Apoplastic loading in the rice phloem supported by the presence of sucrose synthase and plasma membrane-localized proton pyrophosphatase

Kamesh C. Regmi, Shangji Zhang and Roberto A. Gaxiola*

School of Life Sciences, Arizona State University, 427 E. Tyler Mall, Tempe, AZ 85287, USA

*For correspondence. E-mail roberto.gaxiola@asu.edu

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• Background and Aims Although Oryza sativa (rice) is one of the most important cereal crops, the mechanism by which sucrose, the major photosynthate, is loaded into its phloem is still a matter of debate. Current opinion holds that the phloem loading pathway in rice could involve either a symplasmic or an apoplasmic route. It was hypothesized, on the basis of a complementary body of evidence from arabidopsis, which is an apoplasmic 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, it was contended that the presence of sucrose synthase in the SE-CC complexes would be consistent with an apoplasmic sucrose loading route in rice.

• Methods Conventional chemical fixation methods were used for immunohistochemical localization of H\(^+\)-PPases and sucrose synthase at the light microscopy level, while ultrastructural immunogold labelling of H\(^+\)-PPases and sucrose synthase was performed on high-pressure frozen source leaves of rice.

• Key Results Using immunogold labelling, it was found that OVPs predominantly localize at the plasma membrane (PM) of the SE-CC complexes in rice source leaf minor veins, while in the root meristematic cells, OVPs preferentially localize at the vacuoles. The 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 labelling at the SE-CC complexes of rice source leaves.

• Conclusion These findings are consistent with the idea that, in the same way as in arabidopsis and a majority of grasses, sucrose is actively loaded into the SE-CC complexes of rice leaves using an apoplastic step.

Key words: Rice, sieve element–companion cell complex, phloem loading, symplasmic loading, apoplastic loading, proton pyrophosphatase, sucrose synthase.

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 photosynthetically active source leaves to the heterotrophic tissues such as 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 apoplastic – is still a matter of debate (Kaneko et al., 1980; Chonan et al., 1984; Lim et al., 2006; Scofield et al., 2007; Eom et al., 2012; Braun et al., 2014).

On the basis of the concentration and form of transported sugars, the 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 the passive symplasmic Suc loading strategy. 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 (OsSUT2s) 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 apoplastic loading strategy (Braun et al., 2014).

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 apoplas (Eom et al., 2012). Consequently, there would be no need to maintain a plasma membrane (PM) ATPase-dependent transmembrane 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 load Suc actively from the apoplas 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 (Geigenberger et al., 1993; Lerchl et al., 1995). Two of the enzymes involved in this so-called SUS pathway are pyrophosphate (PPi)-dependent fructose 6-phosphate 1-phosphotransferase (FPP) and the UDP-glucose pyrophosphorylase (UGPase) that work near equilibrium (Geigenberger et al., 1993). A decrease in the cytosolic concentration of PPi 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, PPi, 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 PPi-driven proton pump that takes part in cellular PPi, homeostasis (Ferjani et al., 2011; Pizzio et al., 2015). Ubiquitous in the plant kingdom, this highly evolutionarily conserved protein (Seufferlein et al., 2011) can either use the energy derived from PPi, hydrolysis to pump protons across membranes (Maeshima and Yoshida, 1989; Maeshima, 2000) or, under a favourable proton gradient, work reversibly to synthesize PPi (Rocha Façana and de Meis, 1998; Marsh et al., 2000). Vacular localization of H\(^+\)-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 PPi, liberated as a by-product of macromolecular biosynthesis would not only be scavenged by membrane-bound H\(^+\)-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 built up over the years to demonstrate a PM localization of H\(^+\)-PPase in the SE-CC complexes (DeWitt et al., 1991; Long et al., 1995; Langhans et al., 2001; Paez-Valencia et al., 2011), wherein the thermodynamic limitations imposed by the prevailing PMF preclude the proton pumping activity coupled to PPi, hydrolysis (Davies et al., 1997). The thermodynamic conditions are instead conducive to PPi 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 (Rocha Façana and de Meis, 1998; Marsh et al., 2000). Added to this is the indispensability of PPi 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\(^+\)-PPase could function as a PPi 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 multigene family in rice with six paralogues (Oryza sativa vacuolar pyrophosphatases; OVP1–OVP6) hitherto identified (Sakakibara et al., 1999; Choura and Rebai, 2005; Liu et al., 2010; Muto et al., 2011). 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 such as 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 the OVP3 promotor 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 the 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 many seeds as 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 the 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 PPi, and acidify the vacuolar lumen. In contrast, 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 PPi, 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 labelling 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 veins, 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 medium (Yang et al., 2007) on Petri plates at 37 °C in the dark for 48 h. Once the post-germinative process, characterized by the emergence of the radicle, had begun, the plates were transferred to a growth chamber with a 16 h light and 8 h 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 (approx. 3–4 weeks). The tissues for subsequent immunohistochemical, immunogold and immunoblot
analysis were harvested either from soil-grown (leaves, leaf sheaths and flowers) or from plate-grown (root tips and 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 h at 4 °C and allowed to grow until 45 d old or until inflorescent stems had fully developed flowers (rosette leaf/stem/flower immunohistochemistry). Sterilized Col-0 seeds were sown into liquid 1/2 strength Murashige and Skoog (MS) medium supplemented with 1 % sucrose, grown for 7 d in a flask with constant agitation and subsequently used for protein extraction (see below).

Wild-type Col-0 arabidopsis seeds bought from a local grocery store were germinated on wet soil, and allowed to grow in a growth chamber with a 16 h light and 8 h dark regime until the fifth leaf had emerged and the first leaf was fully grown.

Wild-type Physcomitrella patens (Hedw.) were grown on wet soil, with constant fertilization with PPNH 4 liquid medium in a growth chamber with a 16 h light and 8 h dark regime. Growth medium was prepared according to: http://www.bezanillalab.com/Moss%20Manual.pdf.

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

**Light microscopy and immunohistochemistry**

Once the rice plants grew the fifth leaf and the flag leaf had fully grown, the flag leaves and leaf sheaths of rice were excised. After approx. 2 weeks on vertical solid rice medium in Petri plates, the rice seedlings had grown crown and multiple adventitious roots. The crown roots were excised. Once adult plants grown in the greenhouse had developed flowers these were excised.

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

Once the first leaf of maize had fully developed (approx. 4 weeks), it was excised. Two leaves were excised directly from an adult P. fremontii tree.

For immunohistochemistry, the tissues excised were cut into small pieces and immediately immersed in at least a 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 min 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 the desired orientations. Using a Leica RM2155 rotary microtome (Leica Microsystems, Germany; www.leica-microsystems.com), the tissue blocks were cut into 10 μm thick sections, and mounted on poly-l-lysine-coated slides. The tissues were then deparaaffinized, rehydrated and exposed to an antigen retrieval buffer (Biogenex, USA; www.biogenex.com) in a 65 °C water bath for 20 min. After washing in water, the endogenous peroxidase activity was quenched using 3 % (v/v) H 2O 2. 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 min 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 h 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 the manufacturer’s instructions. The tissues were then run through a dehydrating ethanol series, and were permanently mounted. Images were acquired with a Zeiss Axioskop light microscope (Leica Microsystems, Germany) equipped with ×10 Achroplan 0.25 NA Zeiss, ×40 Plan Neo-Fluar 0.75 NA Zeiss and ×100 1.30 NA Oil Plan-Neo-Fluar objective lenses, phase and differential interference contrast optics, an 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 a fine powder with liquid N 2 and transferred in approx. 200 μL aliquots to screw-cap Eppendorf tubes. To each tube, 1 mL 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 10000 g for 30 min 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 10000 g for 10 min 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 approx. 30 min. 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 5000 g for 5 min at 4 °C three times.

**Western blot**

The solubilized protein extracts from maize, 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 dilutions overnight at 4 °C. After washing in TBST, the membrane was developed using a Bio-rad Alkaline Phosphatase Immun-Blot Kit (Bio-Rad Inc., USA; www.bio-rad.com) according to the manufacturer’s instructions.

**Immunogold labelling and transmission electron microscopy**

For high-pressure freezing (HPF) and freeze-substitution (FS), 15-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 150 mm Suc, and quickly
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). A previous in situ hybridization study showed that AVP1 expression at the transcriptional level was maintained in the root pericycle, shoot apical meristem (SAM), floral meristem, leaf and floral primordia, and procambium cells in arabidopsis (Li et al., 2005), while green fluorescent protein (GFP)-tagged AVP1 was observed in ovules, funicule, 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 the 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 CTKAADYGADLVGIE 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. 1A–F). Specifically, active sink tissues of arabidopsis such as the SAM (Fig. 1A), leaf primordia (Fig. 1A), vascular cambium (Fig. 1B, C), ovules (Fig. 1E) and pollen (Fig. 1F) displayed the strongest signal. AVP1 was also prominently expressed in the phloem of leaves and inflorescent stem (Fig. 1A, C, D). Using arabidopsis as a reference, we also performed a parallel immunohistochemical survey of the OVP expression pattern in various tissues of rice. As expected, the OVPs were distinctly present in young proliferative sink tissues such as the SAM (Fig. 1G), emergent leaf sheaths (Fig. 1G, I), root primordium (Fig. 1H), growing embryo (Fig. 1K) and pollen (Fig. 1L). OVPs were also distinctly localized in the vascular tissues of rice source leaves (Fig. 1J) and leaf sheaths (Fig. 1I). Representative negative controls using pre-immune serum (Fig. 1M–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. 1G–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. 2A, C), and also in the lateral root primordium (Fig. 2A, B). Given the amenability of root tips to HPF, we proceeded to immunolocalize OVPs in the root apical meristematic cells of rice roots at the ultrastructural level. Unsurprisingly, upon immunogold labelling, it was found that the OVPs preferentially localized at the vacuoles (Fig. 2E–G, J) in these metabolically active cells characterized by dense, ribosome-rich cytoplasm, an extensive endomembrane system and turgescent nuclei. Negative controls performed with pre-immune serum showed that the immunogold PM labelling was OVP specific (Fig. 2H, I).
FIG. 1. 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 (SAM; 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 labelled. (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 SAM (asterisk), and the leaf sheath primordia (p1 and 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 with older leaf sheaths (p4 and 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–O) Phase-contrast micrographs of representative negative controls using pre-immune sera for Arabidopsis (M) and rice (N and O). SAMs in (M) and (N) are marked with an asterisk, while leaf primordium is labelled 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.
OVPs preferentially localize to the plasma membrane in SE–CC complexes in rice source leaves

To present a coherent model of OVP action in rice, we 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 apoplasmic loader, is consistent with the model that implicates AVP1 as a PM-localized protein working reversibly to function as a PPi synthase rather than the canonical proton-pumping PPase at the SE–CC complexes (Gaxiola et al., 2012).

The current opinion on the phloem loading mechanism in rice is divided (Eom et al., 2012; Braun et al., 2014). While Eom et al. (2012) proposed a novel ‘revised diffusion model’ that postulates a modified passive symplasmic route of Suc loading, Braun et al. (2014) advanced the idea that rice uses an apoplasmic loading mechanism instead. Herein, a vacuolar localization of OVPs in the SE–CC complexes of rice source veins would be expected under the former model, while PM localization of OVPs would favour 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

FIG. 2. Immunolocalization of OVPs in rice roots at both the light (A–C) and electron microscopic (E–G) level. (A) Transverse section through a rice root showing OVP localization in the lateral root primordium (arrow) and the vascular cylinder. (B and 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 labelled 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 (mean = 2.17, s.d. = 0.98) than at the PM (mean = 0.14, s.d. = 0.24); t(15) = 8.52, p = 3.9 x 10⁻⁷. Scale bars: (A) = 100 µm; (B–D) 20 µm; (E) 2 µm; (F) 250 nm; (G) 1 µm; (H) 5 µm; (I) 1 µm, mx, metaxylem, m, mitochondrion; n, nucleus.
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 PP₁-dependent, ATP-conserving 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-immunolocalize SUS in the SE-CC complexes of rice source ATP-conserving SUS pathway. Therefore, we proceeded to labelling was OVP specific (Supplementary Data Fig. S1). These pre-immune serum showed that the observed immunogold labelling was OVP specific (Supplementary Data Fig. S1). These results are consistent with the idea that rice uses an apoplastic Suc loading strategy (Braun et al., 2014).

DISCUSSION

The salient feature of the H⁺-PPase expression pattern in angiosperms, at both the transcript and protein level, is the conspicuous localization observed at both the actively dividing regions (e.g. apical meristems, and leaf and root primordia) and the phloem (DeWitt et al., 1991; Long et al., 1995; Ratajczak et al., 1999; Gaxiola et al., 2001; Langhans et al., 2001; Li et al., 2005; Yang et al., 2007; Paez-Valencia et al., 2011; Viotti et al., 2013; Segami et al., 2014). Additionally, when driven by either a 1-4 or a 1-7 kb AVP1 promoter, β-glucuronidase was also strongly expressed in the phloem and sink tissues in arabidopsis (Mitsuda et al., 2001; Pizzio et al., 2015). 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. 1A–F) and rice (Figs 1G–L and 2A–C), respectively. The immunohistochemical localization of H⁺-PPase was closely correlated between arabidopsis and rice (Fig. 1) 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 and Yoshida, 1989; Rea et al., 1992; Maeshima, 2000). This notion is in agreement 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 PP₁ is produced as a by-product of these anabolic processes (Maeshima, 2000; Heinonen, 2001). PP₁ accumulation in the cytosol in high concentrations would inhibit these polymerization reactions (Shiratake et al., 1997; Maeshima, 2000). The membrane-bound H⁺-PPase scavenge the PP₁ 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 (Figs 1 and 2A–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. 2E–G).

In contrast, the role of H⁺-PPase in the phloem has remained mostly enigmatic. At least in arabidopsis, an apoplastic loader, the importance of H⁺-PPase in phloem function and photosynthetic 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 (Scofield et al., 2007; Eom et al., 2012; Braun et al., 2014). 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 apoplastic loading strategy.

The phloem, composed of Suc-translocating, but apparently inactive SEs, and their metabolically active, and ontogenetically linked sister CCs, is the site 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,
FIG. 3. Immunogold labelling of OVPs in the sieve element–companion cell (SE-CC) complexes of rice source leaf minor veins through both longitudinal and transverse sections. (A–C) Serial section electron micrographs showing longitudinal section through the SE-CCs. (A1–C1) Higher magnification micrographs of the boxed regions from (A), (B) and (C), respectively, showing 10 nm gold particles decorating the plasma membrane (PM) of both SEs and CCs (arrows). (A2–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 and E) Transverse section through the rice source leaf minor vein corroborates immunogold labelling from (A1–C1; A2–C2), with distinct OVP localization at the PM of both SEs (E) and CCs (D). (D1 and E1) Higher magnification insets of the boxed regions in (D) and (E), respectively, showing immunogold labelling of the PM. (F) Quantification of gold labelling 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 (mean = 6.53, s.d. = 2.49) was not significantly different from the mean number of gold particles per µm of CC PM (mean = 5.43, s.d. = 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 (mean = 0.78, s.d. = 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.
apoplastic process, or a passive, symplasmic one (Rennie and Turgeon, 2009). Within this scope, the immunogold localization of AVP1 at the PM of SE-CC complexes in arabidopsis, an apoplastic loader (Paez-Valencia et al., 2011), suggested a rather different role for 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; Long et al., 1995; Langhans et al., 2001), the canonical role of this protein as a proton pumping PP\(_i\) 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 PP\(_i\) synthase was instead postulated (Davies et al., 1997), and was later demonstrated in vitro in maize and oranges (Rocha Fac¸anha and de Meis, 1998; Marsh et al., 2000).

Fig. 4. Immunohistochemical localization of sucrose synthase (SUS) in maize, rice, arabidopsis and poplar source leaves, and immunogold labelling of SUS in the SE-CC complexes of rice source leaf minor veins. (A, C, E and G) Transverse sections through source leaves of maize (A), rice (C) and arabidopsis (E) showing distinct localization of SUS in the phloem tissue (arrows). In contrast, a known symplasmic loader, poplar (Populus fremontii), has distinct SUS localization in the xylem (arrows). (B, D, F and 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 shown (arrowheads). The molecular weight (MW) is marked, and the two bands seen correspond to 102 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 plasm membrane, 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.
Additional evidence showed the requirement for PP, in sustaining the 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 PP\(_i\) synthase in the phloem (Gaxiola et al. 2012). Using arabidopsis, it was recently shown that the phloem-specific knock-down of the AVP1-encoding gene resulted in the stunted growth of both root and shoot, and the phloem expression of soluble PPase triggered a dwarf phenotype (Pizzio et al. 2015). This supports the idea that the PM-localized AVP1 and PP\(_i\) homeostasis are crucial in the arabidopsis phloem, an apoplastic loading species (Pizzio et al. 2015).

In lieu of the complementary set of evidence from apoplasmically 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 (Eom et al. 2012). We discounted the idea that rice harbours a polymer-trapping mechanism on the basis that neither we nor previous anatomical studies found specialized intermediary cells in the rice phloem (Kaneko et al., 1980; Chonan et al., 1984). OVP-specific immunogold labelling on cryofixed rice source leaves revealed a predominantly PM localization at the SE-CC complexes (Fig. 3; Supplementary Data 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 paralogues in rice have been identified to date in the rice genome (Aoki et al., 2003), of which 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 (Sun et al., 2010; Braun et al., 2014). In addition, the rice genome also the contains SWEET sucrose effluxer family of approx. 21 paralogues (Chen et al., 2010) that would be unnecessary if rice used a symplasmic loading mechanism (Braun et al., 2014).

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 harbour an ATP-conserving, PP\(_i\)-cycling, SUS-dependent pathway. It has long been known that the energy status of the CCs in apoplastic loaders such as Ricinus is primarily regulated by the reversible throughput of Suc into UDP-glucose and fructose – a reaction catalysed 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. 4A, E). The tissue-specific transcriptomic profiles of the six SUS paralogues in rice have also been previously characterized, and of particular interest is the finding that the SUS2 paralogue was ubiquitously expressed, including in the source leaf blades (Hirose et al., 2008).

In contrast, the mRNA expression profile of three SUS paralogues (PtSUS1–PtSUS3) 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 agreement with this, immunohistochemical localization of SUS in P. fremontii source leaves showed a distinct localization in the xylem, but visibly less so in the phloem (Fig. 4G), especially when compared with arabidopsis, rice and maize (Fig. 4A–F). Interestingly, transgenic poplar engineered to overexpress constitutively a H\(^+\)-PPase orthologue (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 upregulation 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 such as arabidopsis (Gaxiola et al., 2012). No gain in biomass in PtVP1.1-overexpressing poplar suggests that non-polymer trapping, passive symplasmic loaders such as poplar do not shelter an 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 labelling, SUS was partiael localiized in proximity to the PM of the SE-CC complexes (Fig. 4J–L). This result gave 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 correlaive, and a confirmation of an apoplastic loading mechanism in rice would require further experiments including, but not limited to, p-chloromercuribenzenesulphonic acid (PCMS) assay (Giaquinta, 1979), and generating rice plants with phloem-specific knock-down of OVPs, overexpression of cell wall invertase and phloem-specific overexpression of soluble pyrophosphatase.

**SUPPLEMENTARY DATA**

Supplementary data are available online at www.aob.oxfordjournals.org and consist of the following. Figure S1: negative controls for the immunogold labelling of OVPs in SE-CC complexes of rice source leaves and, upon immunogold labelling, SUS was parietally localized in proximity to the PM of the SE-CC complexes (Fig. 4J–L). This result gave 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 an apoplastic loading mechanism in rice would require further experiments including, but not limited to, p-chloromercuribenzenesulphonic acid (PCMS) assay (Giaquinta, 1979), and generating rice plants with phloem-specific knock-down of OVPs, overexpression of cell wall invertase and phloem-specific overexpression of soluble pyrophosphatase.

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