Plant Proton Pumps: Regulatory Circuits Involving H^+-ATPase and H^+-PPase

A.T. Fuglsang, J. Paez-Valencia, and R.A. Gaxiola

Abstract Proton gradients are crucial for the transport of ions and solutes across the different membranes in plant cells. Several important developmental processes require a tightly controlled proton gradient across cellular membranes. This chapter focuses on two of the three primary proton transport proteins: the plasma membrane H^+-ATPase and the H^+-PPase.

This chapter is divided into two sections. The first section describes the state of plasma membrane H^+-ATPase research,with emphasis on the regulation by physiological stimuli, and proposes a novel mechanism of H^+-ATPase regulation. The second section focuses on the H^+-PPase and new evidence consistent with the involvement of H^+-PPases in plant growth and development. A hypothetical model is discussed.

1 P-Type H^+-ATPases

P-type H^+-ATPases are active transporters that utilize ATP as an energy source to transport H^+ across the plasma membrane. This, in turn, creates an electrochemical gradient that energizes channels and co-transporters (Duby and Boutry 2009; Gaxiola et al. 2007; Sondergaard et al. 2004). The plasma membrane H^+-ATPases belong to a large family of pumps, P-type ATPases, all of which are energized by ATP and form a phosphorylated aspartyl intermediate during the reaction cycle, therefore the name P-type. The P-type ATPase family is further divided

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phylogenetically into subgroups dependent on their substrate specificity (Axelsen and Palmgren 1998). In this book you will also find information about some of the other P-type ATPase subgroups, namely calcium and lipid pumps.

The plasma membrane H^+-ATPase is a single subunit protein at approximately 950 amino acid residues. The protein contains ten trans-membrane helices and a large cytoplasmic domain. The cytoplasmic domain consists of four domains: the nucleotide binding domain (N-domain), the phosphorylation domain (P-domain), the actuator domain (A-domain) and the regulatory domain (R-domain). The N domain is a built-in protein kinase that phosphorylates the conserved aspartate residue on the P domain. The A domain is an intrinsic protein phosphatase that dephosphorylates the same aspartyl group. In 2007 a crystal structure at 3.6 Å of the *Arabidopsis thaliana* isoform 2 (*AHA2*) was published (Pedersen et al. 2007). The crystal structure includes the A, P, and N domains, but does not provide information on the structure of the R domain.

The R domain consists of the C-terminal of the protein and includes approximately 100 amino acids. Further, about 20 N-terminal residues might contribute to the function of the R-domain (Ekberg et al. 2010) (see below). The role of the regulatory domain was first recognized when it was observed that the removal of the C-terminal by trypsination results in an activated form of the H^+-ATPase (Palmgren et al. 1990). The regulatory domain forms an auto-inhibitory domain by binding to the large cytoplasmic domain and thereby inactivating the H^+-ATPase. By a systematic mutagenesis approach two regions have been pinpointed as important for the intramolecular interaction (Axelsen et al. 1999). These domains are called Region I and Region II (RI and RII, respectively). Mutations within these two regions cause a constitutive active pump, most likely because they affect the interaction with the intramolecular receptor for the C-terminal domain. In the inhibited state the protein is thought to exhibit a closed compact structure in contrast to the activated state where the C-terminal is released from the core part of the protein.

Recently it has been demonstrated that the N-terminal end is directly involved in controlling the pump activity state, and that N-terminal displacements are coupled to secondary modifications taking place at the C-terminal end (Ekberg et al. 2010). This suggests an intricate mechanism of *cis*-regulation with both termini of the protein communicating to obtain the necessary control of the enzyme activity state.

1.1 Arabidopsis *Encodes 11 Members of H^+-ATPases*

The *Arabidopsis* genome encodes 11 genes of plasma membrane H^+-ATPases named *AHA1–11* for *Arabidopsis* $H^{\pm}$-ATPase isoform number 1–11. The plasma membrane H^+-ATPase is essential for the plant cell and the large number of genes reflects the expression of different isoforms in different cell types and organs. *AHA1* and *AHA2* are the most abundant isoforms expressed all over the plant, with AHA1 mainly in the leaves and AHA2 mainly in the roots (data obtained from Genevestigator). Reverse genetics have only revealed limited information about the

physiological role of H^+-ATPases most likely because the different isoforms can functionally substitute for each other. Very recently a study of the two major isoforms were published. *aha1* or *aha2* single knock out plants or plants with reduced *AHA1* and *AHA2* transcript does not possess any detectable phenotypes but the *aha1/aha2* double knock out is lethal (Haruta et al. 2010). Analysis of expression patterns based on available micro array data shows that most of the H^+-ATPase isoforms are expressed at a relative constant level and expression level does not change when a related isoform is deleted or reduced as found in the *aha1* and *aha2* plants (Haruta et al. 2010). Interestingly it was found that in the plants with reduced levels of either *AHA1* or *AHA2*, the remaining plasma membrane H^+-ATPase isoforms had a higher degree of phosphorylation of the pen-ultimate Thr residue. This indicates that most regulation of the enzyme activity occurs at the post-translational level.

1.2 Mechanism of Activation by 14-3-3 Proteins

The plasma membrane H^+ pump is subject to regulation by a number of proteins interacting directly with the pumps. The first proteins found to interact with the pump were 14-3-3 regulatory proteins. 14-3-3 proteins belong to a highly conserved protein family that typically bind to phosphorylated target proteins and regulate signaling in eukaryotic cells (Oecking and Jaspert 2009).

14-3-3 proteins bind to the C-terminal regulatory domain of the H^+ pump. Binding of 14-3-3 proteins to the H^+ pump is dependent on the phosphorylation of the penultimate Thr residue (Fuglsang et al. 1999, 2003; Olsson et al. 1998; Svennelid et al. 1999). The phosphorylation site within the very C-terminal end of the H^+-ATPase is an uncommon protein kinase recognition site H/S-Y-T-V. However, a number of similar 14-3-3 binding sites are now identified in other proteins and named mode III (Coblitz et al. 2005). In this study, they demonstrate that binding of 14-3-3 protein to the C-terminal end of several membrane proteins is required for their targeting to the plasma membrane (Coblitz et al. 2005; Shikano et al. 2005). However, earlier studies of AHA2 expressed in yeast did not show a role for 14-3-3 proteins in targeting this pump to the plasma membrane (Jahn et al. 2002), but a reinvestigation of this mechanism might reveal new information.

The fungal phytotoxin fusicoccin (FC) is a commonly used tool in the study of H^+-ATPase activity. FC stimulates H^+ pumping by locking the preformed complex of 14-3-3 proteins and H^+- ATPase in a nearly irreversible manner (Fuglsang et al. 2003). There are no reports if FC on its own can stimulate protein kinases. The penultimate Thr residue is phosphorylated in response to different physiological stimuli and this phosphorylation seems to be to major regulatory mechanism of the H^+ pump since other phosphorylations seem to regulate the 14-3-3 binding. Despite a huge effort in several laboratories the protein kinase responsible for phosphorylation of this particular Thr residue has not yet been identified.

1.3 Phosphoproteomic Studies of Plasma Membrane H^+-ATPases

Phosphoproteomic studies have revealed several phosphosites within the C terminus of several isoforms of the H^+-ATPases. Some are found responding to stimuli others are found in systematic analysis of plasma membrane fractions (Nuhse et al. 2003, 2007; Whiteman et al. 2008). Phosphorylated sites are indicated on the H^+ pump drawn in Fig. 1. The fact that the C-terminal is subjected to such a large number of diverse phosphorylation events suggests a complex mechanism of regulation involving a number of different protein kinases. Only a few of the sites have an assigned physiological role(s), one is the penultimate Thr-947 residue and the other is the Ser-931 residue, as discussed below. One method to link physiological stimuli with specific phosphorylation sites was made in a quantitative phosphoproteomic study (Niittyla et al. 2007). In this study *Arabidopsis* seedlings were grown in hydroponics and the composition of the media could thereby be tightly controlled. By growing the seedling in the dark, followed by the addition of sucrose, the response to sucrose starvation/addition could be monitored. Phospho-peptides were purified and characterized at different time points after sucrose addition and thereby changes in specific phospho-peptides were measured. One phospho-residue in the H^+-ATPase was found to change as response to the sucrose depletion/addition regime namely the Thr947 residue described in relation to 14-3-3 binding, again underlining the importance of this regulatory mechanism.

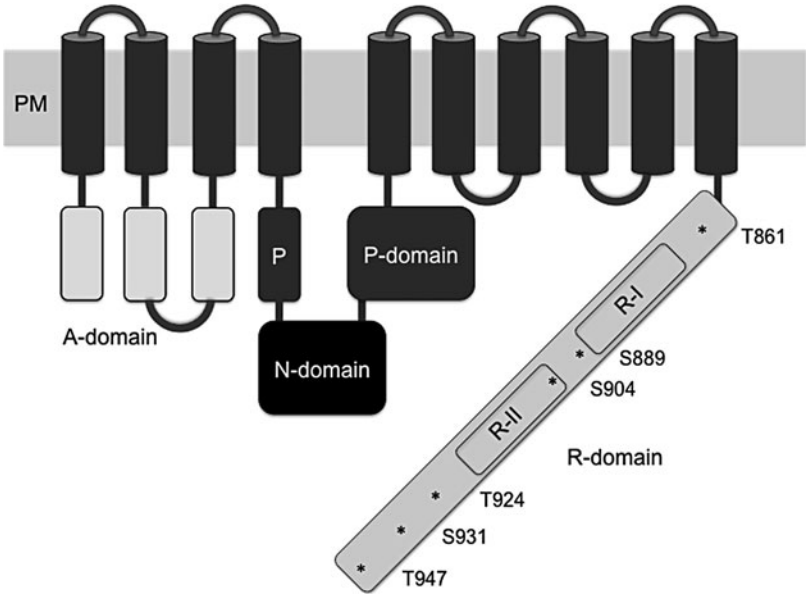

Fig. 1 Schematic drawing of the *A. thaliana* H^+-ATPase isoform 2 (AHA2). The nucleotide binding domain (*N-domain*), the phosphorylation domain (*P-domain*), the actuator domain (*A-domain*) and the regulatory domain (*R-domain*) is indicated with different coloring. In the R-domain the two regions involved in the auto inhibitory regulation is indicated (*R-I* and *R-II*). Phospho-sites identified *in planta* are marked in the R-domain. Numbering is according to amino acid residues in *AHA2*

1.4 Controlling the Size of the Stomatal Pore

1.4.1 Opening of Guard Cells

Pairs of guard cells form stomatal pores and regulate gas exchange between plant cells and the surrounding atmosphere. Light (primarily blue) stimulates stomata opening by activating the plasma membrane H^+-ATPase (Kinoshita et al. 2003; Kinoshita and Shimazaki 1999). Briefly, blue light induces rapid and highly sensitive stomata opening correlated with the phosphorylation of a plasma membrane H^+-ATPase pump and increased H^+ pumping, which results in the activation of voltage-gated K^+ channels by membrane hyperpolarization (reviewed by (Shimazaki et al. 2007)) along with the inhibition of S-type anion channels. H^+-ATPases are phosphorylated upon blue light treatment leading to the binding of regulatory 14-3-3 proteins to the C-terminal end of the H^+ pump (Fig. 2).

Receptors of blue light are phototropins (*PHOT1* and *PHOT2*). Phototropin contains in addition to the light sensing LOV domain(s) a serine/threonine protein kinase domain. In the presence of blue light, it is stimulated and autophosphorylated, resulting in the binding of 14-3-3 proteins. Thus, one consequence of phototropin autophosphorylation is 14-3-3 binding to the PHOT protein. One might speculate that the 14-3-3 proteins may be responsible for the transmission

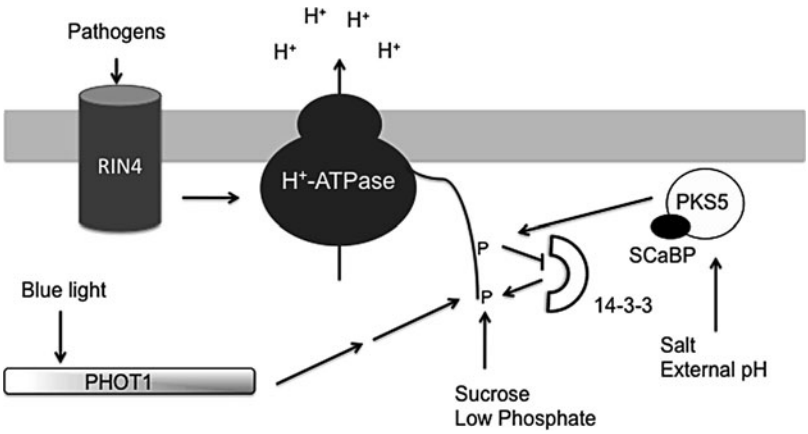

Fig. 2 A model cell showing the regulation of the plasma membrane H^+-ATPase by regulatory proteins. The H^+-ATPase is activated upon binding of 14-3-3 proteins to the phosphorylated penultimate Thr in the R-domain. This phosphorylation is responding to different stimuli. The *left* side of the figure illustrates processes taking place in the guard cells resulting in the opening of the stomatal pore. Here the blue light receptor, PHOT1, initiates an activation cascade activating the H^+-ATPase. Pathogens utilize the RIN4 protein to activate the H^+-ATPase through a direct interaction. The *right* side of the figure illustrates processes taking places in other parts of the plant (including roots). The protein kinase PKS5 is regulated by a combination of salt and high pH and targets a Ser residue upstream of the 14-3-3 binding site. Phosphorylation of this Ser residue prevents 14-3-3 binding

of the signal by facilitating a direct link between phototropins and guard cell H^+-ATPase (Kinoshita et al. 2003; Sullivan et al. 2009). An obvious thought was that the phototropins themselves phosphorylate the H^+-ATPases but several lines of research have demonstrated that other components are implicated in this activation. In *phot1/phot2* double mutants phosphorylation of the plasma membrane H^+-ATPase can be observed upon FC treatment indicating that other protein kinases are involved downstream of the phototropins (Ueno et al. 2005). Very recently the protein phosphatase PP2A was shown to deactivate Phot2 (Tseng and Briggs 2010). PP2A is also implicated in the regulation of H^+-ATPase (Fuglsang et al. 2006) and this suggests a coordinated regulation point in the blue light response.

In addition to activating the H^+ pumps the phototropins also mediate the inhibition of the plasma membrane anion channels (Marten et al. 2007) and as is the case for the activation of the H^+ pump, other players downstream have not yet been identified.

1.4.2 Closure of Guard Cells

Closure of stomata occurs as response to light to dark transition, high CO_2 levels and the hormone abscisic acid (ABA). Studies of mutants with ABA insensitive stomata have revealed that the plasma membrane H^+-ATPases in guard cells are important for the ABA induced closure. One of the mutant loci identified (*Open stomata 2*, *OST2*) were caused by a mutation leading to a constitutive activated form of the *A. thaliana* H^+-ATPase isoform 1 (*AHA1*) (Merlot et al. 2007). Two different *OST2* alleles were identified, one contained two missense mutations $L_{169}F$ and $G_{867}L$, the first one located in the A-domain, the second in Region I in the regulatory domain. The latter easily explains the constitutive activated form. The other allele contained a mutation $P_{68}S$ in the first transmembrane domain. By expression in yeast both alleles were shown to result in activated forms of AHA1.

The open stomata phenotype in *OST2* implies that the R- and S-channel currents are not sufficient to sustain plasma membrane depolarization to close stomata without curtailing the proton pump activity (Merlot et al. 2007). The mechanisms that would inactivate the pumps under normal circumstances are not known, but it has previously been suggested that the protein phosphatases type 2C are involved (Roelfsema et al. 1998).

1.4.3 Pathogens Modulate the H^+ Pumps to Invade Plants Through the Stomatal Pore

Stomata do not only open and close in order to exchange gases, they also form a gateway for pathogens to enter the interior of the leaf. When receptors at the cell surface recognize pathogens, one defense response is, therefore, to close the stomatal pore to prevent bacteria from entering the leaf interior (Melotto et al.

2006). Pathogenic bacteria have evolved strategies to suppress the closure of stomata. One example is the RIN4 protein known to negatively regulate plant response to pathogens (PAMP-triggered immunity, PTI) (Kim et al. 2005; Mackey et al. 2002). Recently it was demonstrated that plants use RIN 4 to regulate H^+-ATPase activity during immune responses, thereby controlling stomatal apertures during pathogen attack. RIN4 binds to the plasma membrane H^+-ATPase (AHA1/AHA2) resulting in activation of the pump (Liu et al. 2009a) (Fig. 2). In resistant plant genotypes the interaction between RIN4 and the H^+-ATPase is prevented, presumable by post-translational modification of the RIN4 protein. RIN4 homologs are found in many plant species and although this mechanism might very well be a general mechanism used by pathogens, it is still unknown if RIN4 plays a role in the opening and closure of stomata under noninfected conditions or if RIN4's role is solely regulated as response to pathogens.

1.5 PKS5: A Protein Kinase Preventing Binding of 14-3-3 Protein

The first protein kinase found as regulator of H^+-ATPase was PKS5. PKS5 belongs to a family of calcium regulated Serine/Threonine protein kinases (PKS/CIPK11) containing 25 members in *Arabidopsis* (Guo et al. 2001; Kolukisaoglu et al. 2004; Kudla et al. 2010). Another member of this protein kinase family SOS2/CIPK24 phosphorylates the Na^+/H^+ antiporter (SOS1) upon salt stress (Qiu et al. 2002), CIPK23 phosphorylates the K^+ channel AKT1 (Laloi et al. 2007; Li et al. 2006; Xu et al. 2006). This indicates a specialized role for this family of protein kinases towards regulation of ion-transporters by phosphorylation.

PKS5 phosphorylates Ser931 positioned between autoinhibitory Region II and the 14-3-3 protein binding site in the C-terminal end of AHA2. Phosphorylation of Ser931 prevents 14-3-3 binding even though the penultimata threonine residue (Thr947) is phosphorylated (Fig. 2). This finding added a second layer to the regulation of the H^+-ATPases in that inactivation of pump activity can occur both by dephosphorylation of Thr947 and by phosphorylation of Ser931. In both cases, 14-3-3 binding is prevented. The mechanism of regulating the binding of 14-3-3 protein was previously also identified in *Nicotiana tabacum* (Duby et al. 2009).

pks5 seedlings exhibit a pH tolerant phenotype tolerating pH in the media up to pH 8.5. The seedlings can adjust pH in the rhizosphere faster than wildtype plants by activating the H^+-ATPase. Growth at pH 8.5 is not physiologically relevant and probably a secondary effect of the *pks5* mutation, the real role of PKS5 is not yet fully understood.

1.5.1 ScaBP1: A Calcium Binding Protein Modulating PKS5 Action

As found for other members of the PKS/CIPK family also, PKS5 interacts with a Ca^{2+} binding protein from the CBL/ScaBP family. By the use of yeast two-hybrid

assays, PKS5 was found to interact with the Ca^{2+} binding protein SCaBP1/CBL2 but not with any other member of the SCaBP/CBL family (Fuglsang et al. 2007). CBL2 has later been located to the tonoplast of the plant cell raising a question about the actual mechanism of interaction between PKS5 and CBL2. The function of the SCaBP/CBL proteins in relation to the PKS/CIPK kinases is still under debate. In some cases the SCaBP/CBL protein seems to regulate the activity of the PKS/CIPK kinase (Tominaga et al. 2010) in other cases the SCaBP/CBL protein possess a role in the recruitment of the protein kinase to the plasma membrane and thereby the phosphorylation target as demonstrated by the *sos1* recruitment system (SRS) for SOS1 (Quintero et al. 2002). Also it has been demonstrated that myristoylation targets CBL1 to the endoplasmic reticulum and that the following S-acylation is crucial for endoplasmic reticulum-to-plasma membrane trafficking (Batistic et al. 2008). When reconstituting the AHA2/PKS5/SCaBP1 signaling pathway in yeast is was found that SCaBP1 was required in order to observe a phenotype related to changed activity of the proton pump AHA2, on the other hand *in vitro* experiments demonstrated that recombinant PKS5 could phosphorylate recombinant AHA2 without the presence of SCaBP1(Fuglsang et al. 2007) (Fig. 2).

1.5.2 DnaJ: A Chaperone Like Protein Repressing PKS5 Activity

In order to understand the physiological role of PKS5 regulation a screen for interacting proteins was performed (Yang et al. 2010). Here a putative Co-chaperone DnaJ-like heat shock protein (AtJ3 homologue 3) was identified. This protein was shown to interact with PKS5 and repressing its protein kinase activity and thereby activating the H^+-ATPase. Environmental stresses often cause protein denaturation, therefore chaperones are key components helping to maintain proteins in their functional conformation during stress conditions. Knock out *atj3* seedlings did not show the same pH resistant phenotype as *pks5*-1 seedlings. Often alkaline conditions are associated with increased soil salinity and the effect of combined salt and high pH was therefore tested. Here atj3 seedlings demonstrated an increased sensitivity compared to wild type plants, at the same conditions *pks5*-1 plants were less sensitive than wild type. Further tests of *pks5*/*atj3* double mutants responded to salt at alkaline conditions in the same way as pks5 seedlings indicating that Atj3 functions upstream of PKS5. These data also suggest that PKS5 might be involved in regulation of H^+-ATPase activity in relation salt stress.

1.6 Nutrient Uptake and Responses to Changes in the Soil

A critical feature distinguishing plants from animals is that plants are sessile and thus have to cope with numerous environmental challenges. For example, plant roots are exposed to soil solutions that are constantly changing in pH as well as in the concentrations of mineral nutrients and toxic ions.

1.6.1 Response to Limited Phosphate

An example of regulation of the plasma membrane H^+-ATPase is found as response to limited amounts of phosphate. White lupin (*Lupinus albus* L.) can grow in soils with sparingly available phosphate (P) by producing specialized structures called cluster roots. To mobilize sparingly soluble P forms in soils, cluster roots release substantial amounts of carboxylates and concomitantly acidify the rhizosphere. It has been demonstrated that, citrate exudation increased transiently and reached a maximum after 5 h. This effect was accompanied by a strong acidification of the external medium and alkalinization of the cytosol. Fusicoccin stimulated citrate exudation, whereas vanadate, an inhibitor of the H^+-ATPase, reduced citrate exudation. The increase in proton secretion was due to both an increased transcription level of a H^+-ATPase gene as well as activating post-translational modifications of H^+-ATPase protein involving binding of activating 14-3-3 protein (Tomasi et al. 2009).

2 Plant H^+-PPases

Prototypical plant H^+-PPases (V-PPase EC 3.6.11) have an overall amino-acid sequence identity of 85% or greater and localize to the vacuolar, Golgi, and plasma membranes (Baltscheffsky et al. 1999; Cleland 1995; Drozdowicz et al. 2000; Jiang et al. 2001; Mitsuda et al. 2001a; Ratajczak et al. 1999). Plants have two phylogenetically distinct types of H^+-PPases: type I and type II. Type I H^+-PPases depend on cytosolic K^+ for their activity and are moderately sensitive to inhibition by Ca^{2+}, and type II H^+-PPases are K^+-insensitive but extremely Ca^{2+}-sensitive. Type I H^+-PPases have been shown to acidify the plant vacuole. The resulting H^+ and electrochemical gradient is instrumental for the storage of sucrose, organic acids, regulation of hydrostatic pressure through the storage of inorganic ions, and cytoplasmic detoxification (Maeshima 2001). H^+-PPases from various sources have been successfully purified and characterized as peptides ranging from 65 to 115 kDa (predicted) and 56–79 kDa (apparent) molecular weight. Variations in its predicted M*r* from the cDNA size and the apparent M*r* from PAGE are common to highly hydrophobic proteins and appear to be related to their extreme hydrofobicity and incomplete saturation by SDS (Maddy 1976). *A. thaliana* has one gene encoding for a type I H^+-PPase (AVP1) and another gene encoding for a type II H^+-PPase (AVP2) (Drozdowicz et al. 2000). Interestingly, *Arabidopsis* web sites report the existence of an *AVP1.2* gene product that results from an alternative splicing of the *AVP1* locus (http://www.arabidopsis.org/). However, there is currently no *in planta* evidence of its expression.

AVP1 is an extremely hydrophobic protein of 770 residues ($MW = 80{,}800$ Da) and its heterologous expression in yeast demonstrated that this polypeptide is sufficient for both H^+ pumping and PPi hydrolysis (Zhen et al. 1994). Although early models favored the presence of 13 transmembrane domains (TMD) for AVP1,

later models suggest either 15 or 16 TMD (Maeshima 2000). Lines of evidence demonstrate that the catalytic pocket of H^+-PPases is facing the cytosolic side and is probably composed of 2–3 conserved segments. A fragment of DXXXXXXXKXE on cytosolic loop 3 (CL3) was suggested as the putative substrate-binding region (Nakanishi et al. 2001). NN′-dicyclohexylcarbomiide-binding residues (Glu-305 and Asp-283 on CL3 and Asp-504 on CL5) have been identified by the combination of site-directed mutagenesis and chemical modification as essential residues involved in enzymatic and proton translocating reaction of H^+-PPases (Kim et al. 1995; Yang et al. 1999; Zhen et al. 1997a). H^+-PPases require Mg^{2+} as a cofactor for the formation of the $MgPP_i$ complex and the resultant active conformation (Gordon-Weeks et al. 1996).

Today over 100 sequences from bacteria, archaea, and eukaryotes are available. Sequences alignments have revealed the existence of only two subfamilies of H^+-PPase described above. Type I family members are K^+-dependent and type II are K^+-independent enzymes (Belogurov and Lahti 2002; Drozdowicz et al. 2000). K^+-dependent H^+-PPases have been found in algae (Takeshige et al. 1988), protozoan, (Docampo et al. 2005) and higher plants (Sarafian et al. 1992). On the other hand, type II H^+-PPases exist in archaebacterium (Drozdowicz et al. 1999), photosynthetic bacterium (Au et al. 2006), fungus (Mimura et al. 2005), and *A. thaliana* (AVP2) (Drozdowicz et al. 2000). Intriguingly, members of the K^+-independent sub-family contain a Lys residue at the position equivalent to the residue 541 of AVP1. Furthermore, substitution of a neutral residue by Lys in the position of K^+-dependent H^+-PPase from *Carboxydothermus hydrogenoformans* confers K^+ independency (Belogurov and Lahti 2002). However, it has also been shown that other residues contribute to the K^+ binding site including G544 and various Cysteine (Cys) residues on the N terminus. In plants, the transmembranal domain 5 (TM5 residues 211–242) of the enzyme is highly conserved. A mutation in the motif GYG (residues from 229 to 231) ceased the cation effect on the H^+-PPase (Van et al. 2005). Alignment of amino-acid sequences demonstrated a relatively high degree of conservation of the C-terminal domains among H^+-PPases. Topological studies using yeast heterologous H^+-PPase expression suggest that both the C-termini and the N-termini face the lumen side and are opposite to the cytosolic catalytic domain that is cytosolic (Maeshima 2000).

Truncation of the C terminus induces dramatic decline in H^+-PPase enzymatic activity, H^+ translocation and coupling efficiency (Lin et al. 2005). In addition, deletion of the C terminus of the H^+-PPase increases its susceptibility to heat stress and apparent K^+ binding constant. Thus, it is likely that the C terminus plays and essential role in sustaining the physiological functions of H^+-PPase.

Unlike the vacuolar H^+/ion pumping ATPases that are large hetero-multimeric complexes, all the catalytic properties of H^+-PPases are imparted by a single polypeptide as demonstrated by the heterologous expression of H^+-PPases in yeast (Kim et al. 1994). However, H^+-PPases could work as homo-dimers or homo-multimeres as judged by native PAGE, cross-linking, and gel filtration data (Zhen et al. 1997b). Radiation inactivation analysis demonstrated that the proper dimeric structure of the H^+-PPase on tonoplast membranes is a prerequisite for both

enzymatic activity and PP_i-supported H^+ translocation. One subunit of the dimeric complex is sufficient for PP_i hydrolysis but proton translocation requires the presence of both subunits (Tzeng et al. 1996). More recent atomic force microscopy (AFM) has been used to observe purified H^+-PPase reconstituted into planar lipid bilayer under physiological conditions. These results reveal a dimeric complex for the H^+-PPase where both the C termini of a dimeric subunit are on the same side of the membrane and are approximately 1.9–2 nm apart. In the proposed mechanistic model, the H^+ channel lies at the interface between the C termini of the H^+-PPase homodimer (Liu et al. 2009b). The high-resolution crystal structure of H^+-PPases is a pending assignment that will expedite the elucidation of the molecular mechanisms involved in the function and regulation of this primary H^+ pump.

2.1 Vacuolar H^+-PPases in Fruits

The vacuoles are organelles that fulfill highly specialized functions depending on tissue, cell type, and/or developmental stage. All vacuoles seem to contain vacuolar H^+-ATPases (V-ATPases) and H^+-PPases that differ in their function depending on the type of vacuole in which they reside (Martinoa et al. 2007). Generally, H^+-PPase activity is high in young tissues whereas V-ATPase activity is relatively constant during growth and maturation. In pear fruit the ratio of H^+-PPase to V-ATPase activity indicated that H^+-PPase is the major H^+-pump of young fruit vacuolar membranes. However, the contribution of the V-ATPase increases with time to become the major H^+-pump during the later stages of fruit development (Shiratake et al. 1997). Growing tissues and exponentially growing cells generate large amounts of pyrophosphate. It is tempting to speculate, that the H^+-PPase could be serving two purposes: the generation of the proton gradient required for vacuolar transport/expansion and the scavenging of PP_i to alleviate its well-documented inhibitory feedback effect. Generally the abundance and activity of the H^+-PPase is high in young tissues. However in some cases such as grape berries, the H^+-PPase is also the predominant vacuolar proton pump in mature cells. Grape berries are very acidic and it is intriguing that in tissues were vacuoles are highly acidic (pH $\geq$3) the H^+-PPase appears to be the predominant pump (Terrier et al. 1997). It has been suggested that the thermostability of the H^+-PPase could be the reason for its abundance in mature grape berries, since they are exposed to the sun and consequently reach high temperatures. In line of this hypothesis, the grape berry H^+-PPase is heat stable and exhibits a temperature optimum of $-50^{\circ}C$ (Martinoa et al. 2007).

In *Prunus persica* (peach), two different full-length clones of H^+-PPase have been isolated from fruit (*PPV1* and *PPV2*). The expression of *PPV1* is very low in contrast with the high expression of *PPV2* in the fruit. *PPV2* presents a clear biphasic pattern of expression during peach fruit development that correlates with the accumulation of citric or malic acid and maturation (Etienne et al. 2002). It has

been suggested that *PPV2* is instrumental for both accumulation of organic acids and sugar storage. Other studies with grape berry revealed the existence of a H^+-PPase that is highly expressed during ripening and appeared to have a synergic behavior with the V-ATPase (Terrier et al. 2001).

2.2 Vacuolar H^+-PPase Is a Key Player for Plant Salt Tolerance

Vacuolar sodium sequestration is a conserved mechanism used by salt tolerant plant species. Overexpression of the type I H^+-PPase AVP1 in *Arabidopsis* resulted in plants with enhanced salt tolerance and drought resistance (Gaxiola et al. 2001). The salt tolerant phenotype of these plants was explained by an increased uptake of Na^+ into their vacuoles. The drought related phenotype was originally attributed to an enhanced vacuolar osmoregulatory capacity (Gaxiola et al. 2001). Since the arrival of this work other groups have subsequently demonstrated that overexpression of this and other plant genes encoding for a type I H^+ PPase can increase both salt- and drought-tolerance in heterologous systems including rice (Zhao et al. 2006), tobacco (Gao et al. 2006), cotton (Lv et al. 2008, 2009), alfalfa (Bao et al. 2008), maize (Li et al. 2008), and creeping bentgrass (Li et al. 2010). Interestingly, a study on the variation of salinity tolerance amongst *Arabidopsis* ecotypes reported a positive relationship between salt tolerance and the levels of AVP1 expression (Jha et al. 2010). Furthermore, an *Arabidopsis* mutant has been characterized in which energization of vacuolar transport solely relies on the activity of the H^+-PPase (Krebs et al. 2010). These lines remain salt tolerant and further confirm our supposition that AVP1 is important for salt tolerance (Gaxiola et al. 2001, 2007).

2.3 Vacuolar H^+-PPases in Maize Aleurone

Cereal endosperm is a model system for cell fate determination in plants. Cells at the outermost layer of the endosperm adopt an aleurone cell fate. An intriguing finding relates to the restricted expression of the maize *Vpp1* gene encoding for a H^+-PPase to the aleurone layer (Wisniewski and Rogowsky 2004). Its expression identifies it as an aleurone cell fate developmental marker, but its physiological role remains obscure. The aleurone layers are rich in lipids and accumulate hydrolytic enzymes in protein bodies during seed maturation. The aleurone cells are less vacuolated than the underlying starchy endosperm cells and they are the only cells in kernels that accumulate storage proteins in their vacuoles. Therefore, it has been suggested that *Vpp1* may play a role in the filling of these storage vacuoles also called aleurone bodies (Wisniewski and Rogowsky 2004). The protein storage vacuoles (PSV) contain three morphologically distinct regions: the matrix, the

crystalloid and the globoid cavities (Weber and Newman 1980). Biochemical and structural characterization of protein storage vacuoles showed that the globoid cavity is defined as a unit membrane that is specifically marked by the presence of H^+-PPase and γ-TIP (Tonoplast Intrinsic Protein) (Jiang et al. 2001). The environment of the globoid cavity is optimal for the formation of phytic acid crystals. Since the globoid membrane contains a H^+-PPase and phytic acid molecules are known to carry pyrophosphate groups (Loewus and Murphy 2000), it has been suggested that the PSV could represent a plant functional equivalency to acidocalcisomes. Acidocalcisomes are the only organelles that have been conserved during evolution from prokaryotes to eukaryotes. Acidocalcisomes have been linked with several functions including the storage of cations and phosphorous, calcium homeostasis, and osmoregulation. Furthermore, its function is essential in the adaptation of parasites to environmental stress (Docampo et al. 2005). H^+-PPases are integral proteins of the acidocalcisomes of many different parasitic protozoa (*T. cruzi, T.brucei, Leishmania donovani, L. amazonensis, Phytomonas francai, Toxoplasma gondi, Plasmodium falciparum* and *Plamsodium berghei*) (Docampo et al. 2005). Considering aleurone cells are the only endosperm cells that maintain a metabolic activity at seed maturity and during its dispersal, it has been suggested that the H^+-PPase in the PSV could help seeds to face stress during germination.

2.4 Subcellular Localization of Plant H^+-PPases

In animal systems lacking H^+-PPases, the vacuolar H^+-pumping ATPases (V ATPases) acidify a wide array of intracellular compartments. In polarized cells such as osteoclast and renal tubular epithelial cells, V ATPases have been localized to the plasma membrane (PM) where they acidify discrete extra cellular compartments. V ATPases have been localized in virtually all the intracellular compartments except for the nucleus. As expected their function is very diverse (Zhou et al. 1999). The subcellular localization of the H^+-PPase in parasites is also versatile and not restricted to the acidocalcisomes. For example the H^+-PPase of *Trypanosome cruzi* has been shown to localize at the acidocalcisomes membrane, golgi apparatus (GA) and PM (Docampo et al. 2005). Furthermore, H^+-PPases can change intracellular localization during invasion of host cells (Drozdowicz et al. 2003). Plant type I H^+-PPases were first isolated from vacuoles and initially considered to be *bona fide* vacuolar markers (Maeshima 1991; Rea et al. 1992). However, density gradient centrifugation and phase partitioning of membrane fractions coupled with immunogold electron microscopy showed the presence of H^+-PPases in the plasma membrane of *Ricinus communis* seedlings and cauliflower inflorescence (Cleland 1995; Ratajczak et al. 1999). Furthermore, proteomic studies confirmed the plasma membrane localization of the *A. thaliana* H^+-PPase AVP1 (Alexandersson et al. 2004).

The *Arabidopsis* type II H^+-PPase encoded by the *AVP2*/*AVP1L* locus has been shown to localize exclusively to the GA (Mitsuda et al. 2001a). The expression of type II H^+-PPase has been documented in young seedlings, cotyledons, rosette-trichomes, sepals and stamen filaments. It has been suggested that the type II H^+-PPase may be required during cell expansion (Mitsuda et al. 2001a). It is likely that the type II H^+-PPase may aid the GA resident V-ATPase in the generation of the acidic environment under conditions where ATP availability is compromised. The existence of a GA H^+-PPase in roots of maize has been demonstrated by immunoelectron microscopy (Oberbeck et al. 1994). Recently, a cDNA encoding a putative AVP2-like maize H^+-PPase gene has been cloned using an elegant suppression subtractive hybridization (SSH) approach (Yue et al. 2008). This gene, named *ZmGPP,* is a good candidate for the GA resident H^+-PPase of *Zea mays*. *ZmGPP* is constitutively expressed in leaves, stems, roots, tassels and ears under normal growth conditions. Interestingly, the expression of *ZmGPP* is up-regulated in both shoots and roots of maize seedlings under dehydration, cold, and higher salt stress: suggesting *ZmGPP* could play an important role in abiotic stress tolerance in *Z. mays* (Yue et al. 2008).

2.5 Are There Other H^+-PPases in Plants?

For years, investigators have been interested in finding out whether endomembrane systems other than the vacuole and GA contain H^+-PPases. Vianello et al. (1991) reported a pyrophosphate dependent H^+ pumping activity from pea stem submitochondrial particles (Vianello et al. 1991). The mass of putative mitochondrial H^+-PPase was shown to be smaller than the vacuolar H^+PPase indicating the possible existence of a new type of H^+PPase. However, another study with radiation inactivation analysis showed that submitochondrial particles from etiolated mung bean seedlings contained a H^+-PPase with an estimated functional size of 170 kDa (Jiang et al. 2000). The discrepancy remains unsolved and to our knowledge no further information has been published. There is an isolated report about the existence of a H^+-PPase present on a endoplasmic reticulum-enriched vesicle fraction from etiolated mung bean seedlings. Antiserum prepared against the vacuolar H^+-PPase did not inhibit the activity of this novel proton pyrophosphatase which excludes a possible contamination of the membrane preparation with tonoplast vesicles (Kuo et al. 2005). Here again, no further information is available.

2.6 Transcriptional Regulation of H^+-PPases

Transcriptional regulatory networks that drive organ specific and cell-specific patterns of gene expression and mediate interactions with the environment represent a fundamental aspect of plant cell signaling. The transcriptional regulation of

gene expression in eukaryotes is mediated by the recruitment of transcription factors (TFs) to *cis* regulatory elements. Transcription factors interact with DNA elements, other TFs, and the basal machinery to regulate the expression of target genes. TF binding sites (or *cis* elements motifs) are the functional DNA elements that influence temporal and spatial transcriptional activity. Multiple *cis*-elements comprise *cis* regulatory modules (CRMs). CRMs integrate signals from multiples TFs that result in a combinatorial control and highly specific pattern of gene expression. Therefore, identifying and understanding the function of *cis* elements and their combinatorial role in CRMs is essential for elucidating the mechanisms by which cells perceive and correctly respond to their environment (Priest et al. 2009). The expression levels of the H^+-PPase are precisely controlled at the transcriptional level in response to various environmental conditions or developmental stages (Maeshima 2000). It has been shown that *cis*-acting regions regulate the expression of AVP1 in pollen. AtCAMTA5 and AtCAMTA 1 (calmodulin-binding TFs) were shown to bind to the pollen-specific *cis*-acting region of *AVP1* promoter (Mitsuda et al. 2003). In the same work, the authors suggested that AVP1 expression in pollen might be regulated via Ca^{2+} signaling (Mitsuda et al. 2003). The *cis*-acting region of the *AVP1* gene was used to identify two novel proteins, AtVOZ1 and AtVOZ2 (*A. thaliana* Vascular plant One Zinc finger protein). The expression of At VOZ1 is restricted to the phloem, while AtVOZ2 expression has been detected in roots, stipules, stamen filaments, and anthers (Mitsuda et al. 2004).

2.6.1 Sugar Starvation

Transient expression assays using a GUS-reporter under the control of a 1,413 bp fragment of the *AVP1* promoter showed that its expression is regulated in response to several energy related stresses (Mitsuda et al. 2001b). The up-regulation of *AVP1* in response to a reduction in light intensity is reminiscent of the behavior of genes involved in sugar starvation. Of note, sugar responsible *cis* elements (i.e., AMY, BOX1, 2 CGACG boxes) are present in the regulatory region of the *AVP1* promoter (Mitsuda et al. 2001b). Up-regulation of H^+-PPase genes has been reported in sucrose-starved cells of *Oryza sativa* (Wang et al. 2007).

Early work with rice seedlings documented anoxia-triggered up-regulation of the H^+-PPase (Carystinos et al. 1995). Furthermore, a recent study showed that among the six rice H^+-PPase genes (*OVP1–6*) only *OVP3* was specifically up-regulated under anoxia (Liu et al. 2010). When the production of ATP drops sharply under anoxia due to decreased oxidative phosphorylation, flood-tolerant species such as rice adapt by switching from the anaerobic respiration to anaerobic fermentation. This results in a cytosolic acidification and inhibition of ATP-dependent proton pump activity (Gibbs and Greenway 2003). Stitt speculated that H^+-PPases could provide the driving force for vacuolar transport during oxygen deficit conditions that limit ATP supply for the function of the vacuolar H^+-ATPase (Stitt 1998).

2.6.2 P_i Starvation

The existence of a complicated transcriptional regulation system involved in plant responses to P_i starvation is well documented (Franco-Zorrilla et al. 2004). A rice TF (OsPTF1) involved in the response to phosphate starvation has been reported (Yi et al. 2005). OPTF1 is expressed in phloem cells of the primary root, leaves, and lateral roots. Overexpression of OsPTF enhances rice tolerance to P_i starvation. Interestingly, microarray data on this OsPTF transgenic rice plants showed a concomitant enhanced expression of H^+-PPases (Yi et al. 2005). These data are consistent with results that showed up-regulation of H^+-PPase activity in *Brassica napus* cell suspensions under phosphate starvation (Palma et al. 2000). In *A. thaliana* P_i starvation triggers increases in transcript and protein abundance of both AVP1 and the plasma membrane H^+-ATPase. Furthermore, the overexpression of AVP1 in *Arabidopsis*, tomato, and rice improves growth under P_i limitation (Yang et al. 2007).

2.7 *Puzzling Phenotypes Triggered by Altering the Expression of H^+-PPases in Plants*

Li et al. reported that the overexpression of the H^+-PPase AVP1 in *Arabidopsis* results in increased cell division at the onset of organ formation, root, and shoot hyperplasia as well as increases in auxin transport. Furthermore, *avp1-1* null mutants display severely disrupted root and shoot development and reduced auxin transport. Intriguingly, changes in the expression of AVP1 affect the abundance and activity of the PM H^+-ATPase that correlate with apoplastic pH alterations and rhizosphere acidification (Li et al. 2005; Yang et al. 2007). Rhizosphere acidification is a central mechanism for plant mineral nutrition. Accordingly, it has been shown that AVP1 transgenic *Arabidopsis*, tomato and rice plants outperform controls when grown under phosphate limitations and accumulate higher contents of potassium under all conditions tested (Yang et al. 2007). Of note, up-regulation of either the *A. thaliana* or *Thellungiella halophila* type I H^+-PPases triggers enhanced growth/biomass and photosynthetic capacity in a variety of agriculturally important crops (Bao et al. 2008; Gaxiola et al. 2001; Li et al. 2008; Lv et al. 2008, 2009; Park et al. 2005; Yang et al. 2007) grown under normal or stressful conditions such as nutrient limitations, water scarcity, and salinity. As described earlier, the salt tolerant phenotypes triggered by the overexpression of the H^+-PPase are consistent with its residence at the tonoplast. However, a vacuolar restricted H^+-PPase complicates the explanation of phenotypes such as an enhanced abundance and activity of the PM H^+-ATPase with the concomitant acidification of apoplast and rhizosphere or an enhanced biomass and photosynthetic capacity. As described earlier, H^+-PPase has been localized to the PM, and its function here warrants more attention.

2.8 Could the H^+-PPase Affect Sucrose Phloem Loading?

2.8.1 PP_i Concentrations Are Essential for Sucrose Phloem Loading

Sonnewald and coworkers suggested that the cytosolic concentration of PP_i in the phloem was essential for sucrose transport (Sonnewald 1992). Lerchl and collaborators further tested this hypothesis via the phloem-specific expression of a soluble pyrophosphatase from *E. coli* (*ppa1*) in tobacco plants (Lerchl et al. 1995). Characterization of these *ppa1* plants revealed that removal of cytosolic PP_i from phloem cells triggered the accumulation of sucrose in source leaves, chlorophyll loss, and reduced shoot and root growth. Interestingly, phloem-specific expression of a yeast invertase (suc2) circumvented the metabolic block of the *ppa1* plants restoring wild type phenotypes (Lerchl et al. 1995). These data are consistent with a model where sucrose phloem loading depends on the levels of cytosolic PP_i in companion cells (Fig. 3). Sucrose must be actively transported from mesophyll cells to companion cells via a sucrose/H^+ symporter that depends on the proton gradient generated by the plasma membrane H^+-ATPase (Srivastava et al. 2008). In order to have an adequate ATP supply for the maintenance of this transmembrane proton gradient, a percentage of the incoming sucrose must be cleaved into fructose and UDP-glucose by sucrose synthase (Lerchl et al. 1995) and subsequently oxidized through the cellular respiration pathway. In this pathway, both the PP_i: fructose 6-phosphate 1-phosphotransferase (PFP) and the UDP-glucose pyrophosphorylase (UGPase) work near equilibrium, so a decrease in the cytosolic concentration of PP_i should prevent the reactions leading to glycolysis and therefore compromise the energy production (Lerchl et al. 1995).

2.8.2 H^+-PPase and H^+-ATPase Localize in Close Proximity at the PM of Sieve Elements

A series of immuno-gold studies with phloem tissue of *R. communis* seedlings prompted the suggestion that the H^+-PPase could be involved in sucrose transport (Long et al. 1995; Robinson et al. 1996). Further work with double- labeling immunolocalization experiments indicated that the H^+-PPase and PM H^+-ATPase localize in close proximity at the PM of the sieve elements in *R. communis* (Langhans et al. 2001). These authors suggested that both H^+-pumps are required for sieve element membrane energization to maintain high sucrose, K^+, and amino acid concentrations. However, a theoretical paper by Julia Davies argued that the sieve tube H^+-PPase could not operate hydrolytically to pump H^+ into the apoplast based on the estimation of the free energy of the H^+-PPase pump action for *in vivo* conditions (apparent PP_i hydrolysis constant and a cytosolic PP_i concentration of 0.011 mM). Interestingly, Davies suggested that a reverse reaction (where the H^+-PPase uses the H^+-gradient at the plasma membrane to synthesize PP_i) was thermodynamically feasible (Davies et al. 1997). Of note, *in vivo* data obtained with

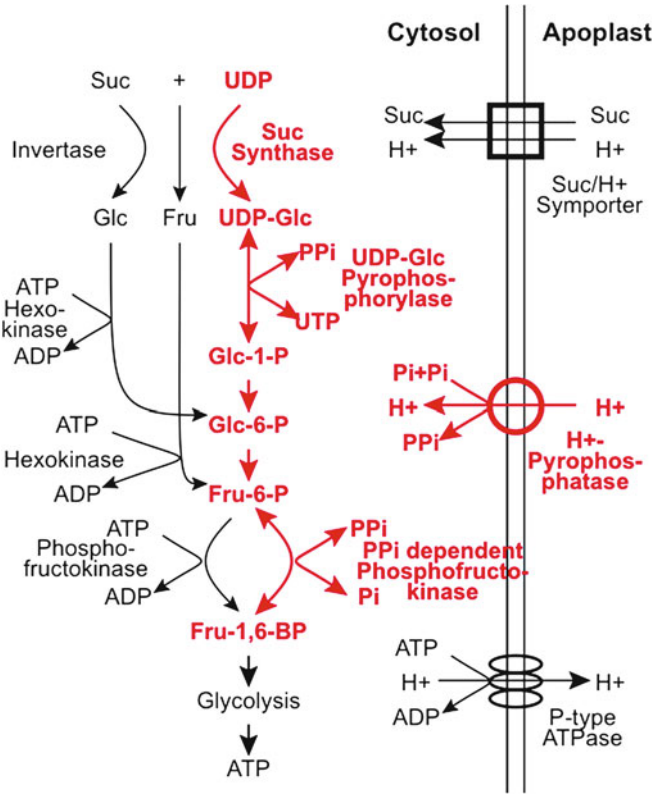

Fig. 3 Model for the function of a PM localized H^+-PPase in the sieve-element/companion cell. complex. In *red*, PP_i dependent ATP conserving pathway. This model is based on work reported elsewhere (Davies et al. 1997; Langhans et al. 2001; Lerchl et al. 1995; Stitt 1998)

the H^+-PPase from *Rhodospirillum rubrum* are consistent with the capacity of this enzyme to play two distinct roles depending on location; it can act as an intracellular proton pump in the acidocalcisomes or as a PP_i synthase in the chromatophore membranes during illumination (Seufferheld et al. 2004). Furthermore, Rocha-Facanha and Meis presented in vitro evidence with tonoplast fractions of maize coleoptiles and seeds consistent with the reverse function of the H^+-PPase (Rocha Facanha and de Meis 1998).

2.8.3 Hypothetical Model

It is tempting to speculate that the plant relative of the *R. rubrum* H^+-PPase can still "remember its prokaryote days" and work as a pump or as a PP_i synthase depending on location or milieu. A PM localized H^+-PPase in the sieve element/companion

cell complex could use the proton motive force (pmf) to maintain cytosolic PP_i levels required for sucrose (Suc) respiration through an ATP-conserving pathway (shown in red in Fig. 3). Companion cells have high respiration rates to maintain the pmf and Suc loading. In the first ATP-conserving step, a portion of the loaded Suc is metabolized to fructose (Fru) and UDP-Glc (glucose) by Suc synthase (Susy), which is well established to have high levels of expression in companion cells (Nolte and Koch 1993; Yang and Russell 1990). Then, UDP-Glc pyrophosphorylase requires PP_i to metabolize UDP-Glc to Glc-1-P and UTP. In the second ATP-conserving step, a PPi-dependent phosphofructokinase uses PP_i and Fru-6-P to create Fru-1,6-BP and P_i. By utilizing this pathway, the companion cells reserve ATP for generating the proton motif force. Since the reactions catalyzed by both UDP-Glc pyrophosphorylase and PP_i-dependent phosphofructokinase are readily reversible, high concentrations of PP_i are needed to maintain the reaction moving toward glycolysis. The required PP_i could be produced as a byproduct of several reactions, whereas the PMF can only be generated by ATP. Therefore, the use of the PMF to regulate and maintain PP_i levels when necessary would help optimize efficient Suc respiration and leave more for transport.

Based on this model, we hypothesize that the upregulation of type I H^+-PPases enhances sucrose fluxes from source to sink tissues by improving phloem sucrose loading capacity. Sucrose produced by photosynthesis, is the cornerstone of higher plant metabolism in both source and sink organs. It is the main substrate for respiration, biosynthesis, and storage. Thus, an enhanced availability of sucrose in the phloem for transport could result in both larger and more energized root systems with an enhanced apoplast and rhizosphere acidification capacity. The latter will result in more efficient nutrient uptake capacity. It is likely that up-regulation of H^+-PPases in the phloem may improve sucrose transport to sink organs, and improve growth through several pathways related to higher availability of reduced carbon. In a sense, it will produce a domino effect for integral plant growth and development.

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