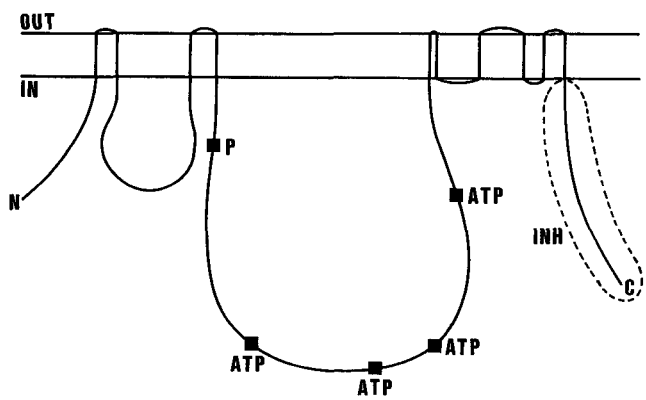

Figure 2. Topology of the plasma membrane H^+-ATPase. The H^+-ATPase is predicted as having 10 membrane-spanning regions that anchor it in the plasma membrane (Wach et al., 1992). The amino (N) and carboxyl (C) termini of the protein are on the cytoplasmic side of the membrane. The aspartate residue phosphorylated during the catalytic cycle is indicated (P), as are four consensus regions (ATP) that are part of an ATP binding site predicted by analogy with known ATP binding sites (Serrano, 1989). An autoinhibitory region (INH) is present at the C terminus (Palmgren et al., 1991); in the yeast enzyme, this has been shown to be involved in regulatory mechanisms involving interactions with other regions of the protein (Eraso and Portillo, 1994).

Tissue-Specific Distribution of H^+-ATPase

The secondary transport activities mediated by the H^+-ATPase seem to be essential in every single cell of the plant, at least at certain stages of development. RNA analysis has shown that the majority of the active H^+-ATPase genes analyzed so far are expressed in most plant organs, although to varying extents (Harper et al., 1990; Perez et al., 1992; Moriau et al., 1993; Ewing and Bennett, 1994), with two exceptions: the expression of the *A. thaliana AHA9* gene (Houlné and Boutry, 1994) and *AHA10* gene (Harper et al., 1994) has been detected only in anthers and developing seeds, respectively. However, as illustrated in Figure 3, the H^+-ATPase is not evenly distributed throughout the plant. Its accumulation in certain cells may provide information about the specialization of these cells in certain specific physiological functions.

Immunodetection and gene expression analysis have been used to study H^+-ATPase distribution at the cellular level. Both of these methods are useful approaches that provide complementary information. Immunodetection can directly demonstrate the presence of H^+-ATPase but probably does not distinguish between different isozymes. One type of gene expression analysis consists of following the expression of the GUS reporter gene (*gusA*) linked to the presumed transcription promoter region of individual H^+-ATPase genes. This approach is gene specific but may not take into account many of the posttranscriptional regulatory changes affecting the gene studied. Only three analyses of H^+-ATPase genes using the *gusA* reporter technique have been reported. Two involved the genes coding for the *A. thaliana* AHA3 and AHA10 isoforms, and the third involved the gene encoding the *Nicotiana plumbaginifolia* PMA1 isoform. It is not known if the pattern of expression of homologous genes in the two species is identi-

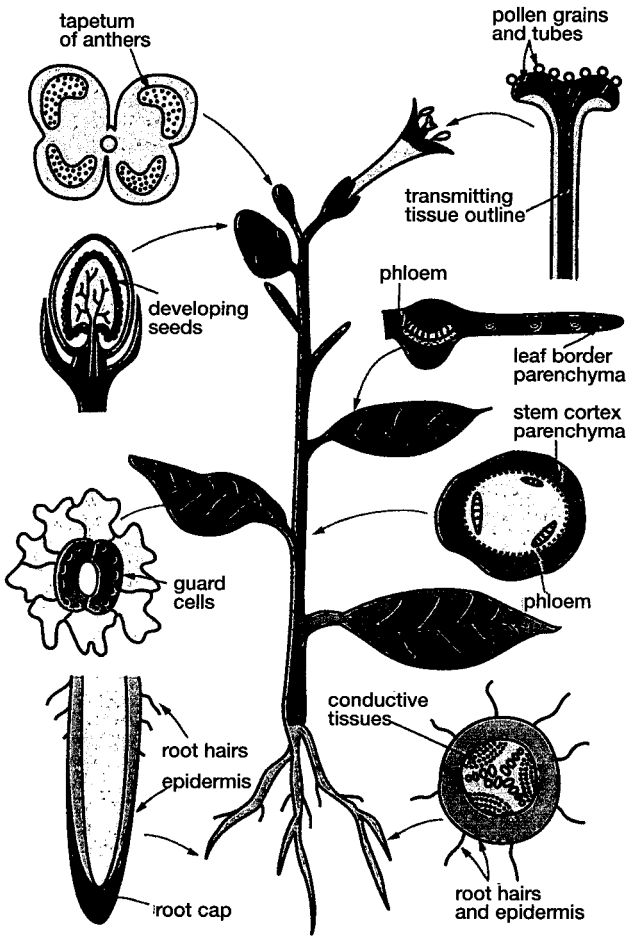

Figure 3. Plasma membrane H^+-ATPase distribution in the plant. Immunocytological and reporter gene studies of the H^+-ATPase have identified a series of tissues and cell types in which this enzyme accumulates. Further studies on other isoforms will probably define additional tissues where the H^+-ATPase is present in large amounts. Physiological functions promoted by H^+-ATPase, discussed in the text, can be correlated with the indicated distribution.

cal, but for each species it is clear that different H^+-ATPase genes have distinct patterns of expression that, at least in *N. plumbaginifolia*, may partially overlap.

In roots, light microscopic immunological studies have shown that the H^+-ATPase is abundant in the root cap and epidermis (including the root hairs) and is present in all cell types of the stele, with an especially high concentration in the companion cells of the phloem (Parets-Soler et al., 1990; Samuels et al., 1992; Stenz et al., 1993). These results have been corroborated by studies of the expression of two H^+-ATPase genes, *AHA3* and *pma1*, the former being expressed in phloem tissues and the latter in the root epidermis, including the root hairs (DeWitt et al., 1991; Michelet et al., 1994). Taken together, these results confirm the important roles of the root epidermis in mineral nutrition and of phloem cells in the transport and loading and/or unloading of solutes. Moreover, H^+-ATPase expression in the root cap correlates with H^+ fluxes and membrane hyperpolarization monitored during gravitropic responses (Stenz et al., 1993).

In stems and leaves, immunodetection experiments show H^+-ATPase to be chiefly present in guard cells and phloem cells (Parets-Soler et al., 1990; Villalba et al., 1991), especially in transfer cells that occur near the ends of phloem bundles (Bouché-Pillon et al., 1994). Expression studies on the *AHA3* and *pma1* H^+-ATPase genes confirm and extend these results, showing that *AHA3* is indeed expressed in phloem tissues throughout the plant (DeWitt et al., 1991) and that *pma1* can be induced in guard cells (Michelet et al., 1994).

In reproductive organs, H^+-ATPase is detected immunologically mainly in the L3 cell layer of the floral meristems, the anthers, the sepal parenchyma, and the ovary mesocarp (Parets-Soler et al., 1993). As expected, the conducting tissues also show high immunoreactivity. H^+-ATPase gene expression studies give better resolution. In addition to being expressed in the phloem tissues, *AHA3* is found in pollen and in the developing seed funiculus, micropyle, and chalaza (DeWitt et al., 1991). *pma1* is expressed in the tapetum, the pollen grain and tube, the layer of cells surrounding the transmitting tissue of the style, the phloem of the fruit placenta, and the developing seed (Michelet et al., 1994). *AHA9* is expressed mainly in the integument tissues surrounding the embryo (Harper et al., 1994). All these tissues and cells have absorptive or secretory roles and would therefore require high levels of H^+-ATPase activity.

Environmental and Developmental Regulation

The expression of *AHA3* and *pma1* is regulated during development. *AHA3* is expressed uniquely in phloem tissue, but only in the fully differentiated state (DeWitt et al., 1991). *pma1* is also expressed in the phloem, but only in the fruit. Moreover, environmental conditions have a marked effect on *pma1* expression in guard cells: under normal growth conditions, expression is often undetectable, but increases dramatically when plantlets are grown in a liquid medium or when leaves from soil-grown plants are immersed for a few hours in a nutritive solution or in water (Michelet et al., 1994).

Translational Regulation

Transcriptional regulation of the H^+-ATPase genes accounts for the tissue-specific distribution of this enzyme. The regulation of at least some H^+-ATPase genes is dependent on environmental and developmental factors, but it seems that translational regulation may also affect the expression of certain isozymes. Indeed, many H^+-ATPase genes produce very unusual mRNAs, whose leader sequence (i.e. that from the 5′ end to the main initiator AUG) is very long and often contains an upstream open reading frame (URF) a few codons in length (Perez et al., 1992). In the case of *pma1* from *N. plumbaginifolia*, it has been shown that these features influence translation (Michelet et al., 1994), which may result in rapid modulation of H^+-ATPase synthesis, e.g. in response to environmental signals.

Posttranslational Regulation

H^+-ATPase is thought to be transported to the plasma membrane via the secretory pathway. It is known that auxin enhances membrane flow from the ER to the plasma membrane (Hager et al., 1991), and it has been suggested that this might be due to the acceleration of exocytotic processes. As auxin also induces a rapid increase in both the amount and activity of H^+-ATPase in the plasma membrane, it seems probable that accelerated transport is involved in this induction process (Hager et al., 1991). However, this implies either that a pool of H^+-ATPase is present in secretory vesicles, awaiting dispatch to the plasma membrane, or that transport is rate limiting. Considering the speed of the auxin response seen in the study by Hager et al. (1991), translational regulation may also be involved.

Protein degradation is another posttranslational regulatory process that may affect H^+-ATPase levels. Indeed, on the basis of data on cycloheximide inhibition of protein synthesis, Hager et al. (1991) suggested that the pool of H^+-ATPase newly inserted in the plasma membrane during auxin treatment has a very short half-life of about 12 min. Whether this short half-life results from indirect destabilization by cycloheximide of an otherwise stable H^+-ATPase isoform or from auxin induction of an unstable isozyme is not known.

Finally, the H^+-ATPase activity of the cell can be posttranslationally regulated by direct modulation of the enzyme. It is known that the plant H^+-ATPase is modulated by an autoinhibitory region located in the C-terminal region of the protein (Palmgren et al., 1991; see our Fig. 2). A similar autoinhibitory region is found in the *S. cerevisiae* and *Neurospora crassa* H^+-ATPases. It is interesting to note that, in the *S. cerevisiae* enzyme, the C-terminal region mediates in vivo regulation in response to growth on Glc. This so-called "Glc effect" increases the activity several-fold, and it has been suggested that this results from the disruption of interactions between different parts of the enzyme after phosphorylation of one, or possibly two, residues in the C-terminal region (Eraso and Portillo, 1994). Modulation by (de)phosphorylation is an attractive hypothesis, since it is known that the yeast and plant H^+-ATPases are naturally phosphorylated by a membrane-associated protein kinase. Although such a modulation has never been proven, circumstantial evidence suggests that a calmodulin-dependent protein kinase may regulate the H^+-ATPase (Shimazaki et al., 1992; reviewed by Sussman, 1994). A family of genes encoding calmodulin-dependent protein kinases has been characterized in *A. thaliana*, and the product of some of these may be targeted to the plasma membrane (reviewed by Sussman, 1994).

In addition to the aforementioned effects of auxin on H^+-ATPase quantity, it may also affect H^+-ATPase activity. Indeed, the protoplast transmembrane potential is increased by auxin (Barbier-Brygoo et al., 1989), as is the in vitro proton-pumping activity of microsomal vesicles (Santoni et al., 1993). Fusicoccin also stimulates H^+-ATPase activity, possibly via the C-terminal inhibitory domain (Johansson et al., 1993).

CONCLUSION

The plant H^+-ATPase is encoded by a multigene family. Those members that have already been studied are expressed in a very specific way, which depends on the cell type, developmental stage, and environmental stimuli. The ability to provide full-expression analysis for all H^+-ATPase isoforms using the methods of immunodetection and *gusA* reporting is only a matter of time. One point of interest will be to determine whether the H^+-ATPase genes of different species have similar patterns of expression. Regulation of activity has already been demonstrated to occur, or is strongly implicated as occurring, at several posttranscriptional steps, namely translation, protein targeting, protein stability and enzyme modulation via the C-terminal region, and possibly by (de)phosphorylation.

The basic enzymatic properties of individual isozymes can be studied using heterologous expression in yeast, but the major objective remains the elucidation of all regulatory mechanisms affecting the expression and activity of each H^+-ATPase in the plant, especially the question of whether the H^+-ATPase is the final step in signal (e.g. auxin) transduction or whether it plays an effector role in certain cases. Approaches involving mutant analysis (gene tagging, antisense inhibition, overexpression) should be particularly useful in relating the information obtained in molecular genetics and biochemical studies of the H^+-ATPase to the physiology of the plant.

Received November 18, 1994; accepted January 16, 1995.

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