Membrane Specificity of Proton Pyrophosphatase and Plasmodesmata Ultrastructure Provide the Structural Basis for Sugar Loading in *Oryza sativa* and *Physcomitrella patens*

by

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ABSTRACT

The remarkable conservation of molecular and intra-/inter-cellular pathways underpinning the fundamental aspects of sugar partitioning in two evolutionarily divergent organisms – a non-vascular moss *Physcomitrella patens* and a vascular cereal crop *Oryza sativa* (rice) – forms the basis of this manuscript. Much of our current knowledge pertaining to sugar partitioning in plants mainly comes from studies in thale cress, *Arabidopsis thaliana*, but how photosynthetic sugar is loaded into the phloem in a crop as important as rice is still debated. Even less is known about the mechanistic aspects of sugar movement in mosses. In plants, sugar either moves passively via intercellular channels called plasmodesmata, or through the cell wall spaces in an energy-consuming process. As such, I first investigated the structure of plasmodesmata in rice leaf minor vein using electron tomography to create as of yet unreported 3D models of these channels in both simple and branched conformations. Contrary to generally held belief, I report two different 3D morphotypes of simple plasmodesmata in rice. Furthermore, the complementary body of evidence in arabidopsis implicates plasma membrane localized Proton Pyrophosphatase (H⁺-PPase) in the energy-dependent movement of sugar. Within this wider purview, I studied the *in situ* ultrastructural localization patterns of H⁺-PPase orthologs in high-pressure frozen tissues of rice and physcomitrella. Were H⁺-PPases neo-functionalized in the vascular tissues of higher plants? Or are there evolutionarily conserved roles of this protein that transcend the phylogenetic diversity of land plants? I show that H⁺-PPases are distinctly expressed in the actively growing regions of both rice and physcomitrella. As expected, H⁺-PPases
were also localized in the vascular tissues of rice. But surprisingly, H\textsuperscript{+}\textsuperscript{-}PPase orthologs were also prominently expressed at the gametophyte-sporophyte junction of physcomitrella. Upon immunogold labeling, H\textsuperscript{+}\textsuperscript{-}PPases were found to be predominantly localized at the plasma membrane of the phloem complexes of rice source leaves, and both the vacuoles and plasma membrane of the transfer cells in the physcomitrella haustorium, linking H\textsuperscript{+}\textsuperscript{-}PPases in active sucrose loading in both plants. As such, these findings suggest that the localization and presumably the function of H\textsuperscript{+}\textsuperscript{-}PPases are conserved throughout the evolutionary history of land plants.
ACKNOWLEDGMENTS

Through my post-pubescent and post-adolescent years, spanning an altogether boarding school life inside the proverbial academic ‘bubble’, I was always in a clockwork grip, at once walking the line between book-strewn paths and paths yet unexplored, of time passing as a string of seemingly endless deadlines – an experience that has been etched in my mind forever since. The upcoming day’s milestones were set clockwise – sleep beckoned only when all the work for the day ahead was complete, alarm-clock was invariably set for six a.m., nine started the meter running full-throttle, at which point I entered the routine regime of multiple hours and half-hours governed by classes, interspersed with assignments, brunch, more classes and laboratory projects, until bedtime when only the deafening midnight whirrs of air-vents were my company.

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Though I have introspectively debated the existence of what we mortal humans call God, and spent my intellectually formative years in college as perhaps a full-blown atheist/agnostic, I had an otherworldly, perhaps divinely inspired, intensely personal revelation more than a year ago. The scientist in me has sought logical explanations for it, but fruitless in that pursuit, I have now personally and internally acknowledged that there is a benevolent, spiritual, suprahuman power that is beyond the grasp of my intellectual sphere. I am soulfully content in my imperfections as a human being, and though I am genetically predisposed to skepticism, I think, deep down, there is something transcendental that permeates the universe. I thank this very universal essence for imbuing life in this polycarbon–skeleton that I deem to call myself.
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1. INTRODUCTION

In his foundational treatise Metamorphosis of Plants, Wolfgang von Goethe declares, “all is leaf”, and in doing so, makes a seminal statement on how, in the natural world, structure often recapitulates function (von Goethe and Miller, 2009). Plants, ranging from distant algae and diminutive mosses to towering sequoia trees, are uniquely morphologically adapted to the specific ecological and climatic niches that they inhabit. In turn, the anatomical architectures of prototypical plant structures like leaves, stems, and roots, reflect in their overall form, the specialized capacity to perform specific function(s). Likewise, the cells that constitute these various plant organs – though similar to some extent in their organellar composition – manifest, within their surrounding cellular context, distinct morphologies that are evolutionarily tailored to their particular task(s). As such, Charles Darwin rightfully remarked that the study of morphology is “the most interesting department of natural history” (Darwin, 1860).

Viewed through this lens, the overarching premise of this dissertation is to look at how photosynthesized sugar is translocated in two evolutionarily distant plants, a monocot rice, and a moss Physcomitrella. Unsurprisingly, and perhaps befittingly, the fundamental nature of sugar movement in these two very divergent organisms shows a remarkable convergence of pathways – both cellular and molecular. The inextricable structure-function relationship is the recurrent refrain throughout this thesis – whether it be in the three-dimensional morphology of plasmodesmata in minor veins of rice, the anatomy of sieve element-companion cell complexes in rice leaves, the cell wall invaginations of transfer cells at the intergenerational junction in Physcomitrella, or in
fact in the crystal structure of proton-pumping pyrophosphatases. Overall, the evolutionarily conserved mechanisms and proteins, framed in the inter/intra-cellular context of sugar movement, form the thematic basis of this manuscript.

Sucrose Loading Mechanisms

Photosynthetic organisms use light energy to reduce atmospheric CO$_2$ to sugars, primarily sucrose (Suc). In higher plants that have evolved long distance sugar transport system in the form of phloem, the photoassimilate (i.e. Suc) is transported from the photoautotrophic “source” tissues (i.e. leaves) to the various heterotrophic “sink” organs such as flowers, seeds, roots and growing leaves. This long-distance mass transport of Suc is initiated by Suc uptake into the phloem tissue of minor veins of source leaves. This process is referred to as phloem loading. Sieve elements (SE), and companion cells (CC) constitute the phloem tissue, wherein the long distance transport occurs in the SEs, which lack a nucleus, have few organelles, and hence depend on the adjoining CCs for their sustenance (van Bel, 2003, van Bel and Knoblauch, 2000, Esau, 1969).

In the phloem tissue, the mechanism of Suc loading can be classified into three major pathways: apoplasmic loading, polymer trapping, and diffusion (McCaskill and Turgeon, 2007, Rennie and Turgeon, 2009). The apoplasmic loading strategy uses the proton motive force as the metabolic energy to actively load Suc from the apoplasm (cell wall space) into the phloem by Suc transporters (SUTs) (Giaquinta, 1983, Van Bel, 1993, Bel and Gamalei, 1992). In the polymer trapping mechanism, Suc diffuses symplasmically from the photosynthetic mesophyll cells into the specialized CCs called intermediary cells (ICs) along the Suc concentration gradient through cell-to-cell connective pores known as plasmodesmata (McCaskill and Turgeon, 2007). Using metabolic energy, Suc is then
polymerized into polysaccharides like raffinose and stachyose that are too large to diffuse back through the plasmodesmata (McCaskill and Turgeon, 2007). This maintains a Suc concentration gradient between the mesophyll cells and the ICs, so that a constant flux of Suc occurs from the source to the sink tissues (McCaskill and Turgeon, 2007, Zhang and Turgeon, 2009). Unlike these two mechanisms that are energy-dependent, symplasmic passive loading mechanism (or diffusion) is energy-independent, and Suc diffuses into the phloem tissues from mesophyll cells along a concentration gradient via plasmodesmata (Rennie and Turgeon, 2009). Many plants including Arabidopsis and maize use apoplastic loading strategy, but the loading mechanism employed by rice is still a contentious issue (Braun et al., 2014, Chonan et al., 1984a, Chonan et al., 1984b, Eom et al., 2012).

The frequency with which plasmodesmata occurs in the phloem tissue of leaf minor veins is supposed to be the diagnostic morphological feature that differentiates symplasmic loaders from apoplastic loaders, where the former are typified by a high plasmodesmatal frequency along the loading pathway (Turgeon, 1996). Ultrastructural analysis of minor veins and the plasmodesmata frequencies in CCs indicate that the symplasmic loaders have much higher plasmodesmatal frequency relative to the apoplastic loaders (Rennie and Turgeon, 2009, Schulz, 2005, Turgeon, 1996, Turgeon and Ayre, 2005).

Apoplastic Loading and Pyrophosphate Homeostasis

A lot of what we know about phloem loading mechanisms comes from studies in model eudicot Arabidopsis. In Arabidopsis, an apoplastic loader, Suc must be actively transported from mesophyll cells to CCs via Sucrose/H\(^+\)-symporters (SUTs) that depend on the proton gradient generated by the plasma membrane (PM) H\(^+\)- ATPase (Srivastava et al., 2008). Some of the incoming Suc must be cleaved into fructose and UDP-glucose
by the action of Sucrose Synthase (SUS) to furnish sufficient ATP for the maintenance of this trans-membrane proton gradient (Lerchl et al., 1995, Geigenberger et al., 1993). Two of the enzymes involved in the cleavage of Suc are PPi:fructose 6-phosphate 1-phosphotransferase (PFP) and the UDP-glucose pyrophosphorylase (UGPase) that work near equilibrium (Geigenberger and Stitt, 1993, Lerchl et al., 1995)(Fig.). A decrease in the cytosolic concentration of PPi should prevent the reactions leading to glycolysis, and thus attenuate ATP production required to maintain the proton motive force generated by the PM H⁺-ATPase (Srivastava et al., 2008). The direction of this so-called SUS pathway is thus dependent on a constant turnover of PPi (Lerchl et al., 1995). Though the importance of PPi in proper apoplastic phloem loading has long been appreciated, the source of PPi has remained enigmatic (Lerchl et al., 1995).

Pyrophosphate and H⁺-PPases

Many biochemical reactions and pathways inside living cells are coupled to the highly exergonic hydrolysis of nucleotide triphosphate (NTP). The hydrolysis of NTP drives these usually endergonic biochemical reactions forward, with a concomitant release of either orthophosphate (Pi) or pyrophosphate (PPi) (Heinonen, 2001). PPi, like its close cousin ATP, is a highly energetic compound because the destabilizing effect of the proximal electrostatic repulsion between the negatively charged groups of the phosphoanhydride is partially relieved by PPi hydrolysis, and also because the electrons of the bridging oxygen atom in PPi are more resonance stabilized upon hydrolysis. The hydrolysis of PPi has an energy output of -23.7 kJ.mol⁻¹ in plant cells (Davies et al., 1993). Two enzymes, soluble pyrophosphatases (sPPases) and membrane-bound H⁺-PPases, very dissimilar both in terms of their amino acid sequences and structures, catalyze the
hydrolysis of PPi. While the former hydrolyzes PPi to release heat energy, only the latter enzyme, hitherto unidentified in animals and fungi, uses the energy to pump protons (H\(^+\)) across biological membranes (Drozdowicz and Rea, 2001, Maeshima, 2000, Sarafian et al., 1992, Rea and Poole, 1985).

Archetypal plant H\(^+\)-PPases are highly conserved with amino-acid sequence identities of 85% or greater (Drozdowicz and Rea, 2001). It was initially thought that all H\(^+\)-PPases are of only one type - the type to which the first H\(^+\)-PPase to be cloned belongs (Sarafian et al., 1992). Later it became clear that H\(^+\)-PPases fall into two clearly delineated clades, namely Type I and Type II enzymes (Drozdowicz et al., 2000). Type I and Type II H\(^+\)-PPases are readily distinguished from each other based on their respective amino acid sequences and the near obligate requirement of the former, but not the latter, for K\(^+\) for activity (Drozdowicz et al., 2000). In model plant Arabidopsis thaliana, two genes, AVP1, AVP2 encode H\(^+\)-PPases. AVP1 encodes the K\(^+\)-dependent, Type I H\(^+\)-PPase (AVP1) which is ubiquitously expressed, and at particularly high levels in developing tissues, while AVP2 encodes the Type II H\(^+\)-PPase that shares only 36% sequence identity with AVP1 (Drozdowicz et al., 2000). Plant Type I H\(^+\)-PPases were first isolated from vacuoles and were considered to be genuine vacuolar markers (Maeshima, 2000, Maeshima, 2001, Rea et al., 1992). Type II H\(^+\)-PPases, on the other hand, localize at the Golgi bodies, and their total amount in tissues is less than 0.2% of that of the Type I H\(^+\)-PPase (Mitsuda et al., 2001a).

The three-dimensional crystal structure of the H\(^+\)-PPase from Vigna radiata (mung bean) shows a homodimeric protein with 16 transmembrane helices constituting the integral membrane domain of each subunit (Lin et al., 2012). The proton-translocation
pathway consists of six core transmembrane helices, and upon PPi hydrolysis, proton pumping is initiated as the core helices undergo conformational changes to translocate H\(^{+}\) ions into the vacuolar lumen through the sequential action of various exposed charged amino acid residues (Lin et al., 2012). Given the “vectorial” K\(^{+}\) dependence of Type I H\(^{+}\)-PPase, this protein was initially thought to also function as a H\(^{+}\)/K\(^{+}\) symporter (Davies et al., 1991), but the crystallographic evidence invalidates this notion altogether (Lin et al., 2012). The overall stoichiometric ratio of H\(^{+}\)/PPi is unity, wherein each H\(^{+}\)-PPase monomer translocates one proton per molecule of PPi hydrolyzed (Lin et al., 2012).
Pyrophosphate and Biosynthetic Reactions

| **Amino Acid activation** (aminoacyl-tRNA synthetase) |
| Amino acid + tRNA + ATP $\rightarrow$ aminoacyl-tRNA + AMP + PPI |

| **RNA synthesis** (RNA polymerase) |
| (NMP)$_n$ + NTP $\rightarrow$ (NMP)$_{n+1}$ + PPI |

| **DNA synthesis** (DNA Polymerase) |
| (dNMP)$_n$ + dNTP $\rightarrow$ (dNMP)$_{n+1}$ + PPI |

| **ADP-glucose formation** (ADP glucose pyrophosphorylase, Starch synthesis) |
| Glucose-1-phosphate + ATP $\rightarrow$ ADP-glucose + PPI |

| **Formation of fatty acyl-CoA** (fatty acyl-CoA synthetase) |
| Fatty acid + CoA + ATP $\rightarrow$ fatty acyl-CoA + AMP + PPI |

Figure 1: Pyrophosphate production in biosynthetic reactions (Adapted from Maeshima, 2000).

The syntheses of all biological macromolecules are coupled to the liberation of Pyrophosphate (PPI) from Nucleotide Triphosphate (NTP). In nucleic acid synthesis, it takes place directly in the incorporation of NMP from NTP to the polynucleotide, but in protein and polysaccharide synthesis, PPI is produced in the formation of the activated precursors aminoacyl-tRNA and NDP-sugar, respectively (Fig. 1) (Heinonen, 2001, Maeshima, 2000).

Actively Dividing Cells and H$^+$-PPases

Root and shoot apical meristems, inflorescence cells, and seedling hypocotyls are typical examples of young growing heterotrophic tissues in plants. These tissues are composed of small actively dividing cells characterized by a round nucleus, small vacuoles, extensive endomembrane systems, and ribosome-rich cytoplasm. The high content of H$^+$-PPase is plausible from a growing cell’s perspective. In young, growing tissues, RNAs,
proteins, and cellulose are actively synthesized for construction of new cells, and as a consequence, a large amount of PPI is produced as a by-product of these anabolic processes (Fig.1) (Maeshima, 2000, Maeshima et al., 1996). Le Chatlier’s principle dictates that PPI accumulation in the cytosol in high concentrations would inhibit these polymerization reactions. The membrane-bound H⁺-PPase scavenges the PPI in the cytosol and uses it as a source of energy for active transport of protons into the expanding vacuoles (Shiratake et al., 1997). By doing so, H⁺-PPase would alleviate competition for ATP between biosynthetic reactions and membrane transport processes (Shiratake et al., 1997). In mung bean seedling hypocotyls, for example, high content and activity of H⁺-PPase in vacuolar membranes of growing tissues on the basis of fresh weight was reported (Maeshima, 1990) that was directly ascribed to the active transcription of the H⁺-PPase gene (Nakanishi and Maeshima, 1998). In fact, the level of H⁺-PPase activity was found to be several fold higher than that of V-ATPase in young seedling hypocotyls (Maeshima et al., 1996). Furthermore, in roots of maize the activity of H⁺-PPase was maximal in the root tips where the cells are young and metabolically active (Chanson and Pilet, 1988).

By contrast, mature cells have lower anabolic activity, and PPI availability is limited. Moreover, the rate of transport of solutes into the vacuole also decreases and the expansion of vacuole ceases. Indeed, the H⁺-PPase activity has been reported to be lower than that of V-ATPase in mature tissues (Maeshima et al., 1996, Shiratake et al., 1997).

H⁺-PPase in Arabidopsis Thaliana

H⁺-PPases are ubiquitous among plants. As more plant genomes have been sequenced, orthologs of H⁺-PPases, putative or confirmed, have been discovered in plants ranging from the earliest bryophytes (mosses) to angiosperms (higher plants) (Seufferheld
et al., 2011). The function of plant vacuolar membrane-bound pyrophosphatase as a proton pump was first proposed by Walker and Leigh (Walker and Leigh, 1981). Only later did Rea and Poole, demonstrate that PPI-dependent and ATP-dependent H⁺-pumping across vacuolar membranes were catalyzed by different enzymes with different kinetic properties and inhibitor sensitivities (Rea and Poole, 1985). Complementary DNA (cDNA) for vacuolar H⁺-PPase was first cloned from Arabidopsis thaliana in 1992 that predicted a highly hydrophobic protein with 770 amino acids and a molecular mass of 80,800 Da (Sarafian et al., 1992). This study, and the subsequent genomic sequencing of Arabidopsis have confirmed that the gene encoding type I H⁺-PPase in Arabidopsis exists as a single copy.

H⁺-PPase Expression and Localization in Plants

Expression patterns of H⁺-PPase in various tissues of Arabidopsis at the mRNA and protein level were investigated by (Li et al., 2005). Their whole-mount indirect immunofluorescence study of wild-type (Col-0) and AVP1 overexpressing line (AVP1OX) roots showed a punctuate localization of H⁺-PPase protein in the root tips, suggesting tonoplast and plasma membrane residence of this protein (Li et al., 2005). Given the diffraction-limited resolution of confocal microscopy, this seemingly dual membrane localization of H⁺-PPase in Arabidopsis root tips could not be established. Recent immunogold evidence demonstrates that the H⁺-PPase localization is, in fact, at the vacuolar and pre-vacuolar membrane in root meristematic cells (Regmi et al., 2015, Viotti et al., 2013). Moreover, in situ hybridization study showed that AVP1 expression at the transcriptional level was maintained in root pericycle, shoot apical meristem, floral meristem, leaf and floral primordia, and procambium cells (Li et al., 2005). As discussed
earlier, high levels of H⁺-PPase expression in these young, proliferative tissues is not surprising (Fig. 2A).

Though H⁺-PPases have been canonically considered to be genuine tonoplast markers, multiple studies have shown a dual membrane localization of the type I H⁺-PPase in plants (Langhans et al., 2001, Long et al., 1995, Paez-Valencia et al., 2011, Ratajczak et al., 1999, Regmi et al., 2015, Pizzio et al., 2015). Immunelectron microscopic observations indicated the presence of a plasma membrane (PM) H⁺-PPase in meristematic cauliflower inflorescence cells (Ratajczak et al., 1999). However, the electron micrographs were distinctly symptomatic of chemical fixation artifacts - with convoluted tonoplast and plasma membranes, and a very diffuse cytoplasm. Double labeling epifluorescence experiments showed that the H⁺-PPase and the PM H⁺-ATPase localized in proximity at the PM of Ricinus communis sieve element companion cell complex (Langhans et al., 2001). Unlike meristematic cells, where vacuolar and plasma membranes are well separated, these two membranes in plant conducting cells are apposed beyond the resolution of light microscopes. These membranes can only be distinctly resolved with an electron microscope, and H⁺-PPase immunogold studies with Ricinus seedlings did show strong PM staining at phloem tissues of cotyledons and roots, whereas in the mesophyll and cortical cells the staining was mainly vacuolar (Long et al., 1995). Furthermore, the co-localization of a bona fide plasma membrane marker Plasma Membrane Intrinsic Protein (PIP1) with H⁺-PPase provided incontrovertible immunogold labeling evidence showing a PM localization of the type I H⁺-PPase in sieve-element and companion cell complexes in minor veins of Arabidopsis source leaves (Paez-Valencia et al., 2011).
H\textsuperscript+-PPase and Apoplastic Loading

The finding that the H\textsuperscript+-PPase localizes at the plasma membrane in the phloem cells in Arabidopsis led Gaxiola et al. (2012) to hypothesize that the up-regulation of \textit{AVP1} has different and seemingly contradictory effects in different tissues (Gaxiola et al., 2012). In non-phloem cells, type I H\textsuperscript+-PPases localize predominantly to and energize the endomembrane system while removing PP\textsubscript{i} produced as a byproduct of metabolism. PP\textsubscript{i} hydrolysis in source leaf mesophyll could promote sucrose synthesis (\textbf{Fig. 2B}) (Ferjani et al., 2011, Gaxiola et al., 2012). In phloem CCs, however, type I H\textsuperscript+-PPases predominantly localize to the PM (Paez-Valencia et al., 2011) and could function as PP\textsubscript{i} synthases regulating sucrose respiration for the generation of ATP and the proton motive force required for phloem loading (Gaxiola et al., 2012).

Recent report of a body of genetic, immunohistochemical, and physiological evidence is consistent with the reverse role of PM-localized H\textsuperscript+-PPases in Arabidopsis, and in so doing, presents tantalizing proof that the PP\textsubscript{i}-synthetic activity of this elegant protein might be the mysterious source of PP\textsubscript{i} in the phloem (Pizzio et al., 2015). In congruence with this idea is the finding that either \textit{CaMV35S} or phloem-specific (\textit{CoYMV}) overexpression of \textit{AVP1} in Arabidopsis elicits significantly higher root and shoot biomass (Pizzio et al., 2015). The roots of \textsuperscript{14}CO\textsubscript{2} photosynthetically labeled \textit{CaMV35S:AVP1} plants accumulate about two-fold higher reduced \textsuperscript{14}C signal relative to Col-0 plants. Accordingly, the roots of \textit{CaMV35S} seedlings had much higher rhizosphere acidification capacity compared to untransformed controls. These results denote that upregulation of the H\textsuperscript+-PPase positively impacts photosynthesis and photosynthate transport from source to sinks.
In stark contrast, phloem-specific $H^+\text{-PPase}$ RNAi ($pCoYMV::RNAiH^+\text{-PPase}$) lines showed a profound growth impairment of shoot and root when grown in the absence of Suc (Pizzio et al., 2015).

Additional clues consistent with an important role for the $H^+\text{-PPase}$ in phloem loading in Arabidopsis came from the activity of a 1.7-kb $AVP1$ promoter fused to the $uidA$ reporter gene in seedlings and adult plants. A prominent phloem-specific expression was evident in both developmental stages. Remarkably, the spatiotemporal expression of the $H^+\text{-PPase}$ gene mimics the distinct sink-to-source transition pattern of the PM-localized Suc-$H^+$ symporter, $SUC2$ (Pizzio et al., 2015). These data imply that the transcription of both $SUC2$ and $AVP1$ genes is closely correlated with sucrose phloem loading capacity in young leaves. Furthermore, the phloem-specific expression of the soluble PPase from *Escherichia coli* in Arabidopsis resulted in a severe dwarf phenotype, that when contrasted with the enhanced biomass triggered by the phloem-specific expression of $AVP1$ (Pizzio et al., 2015), clearly reveals that the lack or reduction of PP$_i$ levels in the phloem system is detrimental for normal growth in apoplastic loaders.

These results support the thermodynamically sound role of $H^+\text{-PPases}$ as PP$_i$ synthases in the PM of SE-CC complexes (Gaxiola et al., 2012, Pizzio et al., 2015, Gaxiola et al., 2015) (Fig. 2C).
Figure 2: Hypothetical models for tissue- and membrane-specific type I H⁺-PPase function.

(A) In active sinks such as meristems, tonoplast-bound H⁺-PPase hydrolyzes cytosolic PPᵢ and energizes expanding vacuoles (Shiratake et al., 1997). (B) PPᵢ scavenging
activity of H⁺-PPase favors sucrose synthesis in cotyledons (Ferjani et al., 2011). (C) PPᵢ synthase activity of plasma membrane-localized H⁺-PPase favors sucrose hydrolysis through the sucrose synthase pathway in sieve element–companion cell complexes (Gaxiola et al., 2012). Herein, the Sucrose synthesized in the chloroplasts in the mesophyll cells are actively loaded in the apoplastic cell wall space, and is, in turn, symported into the companion cells. The activity of H⁺/Suc symporters depends on the H⁺ gradient maintained by the PM H⁺-ATPases. Some of the Sucrose symported into the companion cells are diverted to glycolysis, and ultimately to generate ATP so that the H⁺ gradient can be maintained. (Adapted from Gaxiola et al. 2012).

Structural Basis for PPI Synthase Activity

However much the body of evidence might be consistent with PPI synthase activity of H⁺-PPases in the phloem, it is still reasonable to ask whether there is a sound chemico-structural basis to argue for the H⁺-coupled PPI synthetic activity of this protein. Ultimately, the thermodynamic basis for the reverse reaction of H⁺-PPases has to prove compatible with the protein structure and the underlying chemical mechanism of the protein’s action. H⁻-PPases are part of a group of membrane-bound PPases that also include Na⁺-PPases (found only in prokaryotes), and Na⁺/H⁺-PPases (found only in bacteria) (Kellosalo et al., 2012, Lin et al., 2012, Luoto et al., 2013). The crystal structures of the Thermotoga maritima Na⁺-PPase and Vigna radiata H⁺-PPase have been solved in three catalytic states – substrate-bound state (VrH⁺-PPase:Imidodiphosphate:Mg5 complex), resting state (TmNa⁺-PPase:Ca:Mg), and product-bound state (TmNa⁺-PPase:Pi₂:Mg4), but the protein conformation when the ion exit channel is open is yet unavailable (Fig. 3) (Kellosalo et al., 2012, Lin et al., 2012). On a fundamental level, the remarkable homology among these three membrane-bound
PPases is thought to underlie a common mechanism of action for these three proteins (Kellosalo et al., 2012, Tsai et al., 2014).

Mechanistically, the action of membrane-bound PPases can be divided into two concerted processes: substrate-hydrolysis and ion (\(\text{Na}^+ / \text{H}^+\)) translocation. The binding of the substrate, i.e. highly negatively charged PPi, is complexed in a coordination cage of \(\text{Mg}^{2+}\) ions, and upon attack by a suitably positioned nucleophilic water molecule, substrate hydrolysis occurs. Concomitant with this hydrolysis is the release of a proton (Kellosalo et al., 2012, Lin et al., 2012). While the minutiae of how PPi hydrolysis is accomplished is beyond dispute, the process by which the ion is translocated is still not fully understood. Three different hypotheses have been proposed to explain how the hydrolysis is coupled to the ion pumping activity.

On one hand, Lin et al. (2012) posited that the proton released upon nucleophilic attack is spontaneously translocated through a series of charged amino acid residues constituting a proton wire via the so-called ‘Grotthus mechanism’, and that the proton movement and/or PPi hydrolysis drives the conformational change required for the opening of the proton exit channel (Lin et al., 2012). On the other hand, Baykov et al. (2013) argued for a direct coupling mechanism, wherein the PPi hydrolysis is directly coupled to proton movement, that, in turn, exits the protein without undergoing serial transfers through the proton wire (Baykov et al., 2013). In contrast to these two mechanisms that postulate that PPi hydrolysis drives proton pumping, the third mechanism, also referred to as the ‘Binding Change’ mechanism, conceives that the proton pumping drives PPi hydrolysis instead (Kellosalo et al. 2012; Tsai et al.). Herein, substrate-binding induces the opening of the gate and exit channel through which the
proximally bound Na\(^+\)/H\(^+\) ions can easily exit. The residual negative charge at this site draws a proton from the activated water and initiates PPI hydrolysis (Kellosalo et al., 2012, Tsai et al., 2014).

From a mechanistic standpoint, the model proposed by Lin et al. (2012) is analogous to that for bacteriorhodopsin (Kellosalo et al., 2012, Lin et al., 2012, Tsai et al., 2014). Since bacteriorhodopsin uses light to generate proton gradient, using this mechanistic analogy for membrane-bound PPases would be illogical because no matter what the pH gradient, bacteriorhodopsin can not work in reverse and generate light (Kellosalo et al., 2012). The two forward hydrolytic mechanisms for membrane-bound PPases put forth by Lin et al. (2012) and Kellosalo et al. (2012) therefore only differ in their timing (Tsai et al., 2014). As such, if the driving force for the hydrolytic activity of membrane-bound PPases is the proton gradient, the converse must also be true, i.e. the synthesis of PPI drives ion translocation in reverse (Fig. 4). This is especially true because each monomer of the homodimeric membrane-bound PPases has a single continuous channel only through which ion translocation can occur. The direct implication of this, as visible in Fig. 4, is that the synthesis event is independent of the ion to be pumped. Given that the coupling funnel (depicted in Fig. 4) is 100% conserved in sequence and structure, this also implies that the ion discrimination occurs below the ion gate.

We interpret the post-hydrolysis product-bound state (i.e. \(TmNa^+-\text{PPase}:\text{Pi}_2\text{Mg}_4\)) of Na\(^+\)-PPase to be analogous to the first step in the PPI synthesis (Step 1; Fig. 4), wherein a condensation reaction leads to the production of a water molecule that is coordinated by D731 and D287 (Step 2; Fig. 4). The ion-translocation funnel that lies
below these aspartic acid residues constitutes of serially arranged charged residues – R242, D294, K742, and E301 that are fully conserved in the plant kingdom. The formation of a water molecule draws proton up through these residues leaving a negative charge at the E301, which according to site-directed mutagenesis data, is indispensable for ion translocation (Hirono et al., 2007, Pan et al., 2011, Zhen et al., 1997).

Concomitant with this would be the opening of the gate and the exit channel, through which proton from the lumenal/apoplastic space fills in the residual negative charge on E301 (Step 4; Fig. 4). The proton transmitted to the water molecule gets released as a hydronium ion down the proton gradient (Step 3 – 4; Fig. 4). All told, we concur with the binding change mechanism proposed by Kellosalo et al. (2012), and in accordance, posit that the thermodynamically feasible PPi synthase scheme follows a coherent and continuous pathway depicted in Fig. 4, wherein the PPi synthesis is linked to a concomitant movement of proton down a concentration gradient.
Figure 3: Crystal structure of Vigna radiata H+-PPase and the catalytic site of membrane-bound PPases in different states. (A & B) Three-dimensional ribbon structure of H+-PPase homodimer (blue and red monomers) compared with its hydrophobic surface model (PDB: 4A01). In the latter, blue represents hydrophilic surfaces of the protein exposed to the cytosol, while orange coloration depicts hydrophobic membrane-bound surface. (C & D) Catalytic state of membrane-bound PPase when non-hydrolyzable PPI analog imidodiphosphate (IDP; stick structure) is co-ordinated by
Mg$^{2+}$ ions. (E & F) Catalytic site in product (i.e. 2Pi) bound state (PDB: 4AV6). (G & H) Catalytic site in resting state (PDB: 4AV3).

Figure 4: Step-wise schematic depiction of the coupling funnel of an H$^+$-PPase monomer showing the synthesis of PPI driving H$^+$ pumping. Given sufficient H$^+$ gradient, condensation reaction leads to the synthesis of PPI (Step 2). The concomitant release
of a water molecule draws proton upward through the proton wire constituted by highly conserved charged amino acids (Step 3), and a hydronium (H₃O⁺) ion is consequently released (Step 4). The ion gate (not shown) opens, and the residual negative charge is filled in by an incoming proton from the apoplastic space (Step 4). D = Aspartic acid, R = Arginine, E = Glutamic acid, K = Lysine. It is noteworthy that all these amino acid residues are 100% conserved in organisms including, but not limited to, Rhodospirillum rubrum (a bacterium), Physcomitrella patens (a moss), Arabidopsis thaliana (a model eudicot), Vigna radiata (mung bean), and Oryza sativa (a model monocot rice).

Dissertation Chapter Summaries

Chapter 2

While early investigations in model plant Arabidopsis classified H⁺-PPase as a simple vacuolar proton pump, enough evidence has accumulated over the years that bespeaks a more elaborate model wherein the sub-cellular localization of H⁺-PPase and, by extension, its biochemical roles in different organs are quite distinct (Gaxiola et al., 2015, Gaxiola et al., 2012). It has been observed that H⁺-PPase localizes predominantly in the plasma membrane of the sieve element-companion cell complexes (Langhans et al., 2001, Long et al., 1995, Paez-Valencia et al., 2011). As a plant that employs apoplastic sucrose loading, the reverse function of H⁺-PPase as a PPᵢ synthase in these phloem cells makes thermodynamic sense (Davies et al., 1997, Gaxiola et al., 2012, Paez-Valencia et al., 2011). Lastly, it is logical that in Suc-consuming ‘sink’ tissues like shoot and root apical meristems, H⁺-PPase scavenges cytosolic PPᵢ that could otherwise inhibit several biosynthetic reactions in the metabolically active cells (Shiratake et al., 1997). The hypothesis that the localization of H⁺-PPase in meristematic cells is in the incipient vacuolar membranes gained support from recent immunogold labeling evidence (Viotti et
In light of this, we proceeded to localize H\textsuperscript{+}-PPase in photoautotrophic source leaves and heterotrophic sink meristem in *Oryza sativa* (rice). Though the phloem-loading mechanism in rice is still unclear, a modified symplasmic loading mechanism, termed “Revised Diffusion Model” has been proposed (Eom et al., 2012) that, if true, would immediately imply a vacuolar localization of H\textsuperscript{+}-PPase in the sieve element-companion cell (SE-CC) complexes of source leaves (Fig. 5). If, however, the H\textsuperscript{+}-PPases were localized at the plasma membrane (PM) of SE-CC complexes, it would support the idea that rice employs an apoplastic loading strategy (Braun et al., 2014). In essence, our experiment tests the mechanism predicted in the “Revised Diffusion Model”, with the hypothesis that a PM localization of the H\textsuperscript{+}-PPases would support the existence of an apoplastic sucrose loading strategy in rice source leaves. On the other hand, the apical meristematic cells are characterized by their high metabolic activity that entails a constant output of pyrophosphate as a metabolic byproduct, and lends the hypothesis that H\textsuperscript{+}-PPases would be localized at the incipient vacuolar and endomembrane systems (Fig. 2A).
Figure 5: Schematic representation of the Revised Diffusion Model proposed for *Oryza sativa*. Herein, the Sucrose synthesized in the chloroplast in the mesophyll cells are sequestered in the vacuoles, and the tonoplastic H⁺/SucSymporter (OsSUT2) acts as a “regulatory valve” that controls the concentration of cytosolic Sucrose concentration. How the Sucrose moves into the vacuole, however, is not yet known. This Sucrose then moves down the concentration gradient into companion cells via plasmodesmata, and is ultimately transported to various “sink” tissues of the plant. (Adapted from (Eom et al., 2012)).

Chapter 3

The ubiquity of H⁺-PPases in the plant kingdom, and this proton pump’s remarkably ancient evolutionary origin (Seufferheld et al., 2011), leads us to investigate whether the aforementioned model of H⁺-PPase function in Arabidopsis can be extended to a bryophyte *Physcomitrella patens*. The physiological mechanisms underlying Suc partitioning in mosses is an area of research that is still in its embryonic phase. On the basis of genomic data, however, the basic Suc metabolizing pathways found in higher plants are conserved
in Physcomitrella as well. Given the ancient evolutionary origin of $\text{H}^+\text{-PPases}$, and their distinct localization at the vascular tissues of higher plants, we posit that using a non-vascular model moss Physcomitrella would reveal whether $\text{H}^+\text{-PPases}$ were neofunctionalized into a role in the vascular tissues of higher plants, or whether there are evolutionarily conserved roles of this protein that transcend the phylogenetic diversity of land plants. The sugar loading pathways in Physcomitrella are yet unknown, though from our ultrastructural evidence, at least the gametophyte seems to contain putative food-conducting cells. The end walls of these cells are profusely decorated with plasmodesmata, suggesting a symplasmic mechanism of Suc movement through the gametophyte.

Chapter 4

In photoautotrophic source leaves of plants, the major photosynthate sucrose (Suc) has to be translocated from the sites of synthesis (i.e. mesophyll cells) to the specialized sieve element companion cell complexes prior to long-distance transport. In *Oryza sativa* (rice) source leaves, SE-CC complexes are separated from the mesophyll cells by concentric rings of vascular parenchyma, mestome sheath, and bundle sheath cells. It is via these cells that Suc moves prior to being actively loaded into the SE-CC complexes (Chonan et al., 1984a, Kaneko et al., 1980). While the ultimate Suc loading step involves an energy-consuming apoplasmic mechanism (Chapter 2), the preliminary route of Suc from the mesophyll cells to the vascular parenchyma cells involves symplasmic diffusion through interconnecting plasmodesmatal pores.

While the two-dimensional structure of plasmodesmata has been studied extensively in wide-ranging organisms using transmission electron microscopy, the true three-dimensional nature of these intercellular channels has remained unreported. Using electron
tomography, we investigated the three-dimensional morphology of plasmodesmata in the symplasmic interfaces of the Suc movement pathway in rice minor veins. In doing so, we built tomographic models of simple, twinned, and branched plasmodesmata. Within the vascular bundle of rice leaf minor vein, two unique types of simple plasmodesmata were identified.
2. PHLOEM LOADING IN RICE

Apoplastic loading in the rice phloem supported by the presence of Sucrose Synthase and plasma membrane localized Proton Pyrophosphatase

Abstract

Background and aims

Though *Oryza sativa* (rice) is one of the most important cereal crops, the mechanism by which sucrose, the major photosynthate, gets loaded into its phloem is still a matter of debate. Current opinion holds that phloem-loading pathway in rice could involve either a symplasmic or an apoplastic route. We hypothesized, on the basis of complementary body of evidence from Arabidopsis, an apoplastic loader, that the membrane-specificity of Proton Pyrophosphatases (H⁺-PPases; OVPs) in the sieve element – companion cell (SE-CC) complexes of rice source leaves would support the existence of either of the aforementioned phloem loading mechanisms. Additionally, we contended that the presence of Sucrose Synthase in the SE-CC complexes would be consistent with an apoplastic sucrose loading route in rice.

Methods

Conventional chemical fixation methods were used for immunohistochemical localization of H⁺-PPases and Sucrose Synthase in rice and Arabidopsis at the light microscopy level, while ultrastructural immunogold labeling of H⁺-PPases and Sucrose Synthase was performed on high-pressure frozen source leaves of rice.
**Key results**

Using immunogold labeling, we found that OVPs predominantly localize at the PM of the SE-CC complexes in rice source leaf minor veins, while in the root meristematic cells, OVPs preferentially localize at the vacuoles. PM specificity of OPVs in the SE-CC complexes was deemed to support apoplastic loading in the rice phloem. Further backing for this interpretation came from the Sucrose Synthase-specific immunogold labeling at the SE-CC complexes of rice source leaves.

**Conclusion**

These findings are consistent with the idea that, like in Arabidopsis and a majority of grasses, sucrose is actively loaded into the SE-CC complexes of rice leaves using an apoplastic step.
Introduction

Of the monocot cereal grains that constitute the vast majority of worldwide food consumption, *Oryza sativa* (rice) is one of the most important staple foods for more than half the world’s population (Fresco, 2005). Within this wider purview, our mechanistic knowledge of sucrose (Suc) loading, partitioning, and delivery from the photoautotrophic source leaves to the heterotrophic tissues like seeds, flowers, and roots in rice assumes even greater significance. In this context, it is noteworthy that the mechanism of Suc loading into the sieve element-companion cell (SE-CC) complexes in rice source leaves – whether symplasmic or apoplasmic – is still a matter of debate (Braun et al., 2014, Chonan et al., 1984b, Eom et al., 2012, Kaneko et al., 1980, Lim et al., 2006, Scofield et al., 2007).

On the basis of the concentration and form of transported sugars, function of Suc transporters (OsSUTs), and the minor vein plasmodesmal frequencies in source leaves, Eom et al. (2012) proposed that rice employs a modified version of passive symplasmic Suc loading strategy (Eom et al., 2012). According to this “Revised Diffusion Model,” the Suc synthesized in the mesophyll (chlorenchyma) cells is temporarily sequestered in vacuoles, and transported into the cytosol by vacuolar Suc/H\(^+\) symporters (OsSUT2) to be symplasmically loaded into the phloem via plasmodesmata (Eom et al., 2012). Herein, the OsSUT2s modulate the cytosolic concentration of Suc, and are theorized to function as “regulatory valves” that control the rate of diffusion of Suc into the phloem (Eom et al., 2012). Juxtaposed with this proposition is the counter-hypothesis, based on the re-evaluation of the same set of evidence examined by Eom et al. (2012), which posits that rice, like a majority of grasses, employs an apoplasmic loading strategy (Braun et al.,
If the Revised Diffusion Model of phloem loading in rice were true, Suc loading would occur passively through the symplasmic continuum between photosynthetic mesophyll cells and the SE-CC complexes, independent of the apoplasm (Eom et al., 2012). Consequently, there would be no need to maintain a plasma membrane-ATPase-dependent trans-membrane proton motive force (PMF) to energize the Suc/H\(^+\)-dependent symport of Suc into the SE-CC complexes. On the contrary, the existence of an apoplastic loading pathway in rice source leaves would entail largely symplasmically isolated SE-CC complexes, wherein the PMF is used to furnish the metabolic energy required to actively load Suc from the apoplasm into the phloem by PM localized OsSUTs (Braun et al., 2014).

Sucrose synthase (SUS) is postulated to cleave some of the incoming Suc into fructose and UDP-glucose to supply sufficient ATP for the maintenance of this PMF (Lerchl et al., 1995, Geigenberger et al., 1993). Two of the enzymes involved in this so-called SUS pathway are Pyrophosphate (PP\(_i\))-dependent fructose 6-phosphate 1-phosphotransferase (PFP) and the UDP-glucose pyrophosphorylase (UGPase) that work near equilibrium (Geigenberger et al., 1993). A decrease in the cytosolic concentration of PP\(_i\) should prevent the reactions leading to glycolysis and respiration, and thus attenuate ATP production required to maintain the PMF generated by the PM H\(^+\)-ATPase. Hence, PP\(_i\) homeostasis in the phloem is vital in maintaining a constant flux of Suc in apoplastic loaders (Lerchl et al., 1995).

Type I Proton Pyrophosphatase (H\(^+\)-PPase) is a PP\(_i\)-driven proton pump that partakes in the cellular PP\(_i\) homeostasis (Ferjani et al., 2011, Pizzio et al., 2015).
Ubiquitous in the plant kingdom, this highly evolutionarily conserved protein (Seufferheld et al., 2011) can either use the energy derived from PP\textsubscript{i} hydrolysis to pump protons across membranes (Maeshima and Yoshida, 1989, Maeshima, 2000), or under a favorable proton gradient work reversibly to synthesize PP\textsubscript{i} (Marsh et al., 2000, Rocha Façanha and de Meis, 1998). Vacuolar localization of H\textsuperscript{+}-PPase in actively dividing cells, as has been demonstrated recently in Arabidopsis root meristematic cells (Viotti et al., 2013), is plausible in the sense that the cytosolic PP\textsubscript{i} liberated as a byproduct of macromolecular biosynthesis would not only be scavenged by membrane-bound H\textsuperscript{+}-PPase to transport protons into the expanding vacuoles, but would also alleviate competition for ATP between biosynthetic reactions and membrane-transport processes (Shiratake et al., 1997).

Nevertheless, enough evidence has amassed over the years that demonstrate a PM localization of H\textsuperscript{+}-PPase in the SE-CC complexes (DeWitt et al., 1991, Langhans et al., 2001, Long et al., 1995, Paez-Valencia et al., 2011), wherein the thermodynamic limitations imposed by the prevailing PMF preclude the proton pumping activity coupled to PP\textsubscript{i} hydrolysis (Davies et al., 1997). The thermodynamic conditions are instead conducive to PP\textsubscript{i} synthesis at this interface (Davies et al., 1997), and in fact, the reversibility of the plant enzyme has been demonstrated in vitro in maize and oranges (Marsh et al., 2000, Rocha Façanha and de Meis, 1998). Added to this is the indispensability of PP\textsubscript{i} in the SUS-dependent apoplastic Suc loading regime (Lerchl et al., 1995). In view of these outcomes, Gaxiola et al. (2012) proposed a model in which PM localized H\textsuperscript{+}-PPase could function as a PP\textsubscript{i} synthase regulating Suc respiration for the generation of ATP and the PMF required for phloem loading (Gaxiola et al., 2012).
Genetic, histochemical, and physiological evidence supporting the importance of PPi and PM-localized H⁺-PPase in the Suc loading pathway of Arabidopsis, an apoplastic loader was recently reported (Pizzio et al., 2015).

While the gene encoding type I H⁺-PPase in Arabidopsis, *AVP1*, exists as a single copy, H⁺-PPases constitute a multi-gene family in rice with six paralogs (*Oryza sativa* Vacuolar Pyrophosphatases; *OVP1* - *OVP6*) hitherto identified (Liu et al., 2010, Muto et al., 2011, Choura and Rebai, 2005, Sakakibara et al., 1999). Liu et al. (2010) studied the relative expression profiles of the six *OVP* isoforms at the mRNA level, and found that the *OVP1* and *OVP2* transcript levels were very abundant in young leaf blades, while *OVP2* expression was dominant in growing tissues like root and leaf sheaths (Liu et al., 2010). On the other hand, *OVP5* expression levels were extremely low in most tissues, and the overall mRNA levels of *OVP3*, *OVP4*, and *OVP5* were markedly lower than the levels of *OVP1* and *OVP6* (Liu et al., 2010). Notably, β-Glucuronidase expression driven by *OVP3* promoter was shown to be phloem-specific in the root vascular cylinder when the plants were exposed to anoxic conditions (Liu et al., 2010). However, analysis of tissue-specific protein expression profile of the OVP class of H⁺-PPases has not yet been reported. Intriguingly, rice plants engineered to overexpress *AVP1* developed significantly more robust root systems and twice as much seeds than untransformed controls (Yang et al., 2007), indicating that H⁺-PPase potentially participates in the Suc loading and partitioning in rice as well.

Given the debate surrounding the phloem loading strategy in rice, and the complementary body of evidence gathered from Arabidopsis, we hypothesized that the immunogold localization of H⁺-PPases in the SE-CC complexes of rice source leaves
would help clarify whether rice uses an apoplastic or a symplasmic phloem loading mechanism. Under the scheme of Revised Diffusion Model (Eom et al., 2012), a predominantly vacuolar H\(^+\)-PPase localization in the SE-CC complexes would be expected, wherein the H\(^+\)-PPases scavenge cytosolic pyrophosphate (PP\(_i\)) and acidify the vacuolar lumen. Contrarily, a PM localization of H\(^+\)-PPase at the SE-CC complexes would add credence to an apoplastic Suc loading mechanism in rice, and implicate H\(^+\)-PPases in the maintenance of phloem PP\(_i\) homeostasis. Correspondingly, the Revised Diffusion Model also predicts a SUS-independent phloem-loading pathway at the SE-CC complexes in rice source leaf minor veins.

Using comparable tissues from eudicot Arabidopsis and monocot rice, we present immunohistochemical evidence that demonstrates a closely correlated pattern of H\(^+\)-PPase expression in both plants. To provide a coherent model of H\(^+\)-PPase action in rice, we also investigated the *in situ* localization of OVPs in high-pressure frozen heterotrophic root sink meristems and the loading phloem of photoautotrophic source leaves at the ultrastructural level. On one hand, immunogold labeling revealed a predominantly vacuolar localization of OVPs in the root meristematic cells of rice. On the other hand, OVPs were localized at the PM of SE-CC complexes of rice source leaf minor vein suggesting the existence of an apoplastic loading mechanism. Adding further support to this inference was the finding that SUS-specific immunogold label was also found at the SE-CC complexes of rice source leaves.
Materials and Methods

**Plant growth conditions**

Rice: Seeds were dehulled, sterilized with 10% bleach solution, and germinated on rice growth media (Yang et al., 2007) on petri plates at 37°C in the dark for 48 hours. Once the post-germinative process, characterized by the emergence of the radicle, had begun, the plates were transferred to a growth chamber with 16-hour light, and 8-hour dark regime. Once the coleoptiles had developed, the seedlings were transferred to wet soil, and allowed to grow until the fifth leaf had emerged, and the flag leaf was fully developed (~3 – 4 weeks). The tissues for subsequent immunohistochemical / immunogold / immunoblot analysis were harvested either from soil grown (leaves, leaf sheaths, flowers), or plate grown (root tips, roots) plants at various stages of growth, as detailed below. Adult plants grown in the greenhouse were used to harvest flowers.

Arabidopsis: Col-0 ecotype seeds were directly sown in wet soil, vernalized in the dark for 48 hours at 4°C, and allowed to grow until 45 days old or until inflorescent stems had fully developed flowers (rosette leaf / stem / flower immunohistochemistry). Sterilized Col-0 seeds were sown into liquid ½ MS media supplemented with 1% sucrose, and grown for 7 days in a flask with constant agitation, and subsequently used for protein extraction (see below).

Maize: Wild-type maize seeds bought from a local grocery store were germinated on wet soil, and allowed to grow in a growth chamber with 16-hour light, 8-hour dark regime until the fifth leaf had emerged, and the first leaf was fully grown.

*Physcomitrella patens*: Wild-type *P. patens* (Hedw.) were grown on wet soil, with constant fertilization with PPNH₄ liquid media in a growth chamber with 16-hour light,
8-hour dark regime. Growth media was prepared according to:

*Populus fremontii*: A fully-grown tree from Arizona State University Tempe campus (https://www.asu.edu/map/interactive/) was used to harvest fully-grown leaves.

**Light microscopy and immunohistochemistry**

The following tissues were harvested as following:

Rice source leaf and leaf sheaths: Once the rice plants grew the fifth leaf, and the flag leaf had fully grown, the flag leaves and leaf sheaths were excised.

Rice roots: After approximately two weeks on vertical solid rice media in petri plates, the rice seedlings had grown crown and multiple adventitious roots. The crown roots were excised.

Rice flowers: Once adult plants grown in the greenhouse had developed flowers, the flowers were excised.

Arabidopsis leaves, inflorescent stems, flowers, seedlings: Wild-type Col-0 plants were grown in soil for 45 days, or until inflorescent stems had developed flowers. The fully-grown rosette leaves, stem sections, and entire flowers were excised.

Maize leaves: Once the first leaf had fully developed (~4 weeks), it was excised.

*Populus fremontii*: Two leaves were excised directly from an adult poplar tree.

Immunohistochemistry: The tissues excised were cut into small pieces and immediately immersed in at least 20-fold excess volume of FAA (10% v/v 37% formaldehyde : 5% v/v acetic acid : 50% v/v 200-proof ethanol : 35% v/v water) fixative, and placed under a gentle vacuum for 10 – 15 minutes at room temperature. The fixation was then allowed to continue overnight at 4°C. Following this, the tissues were dehydrated in a graded ethanol
series, exchanged with xylene to clear the tissues, and infiltrated with paraplast. The
tissues were then embedded in paraplast in desired orientations. Using a Leica RM2155
rotary microtome (Leica Microsystems, Germany; www.leica-microsystems.com), the
tissue blocks were cut into 10μm thick sections, and mounted on poly-L-Lysine coated
slides. The tissues were then deparaffinized, rehydrated, and exposed to an antigen
retrieval buffer (Biogenex, USA; www.biogenex.com) in a 65°C water bath for 20
minutes. After washing in water, the endogenous peroxidase activity was quenched using
3% (v/v) H2O2. The tissues were washed with Phosphate Buffered Saline, pH 7.2 with
0.01% (v/v) Tween-20 (PBST), and blocked with 1% (w/v) casein in PBST for 30
minutes at room temperature. After washing with PBST three times, the tissues were
exposed to anti-AVP1 or anti-SUS rabbit polyclonal antibodies at 1:1000 dilutions for 1
hour at room temperature. Negative controls were concurrently performed using pre-
immune serum at 1:1000 dilutions in PBST. After three washes in PBST, the signal was
developed using the SignalStain® Boost IHC HRP anti-rabbit detection system (Cell
Signaling Technology, USA; www.cellsignal.com), following manufacturer’s
instructions. The tissues were then run through a dehydrating ethanol series, and were
permanently mounted. Images were acquired with Zeiss Axioskop light microscope
(Leica Microsystems, Germany) equipped with 10X Achromplan 0.25 NA Zeiss, 40X Plan
Neo-Fluar 0.75 NA Zeiss, and 100X 1.30 NA Oil Plan Neo-Fluar objective lenses, phase
and differential interference contrast optics, Olympus DP72 camera system, and Olympus
cellSens® imaging software.

**Total protein extraction**

Approximately 300 mg of maize, rice leaves, Arabidopsis seedlings, and
Physcomitrella gametophores were immediately ground into fine powder with liquid N₂, transferred in ~200 μL aliquots to screw-cap eppendorf tubes. To each tube, 1mL of 10% (v/v) Trichloroacetic acid in -20°C acetone was added, and proteins were allowed to precipitate overnight at -20°C. The samples were centrifuged at 10,000xg for 30 minutes at 4°C, followed by removal of the supernatant. The pellet was washed with -20°C acetone containing 0.07% β-mercaptoethanol, vortexed, and centrifuged at 10,000xg for 10 minutes at 4°C. The washing, vortexing, and centrifugation steps were repeated four more times. After the final centrifugation step, the supernatant was removed and the pellet dried in a tabletop vacuum for ~30 minutes. Total protein was solubilized by adding Laemmlli’s buffer to the pellet. Prior to storage at -80°C the mixture was vortexed, and centrifuged at 5,000xg for 5 minutes at 4°C three times.

**Western Blot**

The solubilized protein extracts from corn, rice, Arabidopsis, poplar, and Physcomitrella were then run on a 10% SDS-polyacrylamide gel, transferred to a PVDF membrane, blocked with 5% (w/v) non-fat milk in Tris buffered saline with 0.01% Tween-20 (TBST), and probed with SUS-specific polyclonal sera at 1:1000 dilution overnight at 4°C. After washing in TBST, the membrane was developed using Bio-rad Alkaline Phosphatase Immun-Blot® Colorimetric Assay kit (Bio-rad Inc., USA; www.bio-rad.com) according to the manufacturer’s instructions.

**Immunogold labeling and transmission electron microscopy**

High-pressure freezing (HPF) and freeze-substitution (FS): Fifteen-day old rice seedlings grown vertically on petri plates were used to harvest the root tips. Root tips were excised with a sharp double-edged razor blade under 150mM sucrose, and quickly
loaded into interlocking brass planchettes, filled with 150mM sucrose, and subjected to HPF in BalTec HPM 010 high-pressure freezer. To cryofix leaves, Harris unicore 1.2mm punch (Electron Microscopy Sciences, USA; www.emsdiasum.com) was used to punch small leaf discs from fully-grown flag leaves while avoiding the leaf mid-veins. The leaf discs were then immediately transferred to type-B brass planchettes. The empty space was filled with 1-hexadecene, and another type-B planchette was used as a cover, and the sandwich was immediately subjected to HPF. The cryofixed root tips and leaves were then transferred to a pre-chilled -80°C freeze-substitution (FS) cocktail containing anhydrous acetone and 0.2% (w/v) non-methanolic uranyl acetate. The FS was allowed to continue for 96 hours at -80°C, after which the FS solution was allowed to warm to 4°C over a period of 12 hours. The anhydrous acetone was exchanged with anhydrous ethanol several times, and the tissues were gradually infiltrated with medium grade London Resin (LR) White (Electron Microscopy Sciences, USA): 25% (v/v) LR White (12 hours), 50% (v/v) LR White (12 hours), 75% (v/v) resin (12 hours), 100% LR White (12 hours), 100% LR White (12 hours), 100% LR White (12 hours). The root tips and leaves were transferred to gelatin capsules, filled with fresh LR White and polymerized for 24 hours at 50°C in longitudinal or transverse orientations. The polymerized resin blocks were then trimmed, ultrathin sectioned to gold reflectance (90 – 100 nm) using a Leica Ultracut R ultramicrotome (Leica Microsystems, Germany), and mounted on formvar-coated nickel slot grids.

For immunogold labeling, the nickel grids containing the sections were floated on drops of blocking solution (1% w/v casein in PBST) for 1 hour at room temperature. The grids were then sequentially transferred to drops of PBST before being floated on drops...
of anti-AVP1 or anti-SUS polyclonal antibodies diluted 10-fold in the blocking solution. The antibody incubation step was performed at 4°C overnight. Following this, the grids were washed five times with PBST, and then transferred to drops of anti-rabbit IgG conjugated to either 10 nm (leaves) or 20 nm (roots) gold particles (Electron Microscopy Sciences, USA) diluted 25- to 50-fold in the blocking solution. The grids were further washed in PBST five times, and rinsed with double-distilled water three times, before being air-dried overnight. The sections were then post-stained for 5 minutes with 0.5% aqueous uranyl acetate and imaged with a JEOL 1200EX transmission electron microscope (JEOL Corp., Japan) operating at 80 KV. Gold particles were quantified using ImageJ software, and the data was compiled and statistically analyzed using Microsoft Excel 2011 (Microsoft Corp., USA; www.microsoft.com) and its associated plugin Statplus. Linear image adjustments like contrast and brightness were adjusted on grayscale electron micrographs to improve the visibility of gold particles using Adobe Photoshop CS6 (Adobe Inc., USA). All the images were compiled into figure panels using Adobe Illustrator CS6 (Adobe Inc., USA).
Results

Closely correlated expression pattern of AVP1 and OVPs in comparable tissues of Arabidopsis and rice respectively

It has been known that young growing sink tissues such as elongating hypocotyls have significantly higher H\(^{+}\)-PPase activity than mature tissues (Nakanishi and Maeshima, 1998). Previous *in situ* hybridization study showed that *AVP1* expression at the transcriptional level was maintained in root pericycle, shoot apical meristem (SAM), floral meristem, leaf and floral primordia, and procambium cells in Arabidopsis (Li et al., 2005), while GFP-tagged AVP1 was observed in ovules, funicle, root tips, fruit, and vascular tissues of leaf, petal, and roots (Segami et al., 2014). Moreover, when driven by a 1.7 kb *AVP1* promoter, β-Glucuronidase expression was prevalent in the vascular and sink tissues of Arabidopsis (Pizzio et al., 2015). In view of these results, we conducted a comparative study to examine the tissue-specific expression pattern of OVP class of H\(^{+}\)-PPases and AVP1 in various tissues of rice and Arabidopsis, respectively. Previously characterized antiserum (Li et al., 2005, Park et al., 2005, Yang et al., 2007) generated against the highly conserved CTKAADVGADLVGKIE motif (Rea et al., 1992) in H\(^{+}\)-PPases was used in this experiment.

Consistent with the previous outcomes, we found that AVP1 was conspicuously expressed in either the actively growing sink tissues or in the phloem (Fig. 6 A-F). Specifically, active sink tissues of Arabidopsis like SAM (Fig. 6A), leaf primordia (Fig. 6A), vascular cambium (Fig. 6B & C), ovules (Fig. 6E), and pollen (Fig. 6F) displayed the strongest signal. AVP1 was also prominently expressed in the phloem of leaves and inflorescent stem (Fig. 6A, C, and D). Using Arabidopsis as a reference, we also
performed a parallel immunohistochemical survey of OVP expression pattern in various tissues of rice. As expected, the OVPs were distinctly present in young proliferative sink tissues like SAM (Fig. 6G), emergent leaf sheaths (Fig. 6G, and I), root primordium (Fig. 6H), growing embryo (Fig. 6K), and pollen (Fig. 6L). OVPs were also distinctly localized in the vascular tissues of rice source leaves (Fig. 6J) and leaf sheaths (Fig. 6I). Representative negative controls using pre-immune serum (Fig. 6 M – O) showed that the immunohistochemical signal observed was AVP1- and OVP-specific.

**OVPs preferentially localize to the vacuoles in root apical meristematic cells**

As has been demonstrated in Arabidopsis (Viotti et al., 2013), we hypothesized, in accordance with the previous literature (Shiratake et al., 1997), that OVPs would predominantly localize at the vacuoles in actively growing sink tissues (Fig. 7 G-I). We first performed immunohistochemistry on the crown roots of rice seedlings at the light microscopy level, and found that the OVPs were markedly expressed in the phloem in the root vascular cylinder (Fig. 7 A & C), and also in the lateral root primordium (Fig. 7 A & B). Given the amenability of root tips to high-pressure freezing, we proceeded to immunolocalize OVPs in the root apical meristematic cells of rice roots at the ultrastructural level. Unsurprisingly, upon immunogold labeling, it was found that the OVPs preferentially localized at the vacuoles (Fig. 7 E – G & J) in these metabolically active cells characterized by dense, ribosome-rich cytoplasm, extensive endomembrane system, and turgescent nuclei. Negative controls performed with pre-immune serum showed that the immunogold PM labeling was OVP-specific (Fig. 7 H & I).
OVPs preferentially localize to the plasma membrane in sieve element companion cell (SE-CC) complexes in rice source leaves

To present a coherent model of OVP action in rice, we therefore proceeded to immunolocalize OVPs in the SE-CC complexes of photoautotrophic rice source leaf minor veins. The body of immunogold (Paez-Valencia et al., 2011), histochemical, genetic, and physiological evidence (Pizzio et al., 2015) in Arabidopsis, an apoplastic loader, are consistent with the model that implicates AVP1 as a PM localized protein working reversibly to function as a PP$i$-synthase rather than the canonical proton-pumping pyrophosphatase at the SE-CC complexes (Gaxiola et al., 2012).

The current opinion on the phloem loading mechanism in rice is divided (Braun et al., 2014, Eom et al., 2012). While Eom et al. (2012) proposed a novel “Revised Diffusion Model” that postulates a modified passive symplasmic route of sucrose (Suc) loading, Braun et al. (2014) advanced the idea that rice uses an apoplastic loading mechanism instead (Braun et al., 2014, Eom et al., 2012). Herein, a vacuolar localization of OVPs in the SE-CC complexes of rice minor veins would be expected under the former model, while PM localization of OVPs would favor the latter hypothesis. We thus examined the ultrastructural localization of OVPs in SE-CC complexes of rice source leaf minor veins in both longitudinal and transverse sections.

The phloem cells consist of metabolically active CCs, and their energy-dependent partners, the SEs (Esau, 1969). The distinct ultrastructural cytology of these elongated cell complexes are only obvious in longitudinal sections, with the CCs characterized by their dense, ribosome-rich cytoplasm with well-developed nuclei, and multiple
mitochondria, while the SEs, laden with Suc (an endogenous cryoprotectant), are mostly free of organelles (Esau, 1969). These features make vascular tissues particularly suitable for high-pressure freezing.

Upon immunogold labeling, it was evident that OVPs were distinctly localized at the PM of both SEs and CCs (Fig. 8 A-E). Quantification of gold particles showed that the distribution of OVPs was preferential at the PM of CCs rather than the vacuoles, and statistically similar between the PM of SEs and CCs (Fig. 8F). Negative controls performed with the pre-immune serum showed that the observed immunogold labeling was OVP-specific (Fig. S1). These results are consistent with the idea that rice uses an apoplastic Suc loading strategy (Braun et al., 2014).

Sucrose Synthase is also localized at the SE-CC complexes of rice source leaf minor veins

The direct corollary of the inference that rice uses an apoplastic loading mechanism is the existence of a PP\textsubscript{i}-dependent, ATP-conserving Sucrose Synthase (SUS) pathway. Therefore, we proceeded to immunolocalize SUS in the SE-CC complexes of rice source leaf minor veins. Using a Western blot, we first tested the cross-reactivity of the polyclonal antibodies generated against a combination of Sh1 and Sus1 gene products from Zea mays (maize) kernels (Nolte and Koch, 1993, Koch et al., 1992) against total protein extracts from multiple plants including Zea mays (positive control), Arabidopsis, rice, Populus fremontii and Physcomitrella patens (Fig. 9 I). As previously observed for maize, bands representing homotetramers and heterotetramers of SUS with varying proportions of SH1 (upper bands) and SUS1 (lower bands) were found (Koch et al.,
Upon performing BlastP searches (www.gramene.org), orthologous SUS proteins with varying percentages of amino acid sequence identities to SH1 and SUS1 were recognized in all the plant species tested (Fig. 9 I; Table S1). Subsequent immunohistochemical localization of SUS in the source leaves of maize, Arabidopsis, and rice at the light microscopy level showed the distinct presence of this protein in the phloem (Fig. 9 A – C), while a prominent signal was evident in the xylem of Populus leaves (Fig. 9 D). The diffraction-limited resolution of light microscopy did not allow us to distinguish which phloem cells in the rice vascular bundle SUS was localized at (Fig. 9C). Hence, we performed immunogold labeling of SUS in high-pressure frozen rice source leaf minor veins, and found that SUS was parietally localized in proximity to the PM at the SE-CC complexes (Fig. 9 J – L). This result supported our interpretation, congruent with the previous postulation (Braun et al., 2014) that a SUS-based apoplastic Suc loading pathway exists in the SE-CC complexes of rice leaf minor veins.
Discussion

The salient feature of H⁺-PPase expression pattern in angiosperms, both at the transcript and protein level, is the conspicuous localization observed at both the actively dividing regions (e.g. apical meristems, leaf and root primordia) and the phloem (Gaxiola et al., 2001, Langhans et al., 2001, Li et al., 2005, Long et al., 1995, Paez-Valencia et al., 2011, Ratajczak et al., 1999, Segami et al., 2014, Viotti et al., 2013, Yang et al., 2007, DeWitt et al., 1991). Additionally, when driven by either a 1.4kb or a 1.7kb AVP1 promoter, β-Glucuronidase was also strongly expressed in the phloem and sink tissues in Arabidopsis (Pizzio et al., 2015, Mitsuda et al., 2001b). In view of these outcomes, it was not surprising that both AVP1 and OVPs were distinctly expressed in the active sink regions and vascular tissues of Arabidopsis (Fig. 6 A – F) and rice (Fig. 6 G – L; Fig. 7 A – C) respectively. The immunohistochemical localization of H⁺-PPase was closely correlated between Arabidopsis and rice (Fig. 6) denoting that this highly evolutionarily conserved protein (Seufferheld et al., 2011) retains tissue-specificity and presumably function even through the divergence of eudicots and monocots in angiosperm phylogeny.

The traditional model of H⁺-PPase action dictates that this protein is strictly vacuole-specific (Maeshima, 2000, Maeshima and Yoshida, 1989, Rea et al., 1992). This notion is in congruence with the high activity of this protein found in metabolically active cells (Nakanishi and Maeshima, 1998). In young, growing tissues, RNAs, proteins, and cellulose are actively synthesized for the construction of new cells, and as a consequence, a large amount of PPᵢ is produced as a by-product of these anabolic processes (Heinonen, 2001, Maeshima, 2000). PPᵢ accumulation in the cytosol in high concentrations would
inhibit these polymerization reactions (Maeshima, 2000, Shiratake et al., 1997). The membrane-bound H⁺-PPase scavenges the PPi in the cytosol and uses it as a source of energy for active transport of protons into the expanding vacuoles (Shiratake et al., 1997). By doing so, H⁺-PPase would alleviate competition for valuable ATP between biosynthetic reactions and membrane transport processes (Shiratake et al., 1997). Given that our light microscopy based observations showed that OVPs were prominently localized in young sink tissues (Fig. 6; Fig. 7 A–C), we proceeded to investigate the ultrastructural localization of OVPs in the root meristematic cells of rice. In accord with the earlier evidence that AVP1 was localized at the vacuoles of root meristematic cells in Arabidopsis (Viotti et al., 2013), we found that the OVP specific immunogold label was also predominant at the vacuoles in the corresponding root meristematic cells in rice (Fig. 7 E–G).

By contrast, the role of H⁺-PPase in the phloem has remained mostly enigmatic. At least in Arabidopsis, an apoplasmic loader, the importance of H⁺-PPase in phloem function and photosynthate partitioning was recently demonstrated (Pizzio et al., 2015). The phloem loading strategy used by a crop as important as rice, however, still remains obscure. Currently, there are two schools of thought that postulate different and diametrically opposite hypotheses (Braun et al., 2014, Eom et al., 2012, Scofield et al., 2007). Eom et al. (2012) proposed a novel “Revised Diffusion Model” that is a modified version of the passive symplasmic Suc loading scheme, while Braun et al. (2014) postulated an apoplasmic loading strategy (Braun et al., 2014, Eom et al., 2012).

The phloem, composed of Sucrose (Suc) translocating, but apparently docile sieve
elements (SEs), and their metabolically active, and ontogenetically linked sister companion cells (CCs), are the sites for Suc loading, transport, and delivery in plants (Esau, 1969). The mechanism of Suc loading into the SE-CC complexes is either an energy-dependent, apoplastic process, or a passive, symplasmic one (Rennie and Turgeon, 2009). Within this scope, the immunogold localization of AVP1 at the plasma membrane (PM) of SE-CC complexes in Arabidopsis, an apoplastic loader, (Paez-Valencia et al., 2011) suggested a rather different role of this protein at the phloem. Though previous reports of H⁺-PPase localization at the PM of the SE-CC complexes existed (DeWitt et al., 1991, Langhans et al., 2001, Long et al., 1995), the canonical role of this protein as a proton pumping PPᵢ-hydrolase at the Suc-loading apoplastic interface of the phloem was dismissed on thermodynamic grounds (Davies et al., 1997). The reverse function of H⁺-PPase as a PPᵢ-synthase was instead postulated (Davies et al., 1997), and was later demonstrated in vitro in maize and oranges (Marsh et al., 2000, Rocha Façanha and de Meis, 1998). Additional evidence showed the requirement of PPᵢ in sustaining SUS-based Suc loading scheme in apoplastic loaders (Lerchl et al. 1995). Taking all these key results and their mechanistic implications into account, it was hypothesized that the H⁺-PPase works as a PPᵢ-synthase in the phloem (Gaxiola et al., 2012). Using Arabidopsis, it was recently shown that the phloem-specific knockdown of AVP1 encoding gene resulted in the stunted growth of both root and shoot, and the phloem expression of soluble pyrophosphatase triggered a dwarf phenotype (Pizzio et al., 2015). This supports the idea that the PM localized AVP1 and PPᵢ homeostasis are crucial in the Arabidopsis phloem, an apoplastic loading species (Pizzio et al., 2015).

In lieu of the complementary set of evidence from apoplasically loading plants,
we hypothesized that the PM localization of H⁺-PPase at the SE-CC complexes in rice source leaf minor veins would support the apoplastic loading mechanism proposed by Braun et al. (2014), while a vacuolar localization of H⁺-PPase would support the Revised diffusion model (Braun et al., 2014, Eom et al., 2012). We discounted the idea that rice harbors a polymer trapping mechanism on the basis that neither we, nor previous anatomical studies found specialized intermediary cells in the rice phloem (Chonan et al., 1984b, Kaneko et al., 1980). OVP-specific immunogold labeling on cryofixed rice source leaves revealed a predominantly PM localization at the SE-CC complexes (Fig. 8; Fig. S1). This result was deemed consistent with rice using an apoplastic loading mechanism.

Apoplastic loading in rice should theoretically also entail a key role for proton sucrose co-transporters (OsSUTs) in the phloem. Five SUT paralogs have been heretofore identified in the rice genome (Aoki et al., 2003), wherein OsSUT1 was found to be the most predominantly expressed form in rice tissues (Sun et al., 2012), with conspicuous localization, at both the transcript and protein levels, in the SE-CCs of leaf blades and sheaths (Matsukura et al., 2000, Scofield et al., 2007). However, both knock-down and knock-out mutants of PM-localized OsSUT1 displayed no significant phenotypic changes that would be typically associated with deficiencies in phloem loading (Ishimaru et al., 2001, Scofield et al., 2002, Eom et al., 2012). Given the gene redundancy of OsSUTs, this phenotypic discrepancy could instead be explained by the compensatory effect(s) of other OsSUTs, especially OsSUT5 (Braun et al., 2014, Sun et al., 2010). In addition, the rice genome also contains SWEET sucrose effluxer family of ~21 paralogs (Chen et al., 2010) that would be unnecessary if rice used a symplasmic loading mechanism (Braun et
The direct implication of our aforementioned interpretation that rice is an apoplastic loader is also that the SE-CC complexes in rice source leaf minor veins harbor an ATP-conserving, PP$_i$-cycling, SUS-dependent pathway. It has been long known that the energy status of the CCs in apoplastic loaders like Ricinus is primarily regulated by the reversible throughput of Suc into UDP-Glucose and fructose – a reaction catalyzed by SUS (Geigenberger and Stitt, 1993, Geigenberger et al., 1993). As expected, SUS was immunolocalized to the phloem in the leaves of known apoplastic loaders, maize and Arabidopsis (Fig. 9 A & E). The tissue-specific transcriptomic profiles of the six SUS paralogs in rice have also been previously characterized, and of particular interest is the finding that the SUS2 paralog was ubiquitously expressed including the source leaf blades (Hirose et al., 2008).

By contrast, the mRNA expression profile of three SUS paralogs (PtSUS1-3) in a known symplasmic loader, poplar (Zhang et al., 2014), revealed that these genes were primarily expressed in the mature xylem while their expression in the phloem was minimal (Zhang et al., 2011). In accord, immunohistochemical localization of SUS in Populus fremontii source leaves showed a distinct localization in the xylem, but visibly less so in the phloem (Fig. 9 G), especially when compared to Arabidopsis, rice, and maize (Fig. 9 A – F). Interestingly, transgenic poplar engineered to constitutively overexpress H$^+$-PPase ortholog (PtVP1.1) showed no significant increase in shoot and root biomass when grown under normal conditions (Yang et al., 2015). This is crucial because a direct prediction of the model proposed by Gaxiola et al. (2012) is that the up-regulation of PM localized H$^+$-PPases energizes the SUS-based pathway of respiration.
resulting in increased photosynthate flux and partitioning and, in turn, increased shoot and root biomass in apoplastic loaders like Arabidopsis (Gaxiola et al., 2012). No gain in biomass in PtVP1.1 overexpressing poplar suggests that non-polymer trapping, passive symplasmic loaders like poplar do not shelter a SE-CC specific SUS pathway.

This led us to investigate whether SUS was present in the SE-CC complexes of rice source leaves, and upon immunogold labeling; SUS was parietally localized in proximity to the PM of the SE-CC complexes (Fig. 9 J-L). This result conferred support to the notion that the PM localized OVPs function in empowering the SUS based Suc import into the SE-CC complexes in rice source leaves as well, and as such is consistent with the hypothesis that rice, like other monocot grasses, employs an apoplastic Suc loading strategy (Braun et al., 2014). Nevertheless, the evidence presented here is decidedly correlative, and a confirmation of apoplastic loading mechanism in rice would require further experiments including, but not limited to, p-Chloromercuribenzenesulfonic acid (PCMBS) assay (Giaquinta, 1979), and generating rice plants with phloem-specific knockdown of OVPs, overexpression of cell wall invertase, and phloem-specific overexpression of soluble pyrophosphatase.
Figure 6: Light micrographs showing correlative immunohistochemical localization of AVP1 and OVPs in comparable tissues of Arabidopsis (A-F) and rice (G-L), respectively.

(A) Transverse section through the shoot apex of 15-day old Arabidopsis seedling showing conspicuous localization of AVP1 in the shoot apical meristem (white arrowhead), leaf primordia (black arrowhead), and vascular tissues of the petiole (arrows). (B) Transverse section of Arabidopsis seedling showing AVP1 localization in the upper hypocotyl region where the vascular cambium (arrow) converges. (C) Cross-section through Arabidopsis inflorescent stem showing distinct AVP1 localization in the vascular tissues (arrows). (D) Transverse section through Arabidopsis source leaf showing AVP1 localization in the abaxial phloem of the mid-vein (p; arrow) adjoining the central xylem (x). (E) Longitudinal section through the flower of Arabidopsis showing AVP1 localization in the ovules. An egg cell (ec) is labeled. (F) Differential interference (DIC) micrograph showing AVP1 localization in the pollen grains of Arabidopsis. (G) Transverse section through the rice shoot showing the localization of OVPs in the shoot apical meristem (asterisk), and the leaf sheath primordia (p1 & p2). (H) Transverse section through the root-shoot junction in rice showing distinct localization of OVPs in the vascular tissues, and root primordium (arrow). (I) Cross-section through rice leaf sheaths showing distinct OVP localization in the vascular tissue of leaf sheaths. Younger leaf sheaths (p1, p2, and p3) display a stronger signal compared to older leaf sheaths (p4 & p5). (J) Transverse section through a rice source leaf showing OVP localization in the phloem tissue (p; arrow). Note the lack of signal in the bundle sheath cells. (K) Longitudinal section through a rice flower showing a prominent OVP localization in the embryo sac (es) and the growing embryo (e) that the embryo sac encapsulates. (L) DIC micrograph showing OVP localization in a rice pollen grain. (M, N, and O) Phase-contrast micrographs of representative negative controls using pre-immune sera for Arabidopsis (M), and rice (N & O). SAMs in (M & N) are marked with an asterisk, while leaf primordium is labeled with an arrow. Phloem (p) is marked with an arrow in (O). Scale bars: (A – D): 100 µm; (E – I): 30 µm; J: 20 µm; (K – L): 30 µm; (M): 100 µm; (N): 20 µm; (O): 30 µm. p = phloem; x = xylem; ec = egg cell; p1 – p5 = leaf sheaths; e = embryo; es = embryo sac; v = vascular tissue.
Figure 1: (A) Cross-section of a plant cell showing the vacuole (V). (B) Enlarged view of the vacuole showing the membrane-bound structures. (C) High-magnification image of the vacuole with gold particles (GPs) localized within the vacuole (V). (D) Immunogold stain highlighting the distribution of gold particles (GPs) around the plasma membrane (PM).

Legend:
- Vacuole (V)
- Membrane-bound structures (mx)
- Gold Particles (GPs)
- Plasma Membrane (PM)

Graph J: Comparison of gold particle distribution between vacuoles and plasma membrane (PM) using immunolabeling. The bar graph shows significantly higher gold particle density in vacuoles compared to the plasma membrane.

Note: The bar graph includes error bars indicating the standard deviation.
Figure 7: Immunolocalization of OVPs in the rice roots at both light (A–C) and electron microscopic (E – G) levels.

(A) Transverse section through a rice root showing OVP localization in the lateral root primordium (arrow) and the vascular cylinder. (B & C) Higher magnification light micrographs of (A) showing OVP localization in the growing lateral root primordium (B) and the phloem tissue in the vascular cylinder (arrowhead; C). The tissue sections were counterstained with Fast Green, and the OVP signal appears as a brown precipitate. (D): Pre-immune serum negative control. (E) Electron micrograph of root apical meristematic cells in rice immunogold labeled against OVPs. (F) A higher magnification image of the boxed region in (E), and (G), show that the OVPs are predominantly localized at the vacuoles (arrows), as further evidenced in (J). (H and I) Representative negative controls using pre-immune sera. (J) Using Students’ t-test, it was found that OVPs were significantly more prevalent at the vacuoles ($M=2.17, SD=0.98$) than at the PM ($M = 0.14, SD = 0.24$); $t(15) = 8.52, p = 3.9 \times 10^{-7}$. Scale bars: (A): 100 µm; (B – D): 20 µm; (E): 2 µm; (F): 500 nm; (G) 1 µm; (H): 5 µm; (I): 1 µm. mx = metaxylem, m = mitochondrion; n = nucleus.
Figure 8: Immunogold labeling of OVPs in the sieve element companion cell (SE-CC) complexes of rice source leaf minor veins through both longitudinal and transverse sections.

(A, B, and C) Serial section electron micrographs showing longitudinal section through the SE-CCs. (A1, B1, and C1) Higher magnification micrographs of the boxed regions from A, B, and C respectively, showing 10nm gold particles decorating the plasma membrane (PM) of both SEs and CCs (arrows). (A2, B2, and C2) Higher magnification insets of the boxed regions in A1, B1, and C1, respectively, showing the OVP-specific 10 nm gold particle decorations at the PM (arrows). (D & E) Transverse section through the rice source leaf minor vein corroborates immunogold labeling from (A1-C1; A2-C2), with distinct OVP localization at the plasma membrane of both SEs (E) and CCs (D). (D1 and E1) Higher magnification insets of the boxed regions in (D) and (E) respectively, showing immunogold labeling of the PM. (F) Quantification of gold labeling comparing the number of 10 nm gold particles at either the PM of SEs or CCs, or the vacuoles (V) of CCs. Statistically significant differences were found in the distribution of OPV-specific gold label per µm of SE PM, CC PM, and CC V ($F_{2,53} = 26.13; p < 0.0001$). It was inferred from post-hoc pairwise comparisons using Tukey HSD that the mean number of gold particles per µm of SE PM ($M = 6.53, SD = 2.49$) was not significantly different from the mean number of gold particles per µm of CC PM ($M = 5.43, SD = 3.64$), while the mean number of gold particles per µm of CC PM was significantly different from the number of gold particles per µm of CC V ($M = 0.78, SD = 0.72$). Scale bars: (A – C): 5 µm; (A1 – C1 & D – E): 1 µm; (A2 – C2; D1 & E1): 250 nm. cc = companion cell; se = sieve element.
Figure 9: Immunohistochemical localization of Sucrose Synthase (SUS) in maize, rice, Arabidopsis, and poplar source leaves, and immunogold labeling of SUS in the SE-CC complexes of rice source leaf minor veins.

(A, C, E & G) Transverse sections through source leaves of maize (A), rice (C), and Arabidopsis (E) showing distinct localization of SUS in the phloem tissue (arrows). By contrast, a known symplasmic loader, poplar (Populus fremontii) has distinct SUS localization in the xylem (arrows). (B, D, F & H) Corresponding light micrographs of representative pre-immune serum controls are shown. (I) Western blots showing the cross-reactivity of polyclonal sera raised against SH1 and SUS2 maize proteins tested against total protein extracts from maize (Zm; positive control), rice (Os), Populus fremontii (Pf), Physcomitrella patens (Pp), and
*Arabidopsis thaliana* (*At*). The bands representing homo- and heterotetramers of SUS in *Zm* with varying proportions of SH1 (upper bands) and SUS1 (lower bands) are seen (arrowheads). The molecular weight (MW) is marked and the two bands seen correspond to 102 kDa and 60 kDa. (J – L): Immunogold localization of SUS in the SE-CC complexes of rice source leaves. The SUS-specific 10 nm gold label is parietally localized in proximity to the PM, as seen in the higher magnification micrograph in (L) that corresponds to the boxed region in (K) (arrows). (M – O): Pre-immune serum negative controls. Boxed region in (N) shown in (O). Scale bars: (A – H): 100 μm; (J): 5 μm; (K): 1 μm; (L): 100 nm; (M): 1 μm; (N): 2 μm; (O): 250 nm. cc = companion cell; se = sieve element.
Figure S1: Negative controls for the immunogold labeling of OPVs in SE-CC complexes of rice source leaf minor veins. (A) Montage of a companion cell (cc), and (B) sieve element (se) in longitudinal sections showing no immunogold labeling. (C & D) Electron micrographs through the transverse sections of SE and CC, showing no immunogold labeling. Scale bars: (A & B): 1 µm; (C & D): 2 µm. se = sieve element, cc = companion cell.
Table S1: Comparative percentage identities of the amino acid sequences of various orthologs of *Zea mays* (Zm) Sucrose synthases (ZmSUS1 and ZmSH1) in *Oryza sativa* (Os), *Arabidopsis thaliana* (At), *Populus trichocarpa* (Pt), and *Physcomitrella patens* (Pp). BlastP searches were performed in [www.gramene.org](http://www.gramene.org).

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Abstract

*Physcomitrella patens* has emerged as a model moss system to investigate the evolution of various plant characters in early land plant lineages. In higher plants, proton pyrophosphatase (H\(^+\)-PPase) is conspicuously localized at either the actively dividing tissues (like meristems), or the vascular tissues. Given that bryophytes are non-vascular plants, and that H\(^+\)-PPase is a highly evolutionarily conserved protein, we hypothesize that the localization of H\(^+\)-PPases in both the gametophytic and sporophytic generations of *Physcomitrella* would indicate whether the roles ascribed to this protein in higher plants like Arabidopsis are in fact conserved in this evolutionarily distant land plant. Our ultrastructural observations reveal that *Physcomitrella* is an endohydric moss with the leafy gametophyte comprised of a central axial water-conducting strand surrounded by putative food-conducting cells. The end walls of the food-conducting cells are profusely decorated with plasmodesmata, indicating a symplasmic mechanism of sucrose transport. *In situ* immunolocalization of H\(^+\)-PPase in the gametophytic and sporophytic generations revealed that this protein is localized at actively dividing cells, and intriguingly in the transfer cells lining the gametophyte-sporophyte junction. Decades old literature suggests that the transfer of sucrose from the photoautotrophic gametophyte to its nutritionally dependent sporophyte via the transfer cells in mosses uses an invertase-based pathway rather than an ATP-conserving sucrose synthase-based one. We hypothesized that the membrane-specificity of H\(^+\)-PPase, and the localization of sucrose synthase in transfer cells would be
consistent with the latter mechanism. In agreement with this idea, we found dual plasma membrane and vacuolar localization of $\text{H}^+-\text{PPases}$, while sucrose synthase was localized close to the plasma membrane in high-pressure frozen/freeze-substituted transfer cells.

Introduction

The advent of colonization and the subsequent diversification of the early ancestral lineages of Embryophyta (Kenrick and Crane, 1997, Qiu et al., 2006) elicited a period of unprecedented morphological innovations among early land plants. Phylum Bryophyta forms the basal land plant lineage, possesses simple morphology, and represents the ancestral land plant archetype (Renzaglia et al., 2000). Given that these contemporary bryophytes represent distant descendants of early land plants from which modern tracheophytes were also derived (Hébant, 1977), these plants offer a unique prospect to examine the early morphological adaptations requisite for existence on land (Renzaglia et al., 2000). The evolution of vascular tissues is considered one of the most important morphological innovations (Prigge and Bezanilla, 2010).

Among the species constituting mosses, Physcomitrella patens Hedw. belongs to Funariaceae, and was the first bryophyte to have its complete genome sequenced (Rensing et al., 2008). The availability of the complete genome sequence, and a broad repertoire of molecular tools opened a new avenue in comparative genomic (Rensing et al., 2008), and reverse genetic studies using Physcomitrella (Kammerer and Cove, 1996, Schaefer and Zryd, 1997, Strepp et al., 1998). This is particularly relevant because Physcomitrella, as the only representative bryophyte to have its complete genome sequenced, lies at the evolutionary interface between aquatic green algae and vascular plants (Rensing et al., 2008). As such, Physcomitrella is well suited to allow comparisons
that can yield phylogenetic insights into the genomic changes associated with the
occupation of land (Rensing et al., 2008).

Like all mosses, Physcomitrella gametophore lacks true vascular tissue. The
gametophore stem however possesses a relatively complex stem anatomy with a central
water-conducting strand composed of xylem analogs called hydroids (Sakakibara et al.,
2003). Whether the stem of Physcomitrella gametophore also possesses food-conducting
cells is as of yet unknown. Nevertheless, endohydric mosses like Physcomitrella often
also possess specialized food-conducting cells, and these cells have been interpreted to
resemble phloem sieve cells (Behnke, 1975, Behnke and Sjolund, 2012). These food-
conducting cells, also referred to as leptoids (Behnke, 1975, Hebant, 1970, Hébant,
1975), are considered analogs of sieve elements (Ligrone et al., 2000), and have been
postulated to form a symplasmic conduit for the movement of sucrose in the gametophore
(Raven, 2003, Reinhart and Thomas, 1981). Intriguingly, the Physcomitrella genome
harbors orthologs of H\(^+\)-coupled sucrose uptake transporters (SUTs), and given that the
mode of photosynthate transport in mosses is likely to be symplasmic (Raven, 2003), it
has been argued that the SUTs in moss gametophores in fact act to recover sucrose leaked
into the apoplasm (Reinders et al., 2014).

This point of view, however, only accounts for the dominant haploid
gametophytic generation of mosses and ignores the transient diploid sporophytic
generation. This is especially important because it is widely maintained that the largely
heterotrophic sporophyte is nutritionally dependent on the photosynthetic gametophyte
(Ligrone et al., 1993, Ligrone and Gambardella, 1988). This functional dependence is
clearly mirrored in the morphological specializations of the transfer cells that lie at the
interface between the sporophyte and the gametophyte (Ligrone et al., 1993, Ligrone and Gambardella, 1988). These transfer cells are characterized by their extensive cell wall invaginations, dense ribosome-rich cytoplasm, and abundant mitochondria (Browning and Gunning, 1979a, Lal and Chauhan, 1981, Ligrone et al., 1993, Pate and Gunning, 1972). Furthermore, in Physcomitrella, the sporophyte-gametophyte junction presents a diffusion barrier, i.e. the metabolites have to cross an apoplastic boundary (Courtice et al., 1978). This implies that the acropetal translocation of sucrose across the gametophyte-sporophyte interface is likely to involve an apoplastic loading mechanism, with the metabolic energy for this directional movement of sucrose being supplied by the numerous transfer cell mitochondria. Having said this however, the literature is less certain on the mechanism of sucrose movement from the gametophyte to the sporophyte.

The transfer cells are characterized by intense enzymatic activity (Chauhan and Lal, 1982, Hebant and Suire, 1974), and corollarily, Browning and Gunning (1979) showed using excised haustoria of Funaria hygrometrica that both externally applied glucose and sucrose influx into the haustoria was inhibited by metabolic uncouplers (Browning and Gunning, 1979b, Browning and Gunning, 1979c). Conversely, Renault et al. (1992) showed that there is a so-called non-continuity of sucrose at the gametophyte-sporophyte junction, with hexoses being the predominant sugars at this interface in Polytrichum formosum (Renault et al., 1992). In fact, Renault et al. (1989, 1992) claimed that the proton motive force established at the plasma membrane of transfer cells in the haustorium does not play a major role in sugar uptake, but rather in amino acid uptake (Renault et al., 1992, Renault et al., 1989). Their physiological studies instead pointed to a mechanism in which cell wall invertases hydrolyze sucrose into hexoses, with these
hexoses being reconverted to sucrose upon absorption into the haustorium (Renault et al., 1992). In a preceding study, the same authors showed that, at maturity, the apoplastic boundary between the sporophyte and gametophyte in Polytrichum is acidic with a pH of 4.30 (Renault et al., 1989).

Under the scenario proposed by Renault et al. (1989, 1992), the mechanism of sucrose transport from the gametophyte to the sporophyte would be independent of the activity of sucrose synthase in the transfer cells (Renault et al., 1992, Renault et al., 1989). However, a repertoire of enzyme assays in the gametophytic and sporophytic tissues of several mosses revealed substantial activities of all the enzymes associated with the sucrose synthase pathway (Galloway and Black, 1989). Additionally, given that the invertase pathway of sucrose metabolism is twice as costly in terms of ATP consumption compared to the pyrophosphate (PPi)-dependent sucrose synthase pathway (Black et al., 1987), it is plausible that mosses use the more energy-efficient mode of sucrose synthase metabolism to nourish the sporophytic tissue. This, in turn, would entail that PPi homeostasis is crucial in the proper functioning of moss transfer cells, analogous to what is observed in the phloem of apoplasically loading higher plants (Pizzio et al., 2015).

That a balanced turnover of PPi in the phloem of apoplasically loading plants is critical for sucrose flux has been known for quite some time (Lerchl et al., 1995), but the supply source of this PPi had remained enigmatic. Recently, a set of genetic, immunohistochemical, and physiological evidence gave credence to the hypothesis that plasma membrane localized H⁺-PPases act reversibly to synthesize rather than hydrolyze PPi in the Arabidopsis phloem (Pizzio et al., 2015). Given the ancient evolutionary origin of H⁺-PPases, their ubiquity in the plant kingdom, their distinct localization at the vascular

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and actively dividing tissues of higher plants, their role in cellular PPi homeostasis, and their importance in sucrose phloem loading and photosynthate partitioning, we deemed the use of a non-vascular model moss Physcomitrella to explore the function of $H^+$-PPases plausible. Were $H^+$-PPases co-opted into a novel functional role in the vascular tissues of higher plants? Or are there evolutionarily conserved roles of this protein that transcend the phylogenetic diversity of land plants? We posit that a complete tissue- and generation-specific in situ localization study of $H^+$-PPases in Physcomitrella at light and electron microscopic levels would suggest whether the functional role of $H^+$-PPase throughout the evolutionary history of land plants has remained the same, or if the role of this protein has changed with the evolution of vascular land plants.

Immunohistochemical survey of the non-vascular Physcomitrella gametophytes and sporophytes revealed the prominent expression of $H^+$-PPases in either the actively dividing cells, and intriguingly, at the transfer cells lining the haustorial junction. While the former finding was consistent with the traditional notion of $H^+$-PPase activity as a hydrolase, we posited that the latter finding showed a remarkable parallel with phloem loading system in higher plants. Concordantly, we hypothesized that the membrane-specific localization of $H^+$-PPases in the transfer cells would help us understand whether the transfer of sucrose from the gametophyte to the sporophyte followed a sucrose synthase or an invertase-based pathway. Our finding that $H^+$-PPases are localized to both the plasma membrane and vacuoles in the transfer cells was deemed consistent with the sucrose synthase based pathway, with further support gained by sucrose synthase localization in the transfer cells.
Materials and Methods

Culture conditions

*Physcomitrella patens* Hedw. (Buch & Schimp) gametophores were grown on solid BCD media in magenta boxes or petri plates in a growth chamber with 16 hours light and 8 hours dark regime (Cove et al., 2009). Sporophyte growth was induced by growing 4-5 weeks old mature gametophores in a chamber held at 15°C under 8 hours of light and 16 hours of darkness.

Light microscopy

Individual mature Physcomitrella gametophytes were imaged using Olympus SZX7 stereomicroscope (Olympus Corporation, Tokyo, Japan) equipped with Infinity 2 camera system and Infinity Capture imaging software (Lumenera Corporation, Ottawa, Canada).

Resin-embedded naked stems were prepared for transmission electron microscopy (described below) and cut into semi-thin (250 – 500 nm) sections, stained with toluidine blue, and imaged using Nikon Eclipse TE300 inverted microscope (Nikon Corporation, Tokyo, Japan) equipped with Olympus DP26 camera and cellSens digital imaging software (Olympus Corporation, Tokyo, Japan).

Transmission electron microscopy

Individual mature Physcomitrella gametophytes were fixed in 4.0% glutaraldehyde and 1.0% formaldehyde in 0.05M sodium phosphate buffer, pH 7.2 for 2 hours at room temperature. The leaves were gently separated from several stems under a stereomicroscope while still in the fixative fluid, and both leafy and naked stems were
dissected into ~2 mm pieces. The leaves and stems were further fixed in fresh, buffered fixative for 2 hours at room temperature, post-fixed with 1% osmium tetroxide (OsO₄) for 2 hours at room temperature, and en bloc stained with 0.2% uranyl acetate overnight at 4°C. The tissues were then serially dehydrated in acetone, gradually infiltrated with Spurr’s resin over 5 – 7 days, and flat-embedded in molds or on Teflon-coated glass slides in a 60°C oven. The tissues were embedded in various orientations to facilitate longitudinal and transverse sectioning. The resin blocks were trimmed, cut into 70 to 100nm sections with a diamond knife (Diatome Inc., Hatfield, PA) in a Leica Ultracut R microtome (Leica, Vienna, Austria), mounted onto formvar-coated copper slot or 100-mesh grids (Electron Microscopy Sciences, Washington, PA), and post-stained with 2% ethanolic uranyl acetate and Sato’s lead citrate. The sections were then imaged at 80 kV using JEOL 1200EX transmission electron microscope (JEOL Ltd., Tokyo, Japan) equipped with SIA model L3C bottom-mount air-cooled CCD camera (Scientific Instruments and Applications, Inc., Duluth, GA) and Maxim DL5 imaging software (Diffraction Ltd., Ottawa, Canada). All the fixative chemicals were purchased from Electron Microscopy Sciences (Washington, PA).

**Scanning electron microscopy**

Individual gametophores were fixed with 4% glutaraldehyde in 0.05M sodium phosphate buffer, pH 7.2 for 4 – 6 hours at room temperature, post-fixed in 1% OsO₄ for 2 hours at room temperature, serially dehydrated with acetone, and critical point dried (Balzers Union, Principé de Liechtenstein). If the tissues were to be dissected, the critically dried samples were cut in a desirable fashion using a fresh double-edged razor blade under a stereomicroscope. The samples were then mounted onto aluminum stubs, gold-palladium
sputter-coated (Technics, Alexandria, Virginia), and imaged at 15 kV using JEOL JSM-6300 scanning electron microscope (JEOL Ltd., Tokyo, Japan) equipped with IXRF digital acquisition system (IXRF Systems, Inc., Austin, TX). All the fixative chemicals were purchased from Electron Microscopy Sciences (Washington, PA).

The scanning and transmission electron micrographs (SEMs and TEMs respectively) were then processed using Adobe Photoshop Creative Suite 6 software (Adobe Systems Incorporated, San Jose, CA). Linear adjustments (brightness, contrast, and levels) were made to the entire images to enhance clarity.

**Immunohistochemistry**

The tissues excised were cut into small pieces and immediately immersed in at least 20-fold excess volume of FAA (10% v/v 37% formaldehyde : 5% v/v acetic acid : 50% v/v 200-proof ethanol : 35% v/v water) fixative, and placed under a gentle vacuum for 10 – 15 minutes at room temperature. The fixation was then allowed to continue overnight at 4°C. Following this, the tissues were dehydrated in a graded ethanol series, exchanged with xylene to clear the tissues, and infiltrated with paraplast. The tissues were then embedded in paraplast in desired orientations. Using a Leica RM2155 rotary microtome (Leica Microsystems, Germany; www.leica-microsystems.com), the tissue blocks were cut into 10µm thick sections, and mounted on poly-L-Lysine coated slides. The tissues were then deparaffinized, rehydrated, and exposed to an antigen retrieval buffer (Biogenex, USA; www.biogenex.com) in a 65°C water bath for 20 minutes. After washing in water, the endogenous peroxidase activity was quenched using 3% (v/v) H₂O₂. The tissues were washed with Phosphate Buffered Saline, pH 7.2 with 0.01% (v/v) Tween-20 (PBST), and blocked with 1% (w/v) casein in PBST for 30 minutes at room
temperature. After washing with PBST three times, the tissues were exposed to anti-AVP1 or anti-SUS rabbit polyclonal antibodies at 1:1000 dilutions for 1 hour at room temperature. Negative controls were concurrently performed using pre-immune serum at 1:1000 dilutions in PBST. After three washes in PBST, the signal was developed using the SignalStain® Boost IHC HRP anti-rabbit detection system (Cell Signaling Technology, USA; www.cellsignal.com), following manufacturer’s instructions. To develop the signal when mouse anti-serum was used, DAKO EnVISION anti-rabbit anti-mouse system was used according to the manufacturer’s instructions (DAKO Inc.; www.dako.com). The tissues were then run through a dehydrating ethanol series, and were permanently mounted. Images were acquired with Zeiss Axioskop light microscope (Leica Microsystems, Germany) equipped with 10X Achromplan 0.25 NA Zeiss, 40X Plan Neo-Fluar 0.75 NA Zeiss, and 100X 1.30 NA Oil Plan Neo-Fluar objective lenses, phase and differential interference contrast optics, Olympus DP72 camera system, and Olympus cellSens® imaging software.

**Total protein extraction**

Approximately 300 mg of Physcomitrella gametophores were immediately ground into fine powder with liquid N₂, transferred in ~200 µL aliquots to screw-cap eppendorf tubes. To each tube, 1mL of 10% (v/v) Trichloroacetic acid in -20°C acetone was added, and proteins were allowed to precipitate overnight at -20°C. The samples were centrifuged at 10,000xg for 30 minutes at 4°C, followed by removal of the supernatant. The pellet was washed with -20°C acetone containing 0.07% β-mercaptoethanol, vortexed, and centrifuged at 10,000xg for 10 minutes at 4°C. The washing, vortexing, and centrifugation steps were repeated four more times. After the
final centrifugation step, the supernatant was removed and the pellet dried in a tabletop vacuum for ~30 minutes. Total protein was solubilized by adding Laemmli’s buffer to the pellet. Prior to storage at -80°C the mixture was vortexed, and centrifuged at 5,000xg for 5 minutes at 4°C three times.

**Western Blot**

The solubilized protein extracts from were then run on a 10% SDS-polyacrylamide gel, transferred to a PVDF membrane, blocked with 5% (w/v) non-fat milk in Tris buffered saline with 0.01% Tween-20 (TBST), and probed with H+-PPase-specific polyclonal sera at 1:1000 dilution overnight at 4°C. After washing in TBST, the membrane was developed using Bio-rad Alkaline Phosphatase Immun-Blot® Colorimetric Assay kit (Bio-rad Inc., USA; www.bio-rad.com) according to the manufacturer’s instructions.

**Immunogold labeling and transmission electron microscopy**

High-pressure freezing (HPF) and freeze-substitution (FS): Haustoria from gametophytes with fully-grown sporophytes were excised under a drop of 1-hexadecene, and quickly loaded into interlocking brass planchettes, filled with 1-hexadecene, and subjected to HPF in BalTec HPM 010 high-pressure freezer. The cryofixed haustoria were then transferred to a pre-chilled -80°C freeze-substitution (FS) cocktail containing anhydrous acetone and 0.2% (w/v) non-methanolic uranyl acetate. The FS was allowed to continue for 96 hours at -80°C, after which the FS solution was allowed to warm to 4°C over a period of 12 hours. The anhydrous acetone was exchanged with anhydrous ethanol several times, and the tissues were gradually infiltrated with medium grade London Resin (LR) White (Electron Microscopy Sciences, USA): 25% (v/v) LR White (12 hours), 50%
(v/v) LR White (12 hours), 75% (v/v) resin (12 hours), 100% LR White (12 hours), 100% LR White (12 hours), 100% LR White (12 hours). The samples were transferred to gelatin capsules, filled with fresh LR White and polymerized for 24 hours at 50°C in longitudinal orientation. The polymerized resin blocks were then trimmed, ultrathin sectioned to gold reflectance (90 – 100 nm) using a Leica Ultracut R ultramicrotome (Leica Microsystems, Germany), and mounted on formvar-coated nickel slot grids.

For immunogold labeling, the nickel grids containing the sections were floated on drops of blocking solution (1% w/v casein in PBST) for 1 hour at room temperature. The grids were then sequentially transferred to drops of PBST before being floated on drops of anti-AVP1 or anti-SUS polyclonal antibodies diluted 10-fold in the blocking solution. The antibody incubation step was performed at 4°C overnight. Following this, the grids were washed five times with PBST, and then transferred to drops of anti-rabbit IgG conjugated to either Aurion Ultrasmall 0.8 nm (H⁺-PPase) or 10 nm (SUS) gold particles (Electron Microscopy Sciences, USA) diluted 25- to 50-fold in the blocking solution. The grids were further washed in PBST five times, and rinsed with double-distilled water three times, before being air-dried overnight. Silver enhancement of Aurion Ultrasmall immunogold labeled sections was performed for 3 minutes in a dark room by using HQ SILVER™ Enhancement kit (Nanoprobe Inc.) following the manufacturer’s instructions. The sections were then imaged with a JEOL 1200EX transmission electron microscope (JEOL Corp., Japan) operating at 80 KV without post-staining. Linear image adjustments like contrast and brightness were adjusted on grayscale electron micrographs using Adobe Photoshop CS6 (Adobe Inc., USA). All the images were compiled into figure panels using Adobe Illustrator CS6 (Adobe Inc., USA).
**Molecular Phylogenetic analysis**

The evolutionary history was inferred by using the Maximum Likelihood method based on the JTT matrix-based model (Jones et al., 1992). The bootstrap consensus tree inferred from 1000 replicates is taken to represent the evolutionary history of the taxa analyzed (Felsenstein, 1985). Branches corresponding to partitions reproduced in less than 50% bootstrap replicates are collapsed. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches (Felsenstein, 1985). Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using a JTT model, and then selecting the topology with superior log likelihood value. A discrete Gamma distribution was used to model evolutionary rate differences among sites (5 categories (+G, parameter = 1.7951)). The rate variation model allowed for some sites to be evolutionarily invariable ([+I], 0.0000% sites). The analysis involved 6 amino acid sequences. There were a total of 846 positions in the final dataset. Evolutionary analyses were conducted in MEGA5 (Tamura et al., 2011).
Results

Morphology of Physcomitrella gametophyte and sporophyte

Although mosses undergo an alternation of generations like all land plants, they differ from vascular plants in that the haploid gametophytic stage, rather than the diploid sporophytic stage, is the dominant phase of the life cycle. In Physcomitrella, the gametophores (Fig. 12 A) emerge from protonemal filaments, and are affixed to the substrate by basal filamentous rhizoids. There is a single apical meristematic cell that gives rise to young phyllids (Fig. 12 B & C), as the gametophore undergoes vertical growth. At the apex of the monoecious gametophyte, archegonia containing the female gamete or egg (Fig. 12 D) and antheridia containing male gametes, or sperm (not shown) form. Upon fertilization, an apical sporophyte (Fig. 12 E & F) is formed within each archegonium. The diploid tissue in this structure, the capsule undergoes meiosis to form numerous haploid spheroidal spores, with a characteristic exine layer (Fig. 12 G & H).

The Physcomitrella stem is an anatomically complex structure, and possesses a well-developed central column of water-conducting cells or hydroids (Fig. 12 I – K). This observation corroborates the finding previously reported (Sakakibara et al., 2003). These cells are thin-walled, and are devoid of living protoplasm at maturity, presumably due to programmed cytoplasmic lysis (Fig. 12 K). The midrib or costa of leaves, in cross-section, reveals that the central region of the midrib is mostly comprised of thick-walled parenchyma or stereid cells (Fig. 12 L). These stereid cells have narrow lumen, and do not retain a living protoplast at maturity (Fig. 12 M). Given the thickness of their cell walls, it seems plausible that the function of the stereid cells is limited to the mechanical support of the leaf. The thick walls of stereid cells show loose, fibrillar fine structure, and
their function in internal conduction of water can not be ruled out (Fig. 12 M). In the leaf
costa, thin-walled hydroids lie abaxial to the stereids, and are mostly empty (Fig. 12 L).
The leaves penetrate the stem in the cortical layer, just below the epidermis, forming a
leaf trace (Fig. 12 N). The presence of small, thick-walled stereid cells, and thin-walled
empty hydroids characterize the leaf trace (Fig. 12 N). These cells are presumably
contiguous with their respective counterparts in the leaf midrib. The cross-section of
Physcomitrella leaf costa shows a putative food-conducting cell, with plasmodesmatal
connections to the laterally adjoining cells (Fig. 12 O). The stems of Physcomitrella have
elongate putative food-conducting cells with oblique end walls that are profusely
decorated with plasmodesmata (Fig. 12 P & Q). The presence of plasmodesmata
suggests that the mechanism of sucrose translocation via these food-conducting cells is
symplasmic in nature (Raven, 2003).

Phylogenetic analysis of H⁺-PPase orthologs in Physcomitrella

A BlastP search using Arabidopsis type I H⁺-PPase (AVP1) protein sequence as the query, revealed that there are four putative orthologs annotated in the Physcomitrella genome: PP1s346_33V6 (420 aa), PP1s445_15V6 (753 aa), PP1s28_200V6 (806 aa), and PP1s105_42V6.2 (799 aa), hereafter abbreviated as PP1a, PP1b, PP2a, and PP2b respectively. Amino acid sequence alignment using MUSCLE revealed that the latter two shared greater homology with each other than with the former two (Table 1). Using a
maximum likelihood method based on JTT matrix-based model, the molecular
phylogenetic history of these paralogs was inferred using MEGA5. The resulting
bootstrap consensus tree, using AVP1 and VR_HPPase (Vigna radiata H⁺-PPase)
orthologs as outgroups, indicated that the PP1a, PP1b, PP2a, and PP2b paralogs clustered
in two phylogenetically distinct clades (Fig. 13). VR_HPPase was also chosen because it is the only H^{+}-PPase whose crystal structure has been solved (Lin et al., 2012). Given that the Arabidopsis genome harbors two phylogenetically distinct clades of H^{+}-PPases, namely type I and type II, we posited that these two pairs of clustered proteins in Physcomitrella also segregated into type I and type II clades. Credence to this notion was provided by the higher amino acid sequence homology among AVP2, PP2a, and PP2b, than among AVP2, PP1a, and PP1b (Table 2). Conversely, AVP1 showed higher sequence homology with PP1a and PP1b, than with PP2a and PP2b (Table 2).

**Table 1**: Summary of the comparative amino acid sequence homology between pairs of H^{+}-PPase paralogs in Physcomitrella. The percentages of identical residues (%ID), positive residues (%Pos.), and gaps (%Gap) between all the paralog pair combinations are shown.

<table>
<thead>
<tr>
<th></th>
<th>PP1a</th>
<th>PP1b</th>
<th>PP2a</th>
<th>PP2b</th>
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<td>PP1a</td>
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<tr>
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<td>NA</td>
<td>97</td>
<td>98</td>
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<tr>
<td>PP1b</td>
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<td>%Gap</td>
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<tr>
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<td>98</td>
<td>0</td>
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<td>NA</td>
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<tr>
<td>PP2a</td>
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<td>%Gap</td>
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<tr>
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<td>10</td>
<td>41</td>
<td>56</td>
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<td>PP2b</td>
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<tr>
<td>33</td>
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<td>9</td>
<td>41</td>
<td>57</td>
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Table 2: Summary of comparative amino acid sequence homology between type I and type II Arabidopsis H⁺-PPase proteins, AVP1 and AVP2 respectively, and their H⁺-PPase orthologs PP1a, PP1b, PP2a, and PP2b in Physcomitrella. The percentages of identical residues (%ID), positive residues (%Pos.), and gaps (%Gap) are shown.

<table>
<thead>
<tr>
<th></th>
<th>AVP1</th>
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<td>88</td>
</tr>
</tbody>
</table>

Immunohistochemical localization of H⁺-PPases in Physcomitrella

H⁺-PPases are evolutionarily conserved proteins with a pyrophosphate-binding KAADVGADLVGKVE motif that is highly conserved among diverse organisms (Fig. 16) (Rea et al., 1992, Rea and Poole, 1985). The antibody used in this study were raised against this motif in a rabbit (Park et al., 2005), and recognize the H⁺-PPase specific bands when immunoblotted against the total protein extract from Physcomitrella (Fig. 13 N). At the light microscopy level, H⁺-PPases in the gametophytic generation of Physcomitrella were immunolocalized at the actively growing apical regions, including developing leaves called phyllids (Fig. 13 A). The proteins were also distinctly localized at sink reproductive tissues like the male antheridium (Fig. 13 B), egg cell nested inside the archegonium (Fig. 13 E & F), and the developing embryo (Fig. 13 E). Interestingly, the gametophyte stem (Fig. 13 C) and leaves (Fig. 13 C & D) had a relatively very weak signal. Pre-immune sera treated control, as expected, lacked any discernible signal (Fig. 13 G).
In the sporophytic generation, marked by the development of the sporangium, distinct immunohistochemical signal was observed at the sporophyte-gametophyte interface (Fig. 13 H – J). Specifically, localization of H⁺-PPases was evident at the transfer cells that characterize this filial junction (Fig. 13 I). Representative pre-immune and secondary antibody controls lacked any label (Fig. 13 K – M). A parallel immunohistochemical localization of H⁺-PPases using antibodies raised against the KAADVGADLVGKVE motif in mouse (Paez-Valencia et al., 2011) were also used, that further confirmed the transfer cell-specific localization of H⁺-PPases (Fig. 13 J).

Ultrastructure of the transfer cells

In bryophytes, the sporophyte-gametophyte junction called the haustorium, has long been known to harbor a band of transfer cells (Ligrone et al., 1993, Ligrone and Gambardella, 1988). The name of the cells itself implies their presumptive role in the ‘transfer’ of metabolites and nutrients. Light microscopic evidence for the existence of transfer cells at this interface in Physcomitrella exists (Xu et al., 2014), but electron microscopic evidence has been lacking. Since the gametophytic tissues were found not to be amenable to high-pressure freezing/freeze substitution (HPF/FS) protocol, we first analyzed the haustorium using a conventional chemical fixation regime. Using both tannic acid and osmium tetroxide, the distinctive cytological features of transfer cells were evident (Fig. 14 A – E). Saliently, the transfer cells had intricate cell wall invaginations with numerous mitochondria in their immediate vicinity – morphological attributes seen as directly facilitating the energy-intensive nature of nutrient transfer via an increase in the absorptive surface area and proximally located sources of primary energy currency ATP (Fig. 14 A & E). A large central nucleus, several vacuoles, and
plastids were also observed (Fig. 14 B). Given the cytology of these transfer cells, the likelihood that these cells translocate sucrose (an endogenous cryoprotectant), and the need for immunogold labeling of these cells, we cryofixed the excised haustorium samples using HPF/FS. As seen in Fig. 14 D – F, the ultrastructure of the transfer cells was well-preserved under this cryofixation regime.

**Silver-enhanced immunogold localization of H\(^+\)-PPases**

To determine the membrane-specificity of H\(^+\)-PPases in transfer cells, silver-enhanced immunogold labeling was performed on high-pressure frozen haustorial samples, and as seen in Fig. 14 G – I, both the plasma membrane and the vacuoles were profusely decorated with H\(^+\)-PPase-specific label. Negative controls performed with pre-immune sera and/or gold-conjugated secondary antibody alone, showed the specificity of the H\(^+\)-PPase label (Fig. 14 J – M).

**Polysaccharide and sucrose synthase localization in Physcomitrella sporophyte**

Complementary evidence was sought to determine where SUS was localized in the gametophytic and sporophytic tissues, and immunohistochemical localization of SUS at the light microscopy level showed that SUS was prominently localized at the actively dividing regions of the gametophore, and in the band of transfer cells lining the filial interface (Fig. 15 D – G). SUS-specific immunogold labeling revealed that SUS was localized, like in sieve element companion cells complexes in rice (Chapter 2), in close proximity of the transfer cell plasma membrane (Fig. 15 J – L). Histochemical staining using polysaccharide-specific periodic acid schiff’s reagent and starch-specific Lugol’s potassium iodide stain showed that the transfer cells are rich in polysaccharides and starch (Fig. 15 A – C).
Discussion

Like in all plants, sucrose (Suc) is the primary photosynthetic in mosses, and that Physcomitrella has the ability to use Suc as its carbon and energy source is shown by its ability to grow in the absence of light when supplied with exogenous Suc (Thelander et al., 2009). The Physcomitrella genome must therefore harbor all the genes required for Suc metabolism. In fact, two Suc biosynthetic enzymes, sucrose phosphate synthase (SPS) and sucrose-phosphate phosphatase (SPP), and Suc-degrading enzyme sucrose synthase (SUS) are indeed encoded in the Physcomitrella nuclear genome (Thelander et al., 2009).

The entry of carbon from Suc into cellular metabolism in plants can be catalyzed by either sucrose synthase (SUS) or invertase (INV) enzymes, wherein the former catalyzes the reversible conversion of Suc to fructose and UDP-glucose, while the latter catalyzes the irreversible hydrolysis of Suc to glucose and fructose (Black et al., 1987, Huber and Akazawa, 1986). The route of Suc catabolism is an important determinant in energy conservation and carbon allocation in non-photosynthetic cells, because the conversion of Suc to hexose phosphates via the SUS pathway uses half the ATP of what is required upon conversion via INV (Barratt et al., 2009, Koch, 2004). In silico analysis has revealed that Physcomitrella genome contains eight putative neutral/alkaline INVs and six acid INVs (Thelander et al., 2009). Classified according to the pH optima of their enzymatic activities, these INVs also have different sub-cellular localization, with neutral/alkaline INVs generally thought to be located in the cytoplasm, while acidic INVs localize either to the cell wall apoplasm, or in the vacuoles (Roitsch and González, 2004). Interestingly, phylogenetic comparison of the acidic INV family in Physcomitrella with
that of Arabidopsis revealed that all six of these Physcomitrella INVs group closer to their vacuolar orthologs in Arabidopsis (Thelander et al., 2009). Whether, by inference, this means that Physcomitrella does not have cell wall INVs needs further experimental validation.

The genome-based evidence supports the idea that photosynthetic Physcomitrella gametophores primarily allocate and transport carbon in the form of Suc (Thelander et al., 2009). In tracheophytes, the major forms of Suc transport involve energy-intensive apoplastic, passive symplasmic, or polymer trapping processes (Rennie and Turgeon, 2009, Turgeon, 1996, Turgeon and Ayre, 2005). Physcomitrella gametophores, like many other mosses, only retain xylem and phloem analogs in the form of hydroids and food-conducting cells (Fig. 12) (Ligrone et al., 2000). The profusion of plasmodesmatal pores in the end walls of the food-conducting cells (Fig. 12) is consistent with the notion that Suc transport in the haploid Physcomitrella gametophores is predominantly symplasmic in nature (Raven, 2003).

However, the Physcomitrella genome also contains six putative Suc/H⁺ symporters (Reinders et al., 2014) – a finding that at first glance seems to be at odds with the symplasmic mode of Suc transport in the haploid gametophores. But this point of view does not take the transient diploid sporophytic generation of Physcomitrella into account, which the literature suggests to be a strong heterotrophic sink tissue (Ligrone et al., 1993, Ligrone and Gambardella, 1988). Furthermore, given that the photosynthetic gametophyte of Physcomitrella is symplastically isolated from the sporophyte sink (Courtice et al., 1978), it seems plausible that this intergenerational interface is the site for apoplastic transfer of Suc from the gametophyte to the sporophyte. Adding credence
to this notion was our finding that this filial junction in Physcomitrella has a band of transfer cells (Fig. 14).

Transfer cells are found in all plant taxonomic groups, as well as algae and fungi (Offler et al., 2003, Pate and Gunning, 1972), indicating that the genetic tools requisite for transfer cell morphogenesis evolved primitively (Gunning, 1977, Pate and Gunning, 1972). The cell wall invaginations that are distinctive of transfer cells dramatically increase the surface area to volume ratio of the plasma membrane, that, coupled with the mitochondrial abundance, serve to enhance these cells’ nutrient transport capacity. As such, transfer cells have been functionally linked to nutrient acquisition (e.g. in leaves of aquatic plants) (Gunning and Pate, 1969), secretion (e.g. glands) (FARADAY and THOMSON, 1986), loading and unloading from the vascular network for nutrient allocation and partitioning (e.g. leaf minor veins in Angiosperms) (Haritatos et al., 2000, Knop et al., 2001, Turgeon et al., 1993, Watson et al., 1977, Wimmers and Turgeon, 1991, Ligrone and Gambardella, 1988), development of reproductive tissues (Pate and Gunning, 1972), and delivery of nutrients between generations (e.g. gametophyte-sporophyte interface in bryophytes) (Ligrone et al., 1993, Ligrone and Gambardella, 1988). Given the nutrient dependency of the sporophyte sink on the gametophyte source, we considered it reasonable to speculate that the transfer cells at the sporophyte-gametophyte junction in Physcomitrella facilitate the transport of Suc across the filial boundary.

The few physiological studies on moss nutrient transport, however, have yielded contradictory results. While the whole set of Suc-metabolizing enzymes using the SUS pathway have been found to be enzymatically active in the sporophytes of mosses,
Renault et al. (1989, 1992) instead found that the Suc gets irreversibly hydrolyzed into glucose and fructose by INVs at the highly acidic sporophyte-gametophyte apoplast (Renault et al., 1992, Renault et al., 1989). Counterintuitively, these hexoses then get absorbed into the haustorium without the use of proton motive force, and get reconverted into Suc prior to being assimilated (Renault et al., 1992). The proton motive force was found to be empowering the active uptake of amino acids instead. While the acidic pH of the apoplast is amenable to cell wall INV activity, one of the key pieces of evidence supporting Renault et al.’s proposition was the use of p-Chloromercuribenzenesulfonic acid (PCMBS), a non-permeant thiol reagent that modifies sulfhydryl groups, as an INV inhibitor (Renault et al., 1992). INV contains sulfhydryl groups in its active site (Krishnan et al., 1985), and as such PCMBS can act as an INV inhibitor, but this does not preclude the fact that PCMBS is also a potent inhibitor of apoplastic loading (Giaquinta, 1979, Giaquinta, 1983). As stated previously, the INV-based method of Suc uptake into the sporophyte would be twice as energetically expensive (Fig. 10) as a SUS-based one (Fig. 11). Assuming that mosses are not wasteful in their energy allocation, we hypothesized that the transfer cells harbor a SUS pathway for metabolizing Suc instead (Fig. 11). In hypothesizing that there is a SUS-based Suc transport across the gametophyte-sporophyte apoplast in Physcomitrella, we also predicted that this pathway would be pyrophosphate (PPi) dependent.

Given the robust body of evidence implicating $\text{H}^+$-PPases in PPI homeostasis in eudicot Arabidopsis (Pizzio et al., 2015), and the prevalence and highly conserved nature of $\text{H}^+$-PPases in the plant kingdom, we first ran an *in silico* search for putative $\text{H}^+$-PPase orthologs in Physcomitrella. Four orthologs were found, namely, PP1s346_33V6 (PP1a),
PP1s445_15V6 (PP1b), PP1s28_200V6 (PP2a), and PP1s105_42V6.2 (PP2b), of which the first two shared significant sequence homology with the type I $\mathbf{H}^+$-PPase (AVP1) counterpart in Arabidopsis, while the latter two were far more similar to type II $\mathbf{H}^+$-PPase (AVP2) (Table 2). A phylogenetic analysis led us to conclude that like in Arabidopsis, Physcomitrella remarkably harbors both type I (i.e. PP1a and PP1b) and type II (i.e. PP2a and PP2b) $\mathbf{H}^+$-PPases (Fig. 14).

Using antisera generated against a highly conserved motif in $\mathbf{H}^+$-PPases, we then proceeded to immunolocalize $\mathbf{H}^+$-PPases in Physcomitrella gametophytes and sporophytes at the light microscopy level. As suspected, the strongest $\mathbf{H}^+$-PPase-specific signal was found in growing young tissues like phyllids, zygote, and antheridia (Fig. 15). Relatively weak signal was observed in the gametophore stem and leaves that supported a symplasmic mode of Suc transport in these tissues (Fig. 15). Immunohistochemical localization of $\mathbf{H}^+$-PPases in the sporophytes revealed that $\mathbf{H}^+$-PPases were prominently expressed at the transfer cells lining the gametophyte-sporophyte junction (Fig. 15). We posited that this novel observation had parallels with the salient localization of $\mathbf{H}^+$-PPases in the vascular tissues of higher plants, and as such hypothesized that, like in rice (Chapter 2) and Arabidopsis, the membrane-specificity of $\mathbf{H}^+$-PPases would suggest whether the transfer of Suc across the gametophyte-sporophyte interface in Physcomitrella utilized a SUS- or an INV-based pathway.
Figure 10: Schematic depiction of an Invertase-based pathway of sucrose transfer from the gametophyte to the sporophyte, as proposed in Renault et al. (1992).

Upon immunogold labeling followed by silver enhancement, H\(^+\)-PPases were found to be localized at both the plasma membrane and the vacuoles of the transfer cells (Fig. 16), implicating H\(^+\)-PPases in the ATP-conserving SUS-based scheme depicted in Fig 11. Given that the apoplasm separating the gametophyte from the haustorium is highly acidic, thermodynamic constraints should limit the function of the H\(^+\)-PPases at the transfer cell plasma membrane as PPI synthases. Not mutually exclusive to this scenario are the H\(^+\)-PPases localized at the vacuoles that hydrolyze PPI to pump H\(^+\) into...
the vacuolar lumen (Fig. 2A) A direct prediction of this model is that the transfer cells also harbor SUS. Using cross-reactive polyclonal antibodies against SUS (see Chapter 2), we demonstrated at both light and electron microscopy level that SUS is indeed localized in the transfer cells (Fig. 16). Furthermore, H^+-PPases and SUS also showed a highly correlated expression pattern, with both being prominently expressed at actively growing (Fig. 14 & 16) and transfer cells (Fig. 14 & 16). As discussed in Introduction, H^+-PPase localization in anabolically active cells is not surprising. Given that the sucrose cleavage reaction catalyzed by SUS produces UDP-glucose, a substrate for cellulose biosynthesis via cellulose synthases, and given that these mitotically active cells are constantly synthesizing new cell walls, distinct localization of SUS in these cells is plausible.
Figure 11: Schematic depiction of an ATP-conserving SUS-based sucrose loading scheme.

Though our evidence is consistent with SUS-based ATP-conserving pathway as the likely mechanism of Suc transport from the gametophyte to the sporophyte, we must also concede that this phenomenon might be stage-specific. In other words, the sporophytes analyzed in our experiment were fully formed, and it can not be ignored that there might be developmental stages wherein Suc metabolism might involve an INV-based mechanism. For instance, it is known that a high ratio of hexoses to Suc promotes mitosis (Weber et al., 1995, Weber et al., 1996), and as such, at early stages of sporophyte differentiation and development, an INV mediated catalysis of Suc into
glucose and fructose might occur. Further H\textsuperscript{+}-PPase immunolocalization experiments at various time points after photoperiod- and temperature-induced formation of sporophytes is warranted.

The set of evidence described herein is consistent with a role of H\textsuperscript{+}-PPases in Suc metabolism and partitioning that has been more finely elaborated in model eudicot Arabidopsis, and as such implicates H\textsuperscript{+}-PPases as having a conserved function throughout the evolutionary history of plants. Our ultrastructural evidence documenting the dual membrane-specificity of H\textsuperscript{+}-PPases in a basal lineage of land plants decidedly shifts the traditionally held paradigm that only limited this versatile enzyme as a single-purpose vacuolar PPi hydrolase. As an emerging model plant system with a well-tested repertoire of genetic tools, Physcomitrella provides an entirely new avenue for insightful research into the evolution of fundamental physiological mechanisms common to the entire plant kingdom. Currently, work is underway to construct inducible H\textsuperscript{+}-PPase knockdown mutant lines that might shed more light on what phenotypic effects such genetic interference might have on Suc metabolism and partitioning in Physcomitrella.
Figure 12: Morphology of Physcomitrella.

(A) Light micrograph (LM) of a representative mature leafy gametophore used for this study. (B & C) LM of apical region of the gametophore stained with toluidine blue. Note the single apical meristematic cell in (C). (D) Scanning electron micrograph (SEM) of the apical region of the gametophyte. Notice the female archegonia. (E) LM of sporophyte with an apical sporangium. (F) SEM of the sporangium. (G) SEM of a typical haploid spheroidal spore, with prominent exine ornamentation. (H) Transmission electron micrograph (TEM) of a pollen showing the internal ultrastructure. Numerous osmiophilic lipid storage globules are present. (I) SEM of stem in cross-section showing the overall organization of the conducting cells. The distinctive central bundle of hydroids is clearly visible. (J) LM of the near median longitudinal section through the stem showing the general organization of the elongated conducting cells. The centrally located cells are hydroids, while the directly adjoining cells are food-conducting cells. (K) TEM of cross-section of stem showing the centrally located thin-walled hydroids that are virtually empty. (L) TEM of transverse section through leaf costa showing the adaxially located thick-walled stereid cells, with three empty thin-walled hydroids in the center. (M) Higher magnification TEM of a thick-walled stereid cell. The narrow lumen is mostly empty, and the cell wall has a loose, fibrillar structure. (N) TEM of transverse section through the stem showing a leaf trace right beneath the cortical epidermal layer, with thick-walled stereids. (O) TEM of a putative food-conducting cell (also seldom called deuter) in the leaf midrib. (P) Longitudinal TEM section through the stem depicting putative food-conducting cells. (Q) The end walls of such food-conducting cells are often profusely decorated with plasmodesmata. Scale bars: (A) 5 mm; (B) 50 µm; (C) 10 µm; (D) 100 µm; (E) 1 mm; (F) 200 µm; (G) 25 µm; (H) 25 µm; (I) 300 µm; (J) 200 µm; (K) 5 µm; (L) 5 µm; (M) 1 µm; (N) 5 µm; (O) 5 µm; (P) 10 µm; (Q) 500 nm.
PP1S346_33V6.1  227  LFGRVGGGIYTKAADVADLVGKVQNIPEDDPRNPVIANDN
PP1S445_15V6.1  227  LFGRVGGGIYTKAADVADLVGKVQNIPEDDPRNPVIANDN
PP1S105_42V6.2  265  LFACQGGGIYTKAADVADLVGKVQNIPEDDARNPFAVIADL
PP1S28_200V6.1  264  LFACQGGGIYTKAADVADLVGKVQNIPEDDARNPFAVIADL
AVP1            243  LFGRVGGGIYTKAADVADLVGKVQNIPEDDPRNPVIANDN
VR_HPPase      239  LFGRVGGGIYTKAADVADLVGKVQNIPEDDPRNPVIANDN

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0.953

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1

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0.4

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Type I

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PP1S346_33V6.1

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PP1S445_15V6.1

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Type II

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PP1S105_42V6.2

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PP1S28_200V6.1

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1

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AVP1

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VR_HPPase

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0.4
Figure 13: Molecular phylogenetic analysis of H+-PPase orthologs in Physcomitrella by Maximum Likelihood method conducted in MEGA5.

Amino acid sequence alignment of putative H+-PPase orthologs in *Physcomitrella patens* (PP), with H+-PPase from *Arabidopsis thaliana* (AVP1) and *Vigna radiata* (VR_HPPase) using MUSCLE. The highly conserved D(X)\textsubscript{7}KXE motif is shown (black line). The evolutionary history of Physcomitrella H+-PPases was inferred by using the maximum likelihood method based on the JTT matrix-based model, and the bootstrap consensus tree inferred from 1000 replicates is taken to represent the evolutionary history of the taxa analyzed. The percentages of replicate trees in which the associated taxa clustered together in the bootstrap test are shown next to the branches, with the inference that there are two distinct phylogenetic clades of H+-PPases in Physcomitrella – type I and type II, like in its angiosperm counterparts, AVP1 and VR_HPPase.
Figure 14: Localization of H+-PPase orthologs in moss Physcomitrella.

(A & B) H⁺-PPases are localized at the rapidly growing apical regions of moss gametophores, (E) growing embryo, (F) egg cell, but not in the gametophore stem (C), or leaves (C & D). (G) Pre-immune sera showing no label in the gametophore stem. (H & I) H⁺-PPases are conspicuously localized at the gametophyte-sporophyte junction – the haustorium, characterized by transfer cells. (J) Immunolocalization of H⁺-PPases at the haustorium using antisera generated in mouse. (K) Pre-immune sera control, (L) Secondary antibody control, and (M) no labeling control. The brown coloration seen in (K – M) is an endogenous color of the seta as seen in Fig. 15(E). (N) Western blot of Physcomitrella total protein extracts interrogated by anti-H⁺-PPase sera. Scale bars: (A & B) 50 µm (C) 100 µm (D) 25 µm (E) 50 µm (F) 20 µm (G) 100 µm (H) 100 µm (I & J) 20 µm (K – M) 50 µm.
Figure 15: Transfer cell ultrastructure and H⁺-PPase labeling.

(A – C) Haustorium is typified by transfer cells, whose characteristic cell wall invaginations are evident. Chemically fixed (A – C) versus high-pressure frozen/freeze-substituted transfer cells (D – F). (G – I) Immunogold labeling followed by silver enhancement shows both plasma membrane (H) and vacuolar (I) localization of H⁺-PPases in cryofixed transfer cells. (J – M) Pre-immune serum controls. Scale bars: (A) 5 µm (B) 1 µm (C) 500 nm (D) 5 µm (E) 1 µm (F) 500 nm (G) 5 µm (H & I) 250 nm (J) 5 µm (K) 2.5 µm (L) 500 nm (M) 500 nm.
Figure 16: Polysaccharide and sucrose synthase (SUS) localization in Physcomitrella.

(A – B) Lugol’s starch staining of sporangium (A), and haustorium (B), showing starch localization at the spores and transfer cells respectively. (C) Schiff’s staining for polysaccharides including cellulose at the haustorium. (D & E) SUS localization at the apical regions of Physcomitrella gametophores, but lack thereof in the gametophore stem and leaves (F). (G) Prominent SUS localization at the transfer cells lining the haustorium. (H) Non-immune, and (I) secondary antibody controls. (J – L) Immunogold labeling of SUS at the transfer cells showing SUS-specific labeling in close proximity to the plasma membrane. Scale bars: (A – C) 50 μm (D & E) 50 μm (F) 25 μm (G) 50 μm (H) 200 μm (I) 50 μm (J) 5 μm (K & L) 250 nm.
4. ELECTRON TOMOGRAPHY OF PLASMODESMATA

Ultrastructure and three-dimensional morphology of plasmodesmata in rice leaves revealed by electron tomography

Abstract

The ultrastructure of plasmodesma remains as enigmatic today as it was when they were discovered over a century ago – with the literature strewn with diverse interpretations and models based on electron micrographs. In light of the intrinsic three-dimensionality of plasmodesmata, we used electron tomography to elucidate the substructure and overall morphology of these unique plant-specific channels. Specifically, we studied plasmodesmata across cell interfaces along the phloem-loading pathway in minor veins of high pressure frozen Oryza sativa (rice) leaves. Tomographic models of simple, branched, and twinned plasmodesmata illustrated their 3D morphology, and also revealed two distinct morphotypes of simple plasmodesmata. On a broader level, we establish electron tomography as a powerful technique to add the previously missing third dimension in understanding plasmodesmata ultrastructure and morphology.
Introduction

As its name implies, plasmodesma (PD) is a structure that “bonds” (Greek: *desma*) two proto“plasms” (Latin: fluid) together (Robards, 1968b, Zambryski, 2008). Considered evolutionary analogs of animal gap junctions (Roberts, 2005), these are unique and dynamic quasi-organellar (Epel, 1994) nano-pores (Bayer et al., 2014) that interconnect cells constituting the syneytial domains in plant bodies (Lucas and Lee, 2004). The advent of transmission electron microscopy (TEM) opened an entirely new avenue for structural studies of PD (Botha and Cross, 1999, Robards, 1976), and before long, a prototypical PD was resolved as a plasma membrane (PM)-lined linear pore with a central, axially oriented, appressed endoplasmic reticular (ER) cylinder called the desmotubule (DT) (Robards, 1968b, Robards, 1968a, Robards, 1971). The space between PM and ER is referred to as the cytoplasmic annulus or sleeve (Esau and Thorsch, 1985), through which all, if not most of the PD-mediated intercellular transport is thought to occur (Epel, 1994, Kragler et al., 1998, Lucas and Wolf, 1993, Robinson-Beers and Evert, 1991). The internal substructure of PDs, especially that of the wall collar, cytoplasmic sleeve, and DT, however, remains far from resolved – with various models heretofore published.

A widely cited PD model was proposed by Gunning and Overall (1983), that has undergone some revisions and modifications over the years, but fundamentally posits that the cytoplasmic sleeve is composed of particles (Gunning and Overall, 1983, Overall, 1999, Overall and Blackman, 1996). The “mottled” negative staining of putative spherical structures obtained by using tannic acid in the fixative fluid was postulated to be actin, while the electron-dense spoke-like structures connecting the DT to the inner
leaflet of PM were suggested to be actin microfilaments and/or myosin (Overall, 1999). Indirect immunogold studies have corroborated at least the presence, if not the internal structural arrangement of these cytoskeletal proteins in PDs (Blackman and Overall, 1998, Radford and White, 1998, White et al., 1994). The DT, on the other hand, was modeled as a “tightly furled” ER membrane with the osmiophilic phospho- head groups of inner DT membrane forming a central electron-dense spot (Overall, 1999).

Another model proposed by Olesen (1979) put forth the idea that the combinatorial staining obtained by tannic acid and heavy metals revealed a collar of sphincter around the neck regions of PDs that were theorized to comprise of nine radially arranged spherical particles (Olesen, 1979). This deduction was based on the rotational enhancement technique originally used by Markham et al. (1963) to finely resolve and accentuate the manifold symmetry of viruses (Markham et al., 1963). Whether PDs are intrinsically radially symmetrical structures is yet unknown – especially considering that PDs can adopt simple, twinned, or complex branched conformations. Support for the existence of the wall collar and radial arrangement of particles came from field emission scanning electron microscopic imaging of PDs (Faulkner et al., 2008). The wall collars were first hypothesized to act like “plasmodesmal valves” (Olesen, 1979, Olesen and Robards, 1990), whose major component is now known to be callose (β-1,3-glucan) (De Storme and Geelen, 2014, Northcote et al., 1989, Zavaliev et al., 2011). The dynamic homeostasis of callose at the neck sphincters is proposed to regulate the symplasmic flux through the PDs (De Storme and Geelen, 2014, Zavaliev et al., 2011, Burch-Smith and Zambryski, 2012, Overall et al., 2013).
By contrast, Ding et al. (1992) argued for cautious interpretation of tannic acid based negative staining, and put forth a popular alternative PD model (Ding et al., 1992). The authors discounted the idea that PDs have sphincter-like wall collars around the PD neck (Olesen, 1979), and that the cytoplasmic sleeve consists of electron-lucent particles (Overall et al., 1982) – observations they associated with artifactual over-staining by tannic acid (Ding et al., 1992). After post-acquisition image processing, Ding et al. (1992) further conjectured that the cytoplasmic sleeve is in fact a multi-form tortuous channel between two rings of presumably proteinaceous particles embedded/anchored on the outer DT membrane and the inner leaflet of PM (Ding et al., 1992). Electron-dense spokes were also found to connect these DT- and PM-bound particles. Ding et al. (1992) further contended that the central, electron-dense spot in the DT is in fact a “central rod” ostensibly comprised of a series of longitudinally arranged proteinaceous particles, that are, in turn, connected by spokes to the inner DT membrane (Ding et al., 1992).

Overall (1999) questioned the validity of the ultrastructural inferences made by Ding et al. (1992) on the ground that the images were processed to enhance the “particulate nature of plasmodesmata” (Ding et al., 1992), and because of the reported inconsistencies in the biophysical dimensions of the cytoplasmic sleeve (Overall, 1999). Whether the cytoplasmic sleeve consists of a single ring of particles (Overall et al., 1982) or is a convoluted electron-lucent space between two rings of particles (Ding et al., 1992) is still an unresolved issue. The former notion that the cytoplasmic sleeve consists of a single ring of particles rather than two, was seemingly upheld by a relatively recent report of a single ring of 5-6 particles encircling the central DT-like structure in Chara PD using high-resolution scanning electron microscopy (Brecknock et al., 2011).
Resolution of these ultrastructural discrepancies on PD architecture will obviously require molecular tools complemented with newer imaging platforms (Bell and Oparka, 2011). State-of-the-art technology like electron tomography (ET) coupled with high-pressure freezing (HPF)-freeze substitution (FS) holds much promise to study the PD ultrastructure (Burch-Smith and Zambryski, 2012, Overall et al., 2013). Electron tomograms of higher plant PDs have been published (Bilska and Sowiński, 2010, Tian et al., 2007), but as a subsidiary subject rather than the primary one, revealing only the undulations along the length of the PDs (Bilska and Sowiński, 2010, Overall et al., 2013, Tian et al., 2007). In this study, we applied ET coupled with HPF-FS to study the three-dimensional conformations of PD along the symplasmic pathway in the minor vein vascular bundles in *Oryza sativa* (rice) leaves. Specifically, we studied the structure of the PDs between the following three cellular interfaces – bundle sheath and mestome sheath cells, mestome sheath and vascular parenchyma cells, and vascular parenchyma and companion cells.

The comparative structures of specialized monocotyledonous leaf blade PDs have been the subject of limited number of previous studies (Botha, 1992, Evert et al., 1977, Evert et al., 1996a, Evert et al., 1996b, Lopez-Saez et al., 1966, Robinson-Beers and Evert, 1991), but in-depth study of PDs in as important a crop as *Oryza sativa* (rice) has been nonexistent. Furthermore, given the current interest in converting rice into a C4 plant (von Caemmerer et al., 2012), understanding the PD structures in the leaf minor veins assumes greater significance. On a wider level, this study also validates the use of ET – as time-intensive as it might be – as a viable tool to study the three-dimensional architecture of PDs. And unlike two-dimensional projection images of inherently three-
dimensional structures obtained by conventional TEM, tomograms can be optically sliced throughout the depth of the imaged section (Kremer et al., 1996). ET also allows us, most importantly, to build three-dimensional virtual replicas of the cellular sub-structures in question (Kremer et al., 1996).

The models we created based on the electron tomograms of the PDs traversing the intercellular walls between the aforementioned cell types revealed previously unreported three-dimensional morphology of simple, branched, and twinned PDs. Tomographic evidence corroborating the existence of spokes connecting DT-PM and the putative central rod to DT were found that confirmed previous TEM findings. With only osmium tetroxide in the FS cocktail, and without any post-image acquisition enhancements, we found evidence supporting the existence of six presumably proteinaceous electron-dense particles in the outer DT membrane. Without using tannic acid in the FS fluid, an electron-dense halo corresponding to the wall collar was particularly evident when glancing transverse sections of PD necks were examined with a transmission electron microscope, while the existence of neck constrictions was apparent in all types of PDs inspected in longitudinal section by ET.
Materials and Methods

Rice (*Oryza sativa*) growth conditions

Seeds were dehulled, sterilized with 10% bleach solution, and germinated on rice growth media (Yang et al., 2007) on petri plates at 37°C in the dark for 48 hours. Once the post-germinative process, characterized by the emergence of the radicle, had begun, the plates were transferred to a growth chamber with 16-hour light, and 8-hour dark regime. Once the coleoptiles had developed, the seedlings were transferred to wet soil, and allowed to grow until the tenth leaf had emerged, and the first flag leaf was fully developed (~7 – 8 weeks).

Sample preparation for electron tomography and transmission electron microscopy

To cryofix leaves, Harris unicore 1.2mm punch (Electron Microscopy Sciences, USA; www.emsdiasum.com) was used to punch small leaf discs from fully-grown flag leaves while avoiding the leaf mid-veins. The leaf discs were then immediately transferred to type-B brass planchettes. The empty space was filled with 1-hexadecene, and another type-B planchette was used as a cover, and the sandwich was immediately subjected to high pressure freezing (HPF) in BalTec HPM 010 high-pressure freezer. The cryofixed leaves were then transferred to a pre-chilled -80°C freeze-substitution (FS) cocktail containing anhydrous acetone and 2% (w/v) osmium tetroxide. The FS was allowed to continue for 96 hours at -80°C, after which the FS solution was allowed to warm to 4°C over a period of 12 hours. The samples were washed with anhydrous acetone several times, and the leaf discs were gradually infiltrated with Spurr’s resin (Spurr, 1969) according to the following schedule: 10% (v/v) Spurr’s (12 hours), 25% (v/v) Spurr’s (12 hours), 50% (v/v) Spurr’s (12 hours), 75% (v/v) Spurr’s (12 hours), 100% Spurr’s (12 hours).
hours), 100% Spurr’s (12 hours), 100% Spurr’s (12 hours). The leaves were transferred to molds, filled with fresh Spurr’s resin and embedded for 24 hours at 55°C in longitudinal or transverse orientations. The polymerized resin blocks were then trimmed, ultrathin sectioned to gold reflectance (80 – 90 nm) using a Leica Ultracut R ultramicrotome (Leica Microsystems, Germany) or semi-thin sectioned to 200-300nm using a Leica Ultracut UCT (Leica Microsystems, Germany), and mounted on formvar-coated copper slot grids.

**Electron Microscopy and Tilt Series Acquisition**

For transmission electron microscopy, ultrathin sections were post-stained for 5 minutes with 2% (w/v) methanolic uranyl acetate for 5 minutes followed by Sato’s lead citrate, and imaged with a JEOL 1200EX transmission electron microscope (JEOL Corp., Japan) operating at 80 KV. For intermediate voltage electron microscopy, semi-thin, or serial semi-thin sections (200–300 nm), were cut and tilt series were acquired using a FEI Tecnai TF30 microscope operating at 300 kV or FEI Tecnai TF20 microscope operating at 200 kV (FEI Co., Hillsboro, USA). Images (2048 x 2048 pixels) were acquired from +60° to −60° at 1° intervals about two orthogonal axes using a Gatan digital camera (Gatan Inc., Pleasanton, USA).

**Tomogram construction and modeling**

The images were aligned using gold fiducial markers (Ladinsky et al., 1999), and single-axis tomograms were built using eTomo (part of 3DMOD) software, and the single-axis tomograms were combined into dual-axis tomograms (Mastronarde, 1997). Serial-section tomograms were constructed by aligning and then combining individual dual-axis tomograms using eTomo (Austin et al., 2005). The outer and inner leaflet of plasma
membrane and the desmotubule of plasmodesmata were traced using closed contours as self-enclosed structures (Movie S4, S5, S6), and were modeled using the 3DMOD software (Kremer et al., 1996). Meshed volume measurements were computed using IMOD (Kremer et al., 1996). Sub-volumes enclosing the neck regions of the plasmodesmata were modeled using the automatic IMOD rendering tool, ISOSURFACE. Videos were made using IMOD and Adobe Photoshop CS6® (Adobe Inc., USA). All the images were compiled into figures using Adobe Illustrator® software (Adobe Inc., USA).

Results

Here we examined the three-dimensional morphology of plasmodesmata (PDs) across the cellular interfaces between bundle sheath (BS) and mestome sheath (MS), mestome sheath and vascular parenchyma (VP), and vascular parenchyma and companion (CC) cells in cryofixed rice leaf minor vein using electron tomography (ET). We abstained from using tannic acid because the exact underlying chemistry of tannic acid staining is hitherto unknown. All the PDs across these interfaces were investigated in longitudinal sections, and were modeled with closed contours as self-enclosed structures in IMOD (Movie S4, S5, & S6). Given the time-, labor-, and resource-intensive nature of the tomographic modeling process, the volumetric calculations shown below should be taken as a proof of application, rather than as statistical inferences.

**Simple plasmodesmata between bundle sheath and mestome sheath cells**

The conventional wisdom on the structure of simple PDs informs us that these PDs are linear cylindrical pores (Robards, 1971). **Fig. 17(A-E)** depict tomograms of numerous simple PDs connecting the BS and MS cells that largely upholds the established understanding of simple PDs. The serial tomographic slices in **Fig. 17 (A-C)**
show that the neck constrictions (arrowheads) exist at both ends of the PD orifices. Suberin deposit at the middle lamella (ML) has a distinct “tram-line” like appearance (Botha et al., 2008), as seen at the BS-MS interface in C4 sugarcane leaves (Robinson-Beers and Evert, 1991), but only an electron-dense unsuberized middle lamella (ML; yellow lines) is found at the BS-MS interface in rice. In longitudinal section, these simple PDs are symmetrical across the ML, and show a somewhat median enlargement of the desmotubule (DT; magenta; Fig. 17D). When observed end-on, the simple PDs interconnect BS and MS at multiple z-planes (Fig. 17E). From the meshed models generated in IMOD, the average meshed volume enclosed by the cytoplasmic sleeve was calculated to be 4.64 x 10^4 nm^3.

Surprisingly, without using tannic acid as a mordant, and only using osmium tetroxide as the primary staining agent, the electron-dense wall collars in the upper neck region of simple PDs are evident even in regular transmission electron micrographs of transverse section through a pit field (Fig. 17F). The neck constrictions are especially obvious when the sub-volume enclosing the neck region of a simple PD is rendered with IMOD’s Isosurface tool (Fig. 17G). The neck constrictions observed in the longitudinal sections through these PDs likely correspond to the wall collars seen in transverse section – but the particulate sphincter-like structures postulated to exist in these PD collars were not observed.

Single 2.2 nm optical slice through an oblique longitudinal section of simple PD (Fig. 17H) shows the inner leaflet of plasma membrane (IPM) connected to the outer DT membrane via spoke-like extensions, that have previously been reported as electron-dense, fibrillar strands (Botha et al., 1993, Ding et al., 1992, Brecknock et al., 2011).
Figure 17 (I) shows serial 2.2 nm tomogram slices of transverse section through the equatorial region of a simple PD. The DT is enlarged in this PD sub-region, and spoke-like structures are seen to symmetrically connect the center of the DT to the outer DT membrane (Fig. 17I). Furthermore, serial 2.2 nm tomographic slices of the neck region of simple PDs (Fig. 17J) reveal the presence of putative electron-dense particles in the outer DT membrane. A central rod at the core of the DT is also clearly visible (Fig. 17 H, J).

Plasmodesmata between mestome sheath and vascular parenchyma cells

By contrast, the PDs that connect MS and VP cells (Fig. 18), though simple, are visibly wider in the z-plane (Fig. 18B) and more voluminous than the ones that connect BS and MS cells, with an average meshed volume enclosed by the cytoplasmic sleeve calculated to be 3.28 x 10^5 nm³. The average volume occupied by the PDs at the MS-VP interface was 5.88 x 10^5 nm³, compared to 5.86 x 10^4 nm³ for the corresponding PDs at the BS-MS interface. These PDs are also asymmetrical structures (Fig. 18 A – D), with a noticeable neck constriction only at the terminal end contiguous with the VP cell (Fig. 18 A, C – F).

Generally, suberin deposition at the ML contributes to the asymmetry and/or median constriction of the PDs (Botha et al., 1982, Botha et al., 2008, Robinson-Beers and Evert, 1991). However, no discernible suberin layer was observed in the cell wall between the MS and VP cells that could impart the asymmetry observed (Fig. 18). There is no perceptible median enlargement of the DT either (Fig. 18). The distinctly different three-dimensional arrangement and morphology of the simple PDs between BS-MS and MS-VP interfaces can be better appreciated in Movie S1 and S2, respectively.
Upon direct comparison of PDs traversing either BS-MS or MS-VP interfaces, the multimorphic three-dimensional spatial organization of different types of simple PDs is patently obvious (Fig. 17 & 18; Movie S1 & S2). Whether the more capacious PDs at the MS-VP interface is indicative of an advanced developmental status of these PDs, or is correlated with an increased symplasmic flux through this phloem-loading boundary is unknown.

**Plasmodesmata between vascular parenchyma and companion cells**

Unlike the BS-MS and MS-VP interfaces, the cell wall between VP and CC contained simple, twinned, branched, and secondary PDs (Fig. 19). This is congruent with report of branched PDs often being localized in the tangential walls adjoining VP and CC in monocotyledonous leaf blade vascular bundles (Botha et al., 2008). Compared to the total meshed volume of $1.31 \times 10^6$ nm$^3$ occupied by the cytoplasmic sleeve of PDs at the MS-VP interface, the cytoplasmic sleeve of PDs connecting VP and CC was calculated to enclose six-fold more total meshed volume of $7.87 \times 10^6$ nm$^3$. This tells us that the total volume enclosed by the cytoplasmic sleeve increases directionally along the BS-MS, MS-VP, and VP-CC cellular interfaces lining the phloem-loading pathway in minor vein vascular bundles of mature rice leaf blade. This, in turn, implies that the volumetric increase in the cytoplasmic sleeve along the phloem-loading direction potentially accommodates more symplasmic flow at the VP-CC interface than at the BS-MS interface.

Tomographic model of the simple, twinned, and branched PDs through the VP-CC cell have neck constrictions at their orifices (Fig. 19 H). These PDs penetrate the connective tangential wall at multiple z-planes (Fig. 19 G), and when rendered as three-
dimensional models, display a definite non-cylindrical elaborate morphology that the traditional two-dimensional TEM projection images would not have revealed (Fig. 19 D–F; H; Movie S3). A closer inspection of the simple PD in fact reveals that it has what, in effect, looks like an emergent branch (Fig. 19C).

Discussion

As of this writing, multiple interpretations of plasmodesmata (PD) images can be found in the literature (Badelt et al., 1994, Botha et al., 1993, Ding et al., 1992, Olesen, 1979, Overall and Blackman, 1996, Radford and White, 1998, Roberts and Oparka, 2003, Tilney et al., 1991, Turner et al., 1994, White et al., 1994). Botha and Cross (1999) astutely observed, “almost as many interpretations of [PD] structure exist in the literature as there are references (Botha and Cross, 1999).” There are certain consistently observed ultrastructural attributes of PDs – like its trilaminar structure, or the axially oriented desmotubule (DT) – on which there is a definite scientific consensus, but unanimity on the internal arrangement, function, and the chemical nature of the putative molecular subcomponents has remained elusive (Botha and Cross, 1999, Botha and Cross, 2000, Ding et al., 1992, Gunning and Overall, 1983, Olesen, 1979, Overall, 1999, Overall et al., 1982, Overall and Blackman, 1996, Robards, 1968b, Robards, 1968a, Robards, 1971, Roberts and Oparka, 2003, Roberts, 2005).

Specifically, debate persists on the presence of a central proteinaceous rod in the desmotubule (DT), whether there are one or two rings of particles enclosing the cytoplasmic sleeve, and whether a radially symmetrical wall sphincter exists (Ding et al., 1992, Gunning and Overall, 1983, Olesen, 1979, Olesen and Robards, 1990, Overall, 1999, Overall et al., 1982, Overall and Blackman, 1996). The problem lies in reconciling
the information gathered from the PD transections with that interpreted from the longitudinal sections (Botha and Cross, 1999). Despite the possibility that a single universal model might not accommodate the ontogenetic (primary vs. secondary) and morphological (simple vs. branched) variations of PDs (Robinson-Beers and Evert, 1991, Waigmann et al., 1997), it is still believed that these variants are rooted to a common structural PD archetype (Van Bel et al., 1999).

Within this broader purview, we set out to elucidate the three-dimensional morphology of the PDs along the phloem-loading pathway in the rice source leaf minor veins using electron tomography (ET) paired with high-pressure freezing (HPF)-freeze substitution (FS). Given that PDs are ~50 nm in diameter, and coupled to the fact that ET yields approximately 20-fold higher z-axis resolution (6-8 nm) compared to conventional transmission electron microscopy (Donohoe et al., 2006, McIntosh et al., 2005), we considered ET to be the ideal method to resolve the fine structure of PDs.

Electron tomography on the PDs lining the BS-MS, MS-VP, and VP-CC cell trajectory in rice source leaf minor vein revealed previously unreported morphometric variations of PDs in three-dimensions (Fig. 17 – Fig. 19; Movie S1 – S3). Remarkably, we found two distinct simple PD morphotypes that traversed the BS-MS and MS-VP walls respectively (Fig. 17 & 18; Movie S1 & 2). The three-dimensional morphology of simple PDs lining the former interface was structurally congruent with the conventional description of PDs as linear, bilaterally symmetrical, uniform cylindrical pores. Contrarily, the simple PDs lining the MS-VP interface displayed a more complex, asymmetrical, non-uniform configuration. Whether the very dissimilar morphology of the
simple PDs lining the MS-VP vis-à-vis BS-MS interface indicates different ontogenetic origin and/or functional role is not known.

Gamalei (1990) previously suggested that the degree of PD branching (i.e. complexity) in leaf vascular bundles could indicate the course and direction of the bulk sucrose flux (Gamalei, 1990). In accord, we found simple PDs lining the BS-MS and MS-VP cell boundaries, while simple, twinned, and branched PDs were located at the VP-CC interface. Notably, the interior cytoplasmic sleeve volumes enclosed by the PDs increased directionally along these aforementioned cellular interfaces (i.e. VP-CC > MS-VP > BS-MS). Presumably, this gradient of PD volume along the sucrose movement pathway directly correlates with the amount of directional sucrose flux prior to phloem loading.

The ultrastructure of PDs along the sucrose loading pathway from mesophyll cells to the sieve elements in angiosperm leaves harbor subtle variations that are likely associated with specific cellular interfaces or functions (Lopez-Saez et al., 1966, Botha et al., 2008). Several reports also suggest that the PDs along the loading or unloading pathway undergo structural modifications (Botha et al., 2000, Epel, 1994, Lee et al., 2000, Oparka et al., 1999, Robards, 1968b, Robards, 1968a, Robinson-Beers and Evert, 1991, Botha et al., 2008). Many monocot plants are apoplastic loaders, and hence the sieve element-companion cell complexes are relatively isolated (Botha, 1992, Botha and Van Bel, 1992), but a symplasmic continuum normally exists from the mesophyll to at least the vascular parenchyma (VP) cells (Botha et al., 2008).

Previous study on the fine structure of PDs in mature leaves of sugarcane, a C4 monocot, found five morphologically different types of PDs along the mesophyll-
mesophyll, BS-MS, and VP-VP cell trajectory (Robinson-Beers and Evert, 1991). While it is plausible to associate this intra-specific multi-morphology of PDs to their presumptive cell-specific functions, direct evidence has been lacking (Robinson-Beers and Evert, 1991). These authors did note, however, that the presence of external neck sphincters could potentially regulate the molecular weight-exclusion limit of the PDs (Robinson-Beers and Evert, 1991).

One of the distinctive characteristics of the PDs we observed was the presence of neck constrictions at either end of the orifices (Fig. 17 – 19). Previous ultrastructural studies have consistently found electron-dense external wall collar/sphincters when both tannic acid and heavy metals (e.g. osmium tetroxide) were used in the fixation protocol. On the contrary, with only osmium tetroxide in the FS fluid, we found a diffuse electron-dense ring corresponding to the wall collar surrounding the neck region of PDs observed in transverse section (Fig. 17F). The presence of external neck sphincters because of callose deposition (Lucas and Wolf, 1993, Olesen and Robards, 1990) has often been interpreted as a chemical fixation artifact (Hughes and Gunning, 1980, Radford et al., 1998), but our use of HPF-FS protocol should exclude the possibility of this artifact altogether. The presence of callose and the associated neck constrictions have been validated (Northcote et al., 1989), leading to the current belief that the dynamic turnover of callose at the wall collar helps regulate PD size exclusion limit and flux (Burch-Smith and Zambryski, 2012, Radford et al., 1998, Zavaliev et al., 2011).

The VP-CC interface was marked by the presence of primary simple, twinned, and Y-shaped branched PDs, and one secondary PD as well (Fig. 19; Movie S3). It has been hypothesized that Y-, V-, X-, or H-shaped branched PDs represent either the
intermediate state prior to the formation of twinned PDs (Burch-Smith et al., 2011), or
Twinned PDs observed in the VP-CC wall are proximally apposed (within ~10 nm) at
their equatorial region (Fig. 8 A; Movie S1 & S6). Whether this pair formed after the
fission of a H-shaped PD, or is in the incipient fusion stage can not be deduced from the
tomogram.

As a whole, the body of evidence presented here validates ET as a suitable
technique in elucidating both the three-dimensional morphology and the fine substructure
of PDs. Future tomographic analysis of PD structure could benefit from the use of ER-
specific stains like KMNO₄ (Giddings, 2003), or osmium tetroxide-potassium
ferricyanide (Hepler, 1981) in the freeze-substitution cocktail. Methods have been
developed that successfully combine the superior structural preservation obtained from
HPF-FS with the excellent immunolabeling efficiency of Tokuyasu technique (Tokuyasu,
1973, van Donselaar et al., 2007). Use of ET on such immunolabeled cryosections could
potentially reveal the internal arrangement of proteins like myosin, actin, reticulons, and
centrin within PDs. Rigorous application of ET coupled with HPF-FS on a more
ambitious scale will certainly yield yet more fundamental structural insights into the
morphology, ultrastructure, and the molecular composition of PDs.
Figure 17: Electron tomographic analysis of the spatial distribution and morphology of simple plasmodesmata between bundle and mestome sheath cells of rice small vascular bundle.

(A – C) Serial 22 nm thick tomographic slices (average of ten 2.2 nm slices) showing the simple PDs connecting bundle sheath (BS) and mestome sheath (MS) cells. The PDs have neck constrictions at both ends (white arrowheads). The middle lamella (ML) is marked with black arrows. (D) Model depicting six simple PDs across the BS-MS interface, wherein the neck constrictions (white arrowheads), and ML (yellow; white arrow) corresponding to (A – C) are seen (blue: outer plasma membrane leaflet; green: inner plasma membrane leaflet; magenta: desmotubule). (E) End-on view of the PDs showing their spatial arrangement along the z-axis. (F) Transmission electron micrograph showing the presence of an electron-dense halo around the neck region of simple PDs observed in transverse section, without the use of tannic acid (black arrows). (G) Isosurface rendering of the sub-volume of the neck region of a simple PD illustrating the distinct neck constriction (marked with arrows). (H) Oblique 2.2 nm longitudinal section of a simple PD depicting the spokes (arrows) that are continuous with the outer DT membrane (arrowhead). The putative dark “central rod” is also marked (black arrow). (I) Serial 2.2 nm tomographic slices illustrating spoke-like structures between the inner and outer desmotubule (DT) membranes of simple PD in transverse section. Arrow in (A) marks the outer DT membrane, while the arrowhead marks the inner plasma membrane (PM) leaflet. The tomograms depict the equatorial region of a simple PD where the DT is medially enlarged. (J) Serial 2.2 nm tomographic slices showing the arrangement of six electron-dense particles is seen in the outer DT membrane (white arrowheads in). Also see Movie S1 and S4. Scale bars: (A – C) 100 nm; (D – E) 50 nm; (F) 100 nm (G) 10 nm; (H) 50 nm; (I & J) 25 nm.
Figure 18: Electron tomography of simple plasmodesmata traversing the mestome sheath – vascular parenchyma interface in rice small vascular bundle.

(A) A model of simple PDs between MS and VP cells overlaid on the tomogram showing the arrangement of the PDs across the intervening wall between mestome sheath (MS) and vascular parenchyma (VP) cells. (B) An end-on view of the PDs showing their three-dimensional organization in the z-axis. Upon comparison with the FIG. 6(E), it is evident that the PDs at the MS-VP interface are visibly wider in the z-dimension. (C – D) Comparison of automatically generated isosurface rendering of a simple PD (C) with that of a manually generated model (D) showing the congruence of overall PD morpholgy. (E & F) Isosurface renderings of the neck regions of a PD contiguous with the VP cell (E) and the MS cell (F), showing that the neck constriction in F (arrows) is twice as wide as that in E (demarcated with arrows). (G) A model depicting the simple PDs between MS and VP cells (blue: outer leaflet of plasma membrane; green: inner plasma membrane leaflet; magenta: desmotubule). It is obvious that the neck constriction is only confluent at the VP end of the PD orifice. Also see Movie S2 and S5. Scale bars: (A) 100 nm (B – D) 50 nm; (E) 25 nm; (F) 50 nm; (G) 50 nm.
Figure 19: Tomographic images of plasmodesmata between the vascular parenchyma and companion cell interface.

(A – C) Tomographic slices depicting twinned plasmodesmata (PD) separated by less than 10 nm at their equatorial region (A); branched PD (B); simple PD with a branch bud (arrowhead; C). (D, E & F) Corresponding tomographic models of PDs depicted in (A), (B), and (C) respectively. (G) An end-on view of the PDs showing their spatial configuration along the z-axis. Branched PD (arrow) is marked. (H) A model of the PDs at the VP-CC interface (blue: outer leaflet of plasma membrane; green: inner leaflet of plasma membrane; magenta: desmotubule). See also Movie S3 and S6. Scale bars: (A – C) 20 nm; (D – F) 50 nm; (G) 50 nm; (H) 50 nm.

Supplementary Movies (MAC-compatible)

Movie S1: Three-dimensional model of simple plasmodesmata across the bundle sheath – mestome sheath cell interface (blue: outer plasma membrane; green: inner plasma membrane; magenta: desmotubule)


Movie S3: Three-dimensional model of simple, twinned, and branched plasmodesmata across the vascular parenchyma – companion cell interface (blue: outer plasma membrane; green: inner plasma membrane; magenta: desmotubule).


5. CONCLUSION

The evolution of photoautotrophic plants from uni- to multicellularity conferred crucial selective advantages, especially in terms of morphological specialization of specific cell types/tissues to effectively function in the acquisition, assimilation, and transport of nutrients and photosynthates for survival (Lucas et al., 2013). An important feature of early multicellular plants was the acquisition of plasmodesmata, whose cytoplasmic channels provided a uniquely suited symplasmic conduit for the flow and exchange of photosynthates and nutrients between cells (Lucas et al., 1993). However, the diffusion-based constraint of nutrient flux through these inter-cellular channels imposed a penalty on the maximum size a multicellular plant could attain (Fisher and Wang, 1995, Lucas et al., 2013). Hence, the strong selective pressures to overcome the size limitations allowed the evolution of an axially arranged tissue system with a much enhanced assimilatory and conductive capacity (Ligrone et al., 2000, Lucas et al., 2013). The acquisition of these long-distance nutrient and water-conducting cells was thus an important innovation in the terrestrialization of plants (Kenrick and Crane, 1997, Lucas et al., 2013, Prigge and Bezanilla, 2010). Interestingly, the degree of modifications of the cells functionally specialized for long-distance transport increases from distant non-vascular plant lineages (e.g. Bryophytes) onto more advanced lineages of seed plants (Ligrone et al., 2000, Ligrone et al., 2012, Raven, 2003, van Bel, 2003, Pittermann, 2010). As can be inferred from the preceding three chapters, the intra-/inter-cellular, genetic, and molecular pathways underpinning the fundamental aspects of photosynthate
partitioning are seemingly conserved even in organisms as evolutionarily divergent as Physcomitrella and rice.

Firstly, both rice minor vein vascular cells and the non-vascular food-conducting cells of Physcomitrella harbor plasmodesmatal pores that bear striking structural resemblances. In both organisms, these channels form a symplasm through which photosynthesized sucrose is translocated. Remarkably, in these two very dissimilar organisms the pathway for passive diffusion of sucrose is via pores that are seemingly both structurally and functionally homologous. However, as explained in Chapter 2, sucrose movement can also occur via the cell wall space in an energy-dependent process. The companion cells in rice minor veins contain sucrose synthase (SUS), a fully operational pyrophosphate-dependent glycolytic sequence, and a high density of mitochondria – molecular and cytological features that make them well-suited for sucrose concentrating and loading step in the phloem tissue. Is this necessarily a unique feature of advanced lineages of angiosperms?

Bryophytes and the ancestor of vascular plants diverged early in land plant evolution, and despite their divergent morphologies and life cycles, the genomic evidence points to the conservation of molecular pathways implicated in the metabolism of sucrose. The enzymatic and morphological toolkit of companion cells finds a primordial parallel in the transfer cells of Physcomitrella, wherein the same pyrophosphate-dependent SUS pathway and its associated enzymes seem to be implicated in the transfer of sucrose from the photosynthetic gametophyte to its nutritionally reliant sporophyte. Inherent in this ancestrally derived scheme of sucrose metabolism and transport is the pivotal role played by a versatile enzyme Proton Pyrophosphatase (H⁺-PPase) in
maintaining intracellular pyrophosphate homeostasis. Itself an evolutionarily ancient enzyme, H+ -PPases are remarkably well-conserved across the entire plant kingdom, and our results indicate that the dual membrane-specificity of this protein is a feature common to perhaps all plants. The dual location of this enzyme is in fact indicative of its structurally congruent potential to work in a reversible manner – either in the synthesis or hydrolysis of its substrate, pyrophosphate. This dual membrane-specificity and the concomitant reversibility is not a novel plant-specific attribute of this elegant pump, because in *Rhodospirillum rubrum* (a phototrophic purple non-sulfur bacteria), H+-PPase is bound to either chromatophore or acidocalcisome membranes, wherein this protein can function as a pyrophosphate synthase or a hydrolase, respectively (Baltscheffsky et al., 1966, Moyle et al., 1972, Seufferheld et al., 2004, Baltscheffsky et al., 1999).

Overall, this dissertation has attempted to shed some light on how the basic mechanisms of photosynthate transport are conserved even in evolutionarily divergent organisms. In this regard, Physcomitrella is an obvious and attractive candidate for research. Given that plant physiological research in mosses were last seriously performed more than two decades ago, and aided by the technology at our disposal today, makes Physcomitrella ideally poised for physiological investigations with the potential to reveal new and evolutionarily fundamental insights into the early adaptations required for the terrestrial conquest of land by plants. Within this purview, future research directions include generating comparative three-dimensional tomograms of plasmodesmata from Physcomitrella and other advanced lineages of land plants. Currently, work is also underway to generate inducible H+-PPase knockdown mutants in Physcomitrella to test
our nascent hypothesis that the role of this enzyme ascribed to in vascular plants like Arabidopsis is also conserved in a basal lineage of non-vascular plant.
REFERENCES


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APPENDIX A

MOVIE S1: 3D MODEL OF SIMPLE PLASMODESMATA

[Consult Attached Files]

Movies can be viewed with Quicktime Player.

Movie S1: Three-dimensional model of simple plasmodesmata across the bundle sheath – mestome sheath cell interface (blue: outer plasma membrane; green: inner plasma membrane; magenta: desmotubule)
APPENDIX B

MOVIE S2: 3D MODEL OF SIMPLE PLASMODESMATA

[Consult Attached Files]

Movies can be viewed with Quicktime Player.

APPENDIX C

MOVIE S3: 3D MODEL OF COMPLEX PLASMODESMATA

[Consult Attached Files]

Movies can be viewed with Quicktime Player.

Movie S3: Three-dimensional model of simple, twinned, and branched plasmodesmata across the vascular parenchyma – companion cell interface (blue: outer plasma membrane; green: inner plasma membrane; magenta: desmotubule).
APPENDIX D

MOVIE S4: MODELING SIMPLE PLASMODESMATA IN IMOD

[Consult Attached Files]

Movies can be viewed with Quicktime Player.

APPENDIX E

MOVIE S5: MODELING SIMPLE PLASMODESMATA IN IMOD

[Consult Attached Files]

Movies can be viewed with Quicktime Player.

APPENDIX F

MOVIE S6: MODELING COMPLEX PLASMODESMATA IN IMOD

[Consult Attached Files]

Movies can be viewed with Quicktime Player.

Movie S6: Modeling of simple, twinned, and branched plasmodesmata across the vascular parenchyma – companion cell interface as self-enclosed contours (blue: outer plasma membrane; green: inner plasma membrane; magenta: desmotubule)