

Genetic Manipulation of Vacuolar Proton Pumps and Transporters^{1,2}

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Cells expend as much as 50% of their total intracellular energy reserves to maintain gradients of ions across their membranes (Nelson, 1994). These gradients have been associated with the myriad of functions attributed to the membranes of living organisms. In the past, much of our knowledge about the function of the proteins involved in creating these ion gradients came from biochemical and biophysical studies. However, it was often difficult to associate the vast repertoire of membrane functions with particular proteins. Now, with the complete sequence of the Arabidopsis and yeast (*Saccharomyces cerevisiae*) genomes, and the facility with which genes can be engineered and transferred between these two organisms, there are new opportunities to identify each transporter encoded in the genome with a specific set of functions in the organisms. These new tools have also made it possible to examine the basic tenets of the chemiosmotic hypothesis in intact organisms.

Plants and fungi are similar in that they use protons as the "currency" (proton electrochemical gradient [PEG]) with which to mediate ion gradients (Sze et al., 1999), whereas animal cells use Na⁺ ions as the driving force. While plants work at photosynthesis, they are making an important long-term investment by creating H⁺ gradients. The initial "cash reserve" is generated by transport systems that form the H⁺ gradient across biological membranes. Because these pumps invest the plant's energy, they are likely to be tightly controlled (Sagermann et al.,

2001). The accumulation of ions into intracellular organelles (vacuoles, prevacuolar bodies, and Golgi vesicles) against the concentration gradient often requires the "withdrawal" of the H⁺ currency from an intracellular organelle by the secondary transporters. Each of the secondary transporters (e.g. proton/cation antiporters) can be thought of as an individual company that imports and exports goods and services. Thus, a hierarchy is formed between the H⁺ pumps that inject the currency into the intracellular organelles and the secondary transporters that utilize this currency. In this survey, we will consider only the role of the two vacuolar H⁺ pumps in the generation of the PEG, although we are aware that the magnitude of the PEG can be affected by other transporters (i.e. H⁺ cotransporters and electrogenic antiporters and other ion pumps).

In both Arabidopsis and yeast cells, the vacuole is the largest intracellular H⁺ bank (see Fig. 1a). The acidification of the Arabidopsis vacuole is carried out by two systems: the vacuolar H⁺-ATPase and the vacuolar H⁺-pyrophosphatase. The vacuolar H⁺-ATPase is a multisubunit complex whose subunits are encoded by at least 26 genes (Sze et al., 2002). The Arabidopsis H⁺-pyrophosphatase is a single subunit protein. However, the Arabidopsis genome contains three homologs (AVP1–AVP3; Drozdowicz and Rea, 2001). The different structure and energy requirements of the vacuolar H⁺-ATPase and the vacuolar H⁺-pyrophosphatase may offer plants the biochemical and regulatory plasticity with which to generate the PEG in a range of growth conditions. In both Arabidopsis and yeast, many of the genes encoding the secondary transporters that utilize the PEG (Anraku, 1996; Serrano and Alonso, 2001) have been identified. However, the roles these genes play in cell growth and in the response to environmental stresses such as toxic salts and high osmolarity are only beginning to emerge.

The Arabidopsis genome encodes approximately 500 cotransporters, many of which localize to the vacuolar membrane and make use of the PEG either directly or indirectly. These transporters have been

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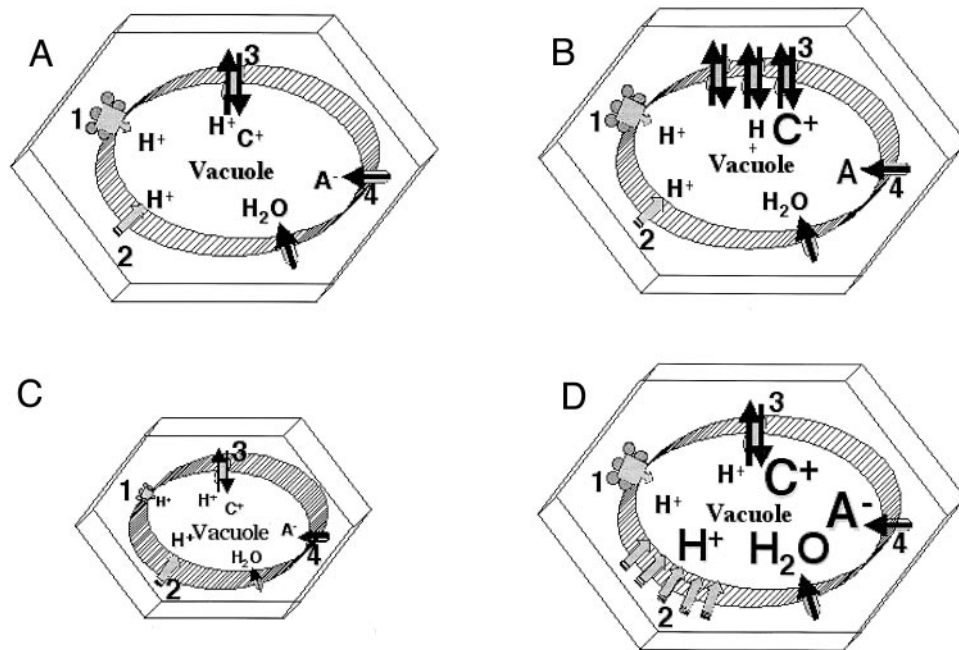


Figure 1. Model of H^+ pumps and transporters found in the plant vacuolar membrane. A, H^+ -ATPase (1) and H^+ -PPase (2) transport protons (H^+) into the vacuolar lumen. Organic and inorganic anions (A^-) enter the vacuole via channels (4) to achieve electroneutrality, allowing the generation of a pH gradient. This PEG drives secondary active accumulation of organic and inorganic cations into the vacuole via H^+ /cation antiporters (3), with osmotically accompanying water. B, Ectopic expression of cation/ H^+ antiporters. Transgenic plants with enhanced expression of cation/ H^+ antiporters (3) in the vacuolar membrane sequester higher amounts of cations through the utilization of the existing PEG generated via the two H^+ pumps (1 and 2). C, Reduced H^+ -pumping activity in the *det3* mutant. H^+ -ATPase activity (1) in the *det3* mutants is diminished. We speculate that there is a reduction in the PEG perturbing transport activities (3 and 4) across the vacuolar membrane. D, Ectopic expression of AVP1. We speculate that transgenic plants with enhanced expression of the AVP1 (2) have an enhanced PEG. This altered PEG is thought to increase transport activities (3 and 4) across the vacuolar membrane.

classified on the basis of both phylogeny and function (Paulsen et al., 1998; Maser et al., 2001; Ward, 2001) as transporters for sugar, cation, C, or N compounds. Manipulating the regulation of the PEG across the tonoplast (vacuolar membrane) by changing the activity of the primary H^+ pumps may be a means of coordinately regulating a plethora of transporters.

In this article, we describe experiments that use genetic manipulations to alter the ion transport across the vacuolar membrane of *Arabidopsis* and yeast (see Fig. 1). Studies have shown that the availability of mutants and the ability to transfer genes between these two organisms set the stage for a powerful method of analyzing a process as complex as ion transport. A key conclusion is that it is possible to alter ion transport broadly by altering the proton gradient. The ability to alter ion transport has both theoretical and practical applications. The ability to manipulate the proteins—pumps, transporters, and ion channels—responsible for the movement of ions across the vacuolar membrane will further our understanding of the role of this organelle in the growth and development of plants. The capability of engineering the level and behavior of these pumps offers the possibility of increasing the tolerance of the plant

to adverse conditions. This technical breakthrough presages engineered plants of agricultural importance capable of growing in soils of high salinity and restricted water availability, as well as plant biofilters capable of detoxifying industrial waste sites containing ions toxic to humans.

THE ARABIDOPSIS PROTON PUMP ELUCIDATES ION METABOLISM IN YEAST

Biochemical and biophysical experiments place the proton gradient at the control center of ion movements. Based on this idea, alterations in the proton gradient could compensate for many defects in specific transporters or increase the tolerance to toxic ions. For example, a mutant with a defect in the transport of a particular ion into the vacuole might be suppressed by overexpressing the vacuolar proton pump. This experiment would be difficult to carry out by overexpressing the *Arabidopsis* or yeast V-type ATPases because both the plant and yeast are multisubunit proteins. However, the *Arabidopsis* AVP1 transporter encodes a single polypeptide capable of enhancing the pumping of protons into the lumen of the yeast vacuole (Kim et al., 1994). The

simplicity of the AVP1 structure makes it an excellent candidate for manipulating the proton gradient.

A clear demonstration of the utility of the Arabidopsis AVP1 protein came from heterologous expression of this protein in yeast (Gaxiola et al., 1999). Yeast mutants lacking the plasma membrane sodium efflux pump *Ena1* are sensitive to low concentrations of sodium that do not inhibit the growth of wild-type strains (Haro et al., 1991). In the absence of the sodium efflux pump, cytosolic sodium builds up to toxic levels. Could manipulation of the proton gradient in the vacuole provide the energy to sequester enough of this toxic sodium in the vacuole to overcome the deleterious effects of the loss of the plasma membrane sodium efflux pump? To answer this question, a gain-of-function allele of the Arabidopsis *AVP1* (*AVP1-D*) was expressed in the yeast *ena1* strain. Two striking results were obtained (Gaxiola et al., 1999). First, heterologous expression of the Arabidopsis *AVP1-D* protein in yeast suppressed the salt sensitivity of the *ena1* mutant. Second, this *AVP1-D*-mediated phenotype required functional ion transporters (*Nhx1* and *Gef1*) on a prevacuolar membrane to mediate salt tolerance. These experiments support the idea that alterations in the vacuolar PEG can enhance the ability of secondary transporters to sequester ions in the lumen of the vacuole.

HETEROLOGOUS EXPRESSION OF ARABIDOPSIS TRANSPORTERS IN YEAST

Because yeast shares many basic transport strategies with plants, it provides a facile system to test the functions of plant transport proteins. Moreover, the recent construction of a library containing a null allele for each of the 6,100 yeast genes means that the function of any Arabidopsis gene can be tested by its ability to complement the defect of one or all of the yeast mutants known to be defective in a transport process. Successful complementation of the yeast mutant by an Arabidopsis gene can be remarkably informative about the function of the plant gene. Of course, in such an experiment, only a positive result is instructive. The key question was whether membrane proteins from Arabidopsis would be able to target the appropriate membrane and function in the heterologous yeast system. There are now many successful experiments showing that heterologous expression works, and is an invaluable tool for assessing the function of Arabidopsis membrane proteins (Tanner and Caspari, 1996; Sze et al., 2000; Barbier-Brygoo et al., 2001).

This heterologous expression system is not only useful in defining the function of plant membrane proteins, but also in resolving complex transport puzzles in yeast. The Arabidopsis chloride channel genes played an important role in understanding the function and phenotypes of the yeast *gef1* mutant. Defects in the yeast *GEF1* gene lead to an iron re-

quirement and cation sensitivity in yeast (Stearman et al., 1996; Gaxiola et al., 1998). These phenotypes initially appeared confusing because the amino acid sequence of the *Gef1* protein indicated that it was a CLC voltage-gated chloride channel homolog.

The solution to the puzzling mutant phenotypes was that the iron requirement is related to chloride uptake into vesicle compartments. High-affinity iron uptake in yeast is mediated in part by the Fet3-Ftr1 oxidase-permease complex. The Fet3 oxidase requires copper to function. Copper loading of the apoprotein Fet3 takes place in late Golgi vesicles where the coordinate activity of a copper ATPase (*Ccc2*), the vacuolar H⁺-ATPase and *Gef1* are required. This model posits that the loading of copper onto the Fet3 apoprotein requires both intravesicular uptake of copper and an acidified environment. The compensatory transport of an anion via *Gef1* will promote electroneutrality. The cation sensitivity of *gef1* mutants can be explained by the requirement of anion (chloride) transport at the vacuole to allow the formation of the PEG required for the uptake of the cations (see Fig. 1a). According to this view, failure to take up chloride would impede sequestration of the cations, and the buildup of these toxic cations in the cytosol would lead to the consequent sensitivity.

Of course, it was possible that the yeast *Gef1* protein was not a chloride channel, and that this explanation was incorrect. However, both of the puzzling *gef1* mutant phenotypes can be suppressed by the introduction of Arabidopsis *CLC-c* and *-d* chloride channel genes (Gaxiola et al., 1998) and the ray *Torpedo marmorata CLC-0* gene, a bona fide voltage-gated chloride channel. The ability of these heterologous chloride channel genes to suppress the yeast mutant phenotypes strongly supports the proposed model. Interestingly, Arabidopsis *CLC-a*, which was unable to suppress *gef1* phenotypes (Gaxiola et al., 1998), has been implicated in nitrate transport in Arabidopsis (Geelen et al., 2000). However, direct evidence of *Gef1*-mediated chloride transport in yeast is missing.

AN ARABIDOPSIS MUTANT ALTERED IN THE V-ATPASE

The phenotypes of mutants defective in the Arabidopsis V-ATPase will be extremely informative about the role of this multisubunit complex in the growth and morphology of the plant. The analysis of Arabidopsis mutants defective in the vacuolar ATPase is in a rudimentary stage compared with studies of the comparable protein in yeast. The main difficulty has been the lack of availability of mutants defective in each of the many subunits that compose the active protein.

Fortunately, there is one mutant whose phenotypes suggest that further studies will be extremely rewarding. The first V-ATPase mutant, *det3*, shows a reduction in subunit C and an approximately 2-fold

reduction in V-ATPase activity (see Fig. 1c; Schumacher et al., 1999). The *det3* mutant has a number of unexpected phenotypes, the most striking of which is that the plants are de-etiolated when grown in the dark (Schumacher et al., 1999). Furthermore, there are defects in hypocotyl cell expansion, shoot apical meristem activity, and response to brassinosteroids. The phenotypes of the *det3* mutant—the inability to properly respond to dark, the growth defects, and the hormone responses—cannot be simply explained, which emphasizes the importance of studies on the V-ATPase mutants in a multicellular organism.

The DET3 function appears to be required for a response to brassinosteroids, but not for a response to auxin (Schumacher et al., 1999). Moreover, the *det3* mutant is defective in stomatal closure induced by high external calcium ions and hydrogen peroxide, whereas stomatal closure induced by ABA and cold was maintained (Allen et al., 2000). One interpretation of these findings is that only a subset of signaling pathways absolutely requires integrity of the vacuolar PEG (Harper, 2001); others may be dependent on specific vacuolar pumps (i.e. Ca^{2+}) instead of antiporters to mediate the transport of signal transduction molecules across the vacuole. Alternatively, the differential phenotypes displayed by the *det3* mutant to ABA and cold could be that these other stimuli signal across the endoplasmic reticulum, rather than the vacuole (Harper, 2001).

The interpretation of DET3 function is hampered by the lack of a loss-of-function allele. Because the *det3* mutant maintains partial V-ATPase activity, it is likely that the complete spectrum of functions carried out by the V-ATPase is not revealed in this mutant. However, a complete loss of DET3 may cause lethality in Arabidopsis. One possible avenue to obtain more severe mutants of the V-ATPase would be to search for them in the background of a strain overexpressing AVP1, the H^+ pyrophosphatase. This protein may be able to functionally replace the reduced activity of V-ATPase in certain tissues and permit the isolation of otherwise lethal V-ATPase mutations.

INCREASING THE PEG ACROSS THE ARABIDOPSIS VACUOLE

An alternative to manipulating the PEG via the V-ATPase is to increase or decrease the activity of the AVP1 pyrophosphatase. A potential advantage of the AVP1 overexpression is that this H^+ pump uses inorganic pyrophosphate, allowing ATP to be conserved and used to improve plant cell performance under a more demanding environment (Stitt, 1998). The ability to alter the PEG across the yeast vacuole through heterologous expression of a single plant H^+ pump, AVP1-D, suggests that high-level expression of this pump in plants may increase the PEG across the vacuole. In fact, transgenic Arabidopsis plants that ectopically express AVP1 exhibit increased tol-

erance to salt. Furthermore, AVP1 transgenic plants are also drought tolerant (see Fig. 2F) and their size is enhanced because of an increase in cell number (Eckardt et al., 2001; Gaxiola et al., 2001). Transport studies show that overexpressing AVP1 causes a 36% increase in plant vacuolar $\text{Ca}^{2+}/\text{H}^+$ transport. AVP1 transgenic plants accumulate more solutes than control plants under a constant water content. These data suggest that AVP1 expression also enhances the activity of various plant secondary transporters, including the Na^+/H^+ exchangers. The inference is that an increase in these exchangers leads to increased solute accumulation in the vacuole and, therefore, an increase in water retention (see Fig. 1d).

Plants overexpressing AVP1 are large, and *det3* plants are small (see Fig. 2E). This observation would suggest that the PEG across the plant vacuole is an important mechanism to regulate cell growth. In agreement with this idea, carrot (*Daucus carota*) plants perturbed in vacuolar H^+ -ATPase activity also have reduced cell expansion and altered leaf morphology (Gogarten et al., 1992). Taken together, these findings suggest that the PEG across the plant vacuole is an important mechanism to regulate cell growth. One prediction of this notion is that ectopic expression of AVP1 might rescue some of the *det3* phenotypes.

These speculations point out how important it is to have loss-of-function mutants in the AVP1 pyrophosphatase. Such plants, if viable, would provide important insights into the functions of this activity that are distinct from those of the V-ATPase. However, there are three such genes in Arabidopsis: AVP1, AVP2, and AVP3. Although they appear to encode proteins with different specificities (Drozdowicz and Rea, 2001), a complete loss of function could require multiple knockouts. The availability of T-DNA insertion lines (for example http://signal.salk.edu/tdna_FAQs.html or http://www.tmri.org/pages/collaborations/garlic_files/GarlicDescription.html) should certainly speed up the search for loss-of-function mutants in both H^+ pumps. Furthermore, it would be extremely useful to have direct measurements of AVP1 activity on the vacuolar, Golgi, and plasma membranes. Various secondary transporters, on all endomembranes, also need to be assayed for changes in activity.

INCREASING EXPRESSION OF PLANT VACUOLAR ANTIPORTERS

Another method for increasing the ion flux is to increase the expression of genes encoding vacuolar antiporters (see Fig. 1b). Transport studies demonstrate that various plants contain tonoplast Na^+/H^+ antiporter activity (Barkla et al., 1994; Ballesteros et al., 1997; Blumwald and Gelli, 1997; Blumwald et al., 2000). The identification and characterization of an intracellular yeast Na^+/H^+ antiporter (Nass et al., 1997; Nass and Rao, 1998) and the completion of the

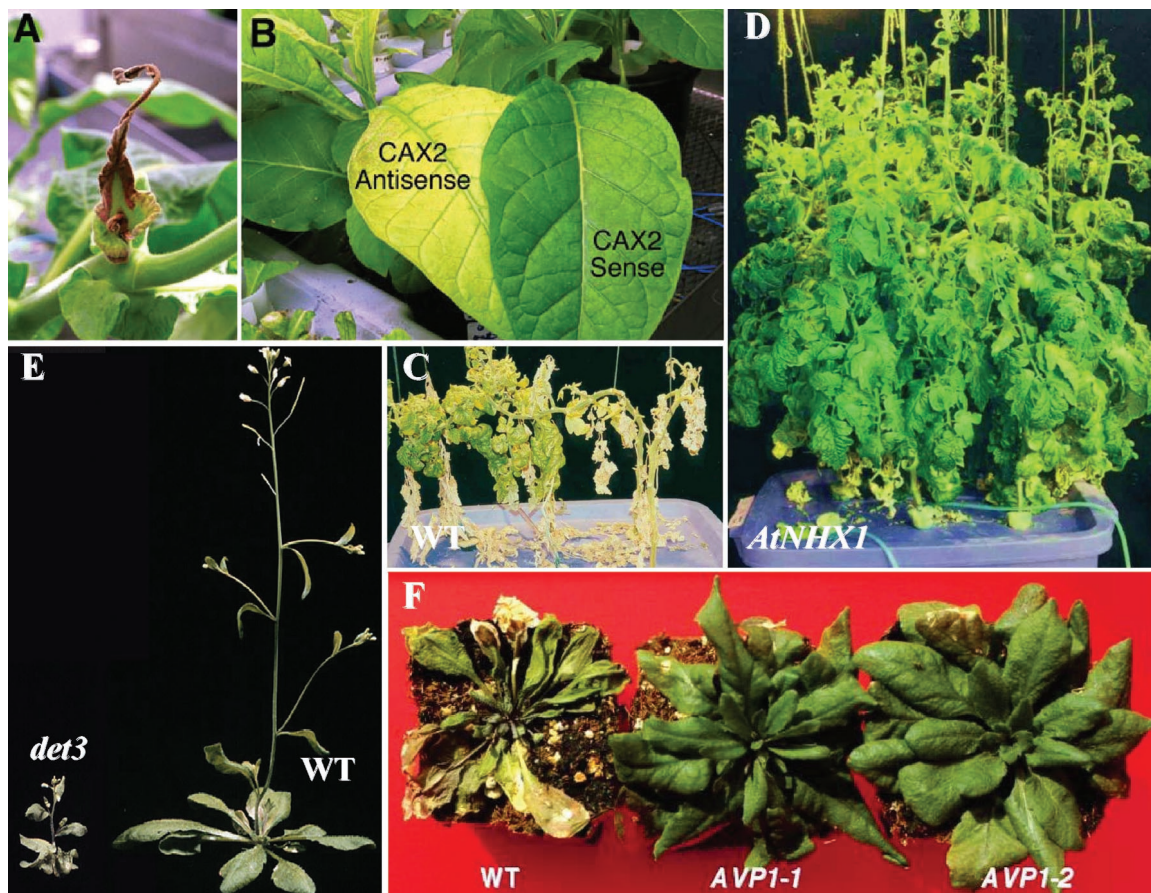


Figure 2. Plant phenotypes of altered expression of H^+ pumps and H^+ /cation antiporters. Expression of the Arabidopsis *CAX1* gene in tobacco (*Nicotiana tabacum*) causes apical burning and other growth defects associated with calcium deficiencies. B, Expression of Arabidopsis *CAX2* gene in the sense orientation makes tobacco plants more tolerant of manganese. The Arabidopsis *CAX2* sense- and antisense-expressing plants shown here were grown for 1 week in a hydroponic solution containing 0.5 mM $MnCl_2$ (figure from Hirschi, 2001, with permission). Control (C) and *AtNHX1* transgenic tomato (*Lycopersicon esculentum*; D) growing in the presence of 200 mM NaCl. E, *det3* and control Arabidopsis plants grown in soil. F, Control and transgenic *AVP1* lines after recovery from 10 d of drought stress.

Arabidopsis genomic sequence facilitated the cloning of the first Arabidopsis Na^+/H^+ (Gaxiola et al., 1999; Aspe et al., 1999).

Plants have been engineered to overexpress this member of the Arabidopsis tonoplast Na^+/H^+ antiporter family (Apse et al., 1999; Zhang and Blumwald, 2001; Zhang et al., 2001). In each case, the plants accumulate more Na^+ in their vacuoles and are more tolerant to Na^+ in the growth media (see Fig. 2, C and D). The Na^+ accumulation occurs mainly in the green parts of the plant, but not in the fruits (Zhang and Blumwald, 2001; Zhang et al., 2001). These results strongly implicate that heightened activity of a single Na^+ transporter on the tonoplast can enhance crop productivity.

Recent work with reconstituted liposomes demonstrates that *AtNHX1* catalyzes low-affinity transport of both Na^+ and K^+ (Venema et al., 2002), making it likely that in normal conditions, without sodium stress, *AtNHX1* is involved in K^+ homeostasis. The fact that overexpression of *AtNHX1* antiporter in-

creases the vacuolar Na^+ sequestration implies that there is enough PEG to support the extra activity. Alternatively, *AtNHX1* overexpression could trigger the activation of any of the vacuolar H^+ pumps to provide the extra PEG required.

Ectopic expression of the tonoplast-localized H^+ /metal transporter *CAX2* in plants causes increased transport of numerous metals into the plant vacuole (Hirschi et al., 2000). However, these plants are only modestly tolerant to more manganese in the media (see Fig. 2B), which suggests that increased *CAX2* activity is only one of several modifications required to increase significantly metal sequestration in plants, and consequently to engineer tolerant phenotypes.

Expression of the Zn^{2+} and Mg^{2+}/H^+ *AtMHX* causes plants to be more sensitive to Zn^{2+} and Mg^{2+} in the growth media without increased accumulation of these ions in the stem tissue (Shaul et al., 1999). Ectopic expression of the Arabidopsis calcium exchanger, *CAX1*, doubles total calcium accumulation but renders plants more sensitive to environmental

perturbations (see Fig. 2A; Hirschi, 1999). The increased sensitivities may be caused by alterations in calcium distribution throughout the plant cell or by altering the intracellular Ca^{2+} pulses required for signal transduction pathways (Allen et al., 2000).

These studies suggest that no unifying principle can be applied to the phenotypic consequences of ectopic expression of vacuolar antiporters. In mammalian cells, Na^+/H^+ exchanges play an essential role in the regulation of intracellular pH, and ectopic expression of this transporter may cause drastic fluctuations in the PEG across membranes (Aharonovitz et al., 2000).

ALTERING THE PEG FOR CROP IMPROVEMENT

The phenotypes caused by ectopic expression of AVP1 in *Arabidopsis* suggest that manipulation of vacuolar proton-pumps in economically important crops holds promise for the reclamation of farmlands lost to salinization and lack of rainfall. In addition, the fact that these transgenic plants are larger than control plants could contribute to the determination of ways to increase plant productivity under all soil conditions. Transgenic plants might be designed that could alter the vacuolar PEG in particular tissues or during certain stress conditions. Moreover, the combination of the pump with various transporters may offer both diversity and amplification of the effects. For example, the combination in one plant of the AVP1 transgene with the ATNHX1 transgene may give additive effects that provide a substantial increase in salt tolerance. Salt-tolerant plants like *Mesembryanthemum crystallinum* naturally utilize high-level expression of both the vacuolar H^+ -ATPase and Na^+/H^+ antiporter to tolerate high-salt conditions (Tsiantis et al., 1996). For phytoremediation purposes, root-specific promoters could simultaneously overexpress AVP1 and the cation/ H^+ antiporter CAX2. These plants may be able to remediate soils contaminated with heavy metals.

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