

A H⁺-ATPase That Energizes Nutrient Uptake during Mycorrhizal Symbioses in Rice and *Medicago truncatula* CWJOPEN

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Most plant species form symbioses with arbuscular mycorrhizal (AM) fungi, which facilitate the uptake of mineral nutrients such as phosphate from the soil. Several transporters, particularly proton-coupled phosphate transporters, have been identified on both the plant and fungal membranes and contribute to delivering phosphate from fungi to plants. The mechanism of nutrient exchange has been studied in plants during mycorrhizal colonization, but the source of the electrochemical proton gradient that drives nutrient exchange is not known. Here, we show that plasma membrane H⁺-ATPases that are specifically induced in arbuscule-containing cells are required for enhanced proton pumping activity in membrane vesicles from AM-colonized roots of rice (*Oryza sativa*) and *Medicago truncatula*. Mutation of the H⁺-ATPases reduced arbuscule size and impaired nutrient uptake by the host plant through the mycorrhizal symbiosis. Overexpression of the H⁺-ATPase Os-HA1 increased both phosphate uptake and the plasma membrane potential, suggesting that this H⁺-ATPase plays a key role in energizing the periarbuscular membrane, thereby facilitating nutrient exchange in arbusculated plant cells.

INTRODUCTION

Arbuscular mycorrhizal (AM) fungi associate with the majority of plant species and facilitate the uptake of mineral nutrients, such as phosphate (Parniske, 2008; Bonfante and Genre, 2010). In addition to improved nutrient supply, the AM symbiosis also provides tolerance to biotic and abiotic stresses (Parniske, 2008; Bonfante and Genre, 2010; Smith and Smith, 2011b). Formation of the AM symbiosis is achieved through the exchange of chemical signals between the plant and the fungus (Bonfante and Requena, 2011; Oldroyd, 2013), with strigolactone release from the plant root acting as an early step in this chemical communication. Strigolactones promote germination of fungal spores and stimulate hyphal branching, precursors of fungal colonization of

the plant (Bonfante and Genre, 2010; Harrison, 2012; Oldroyd, 2013). In turn, the AM fungus produces lipochitoooligosaccharides and chitoooligosaccharides that are recognized by the host plant to activate the symbiosis signaling pathway (Maillet et al., 2011; Genre et al., 2013).

Upon physical contact between the fungus and the surface of the root, swollen fungal infection structures, hyphopodia, are formed and these are facilitated by plant-derived cutin signals (Wang et al., 2012). Once the epidermal cell layer is breached, the fungus grows intercellularly through the root cortex and forms highly branched structures, called arbuscules, within inner root cortical cells (Harrison, 2005; Parniske, 2008). The developing arbuscule remains surrounded by plant cell membrane, the periarbuscular membrane, which is contiguous with, but distinct from the plasma membrane (Pumplin and Harrison, 2009). The arbuscule provides a large interface for nutrient exchange (Parniske, 2008; Bonfante and Genre, 2010) and is the location of specific proton-coupled phosphate transporters within the periarbuscular membrane (Rausch et al., 2001; Harrison et al., 2002; Paszkowski et al., 2002; Yang et al., 2012).

In plant cellular systems, transport of solutes and metabolites into and out of the cell are driven predominantly by an H⁺ electrochemical gradient. This gradient is produced by the activity of plasma membrane H⁺-ATPases, which are found in both plants and fungi (Sondergaard et al., 2004). The extrusion of a positive charge, in the form of protons, results in a membrane potential (negative on the inside) and this can energize the movement of positively charged ions into the cell. H⁺-ATPases play diverse roles in signal transduction during cell expansion, regulation of

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www.plantcell.org/cgi/doi/10.1105/tpc.113.120527

intracellular pH, response of the plant to salt stress, and regulation of stomatal opening (Palmgren, 2001; Sondergaard et al., 2004; Merlot et al., 2007). H⁺-ATPases are also involved in the early stages of pathogen recognition and are associated with the regulation of stomatal opening to hinder entry of bacterial pathogens (Liu et al., 2009; Elmore and Coaker, 2011).

Cytochemical studies have shown proton pumping activity associated with both plant and fungal membranes at the arbuscular interface, consistent with active transport processes occurring at this location (Gianinazzi-Pearson et al., 1991). These results suggest that protons translocated by the pump and accumulating in the periarbuscular space may provide the motive force that drives the uptake of phosphate into the root cortical cells and the uptake of sugars into fungal cells (Gianinazzi-Pearson et al., 1991; Palmgren, 2001; Sondergaard et al., 2004). Arbuscule-specific expression of H⁺-ATPases has been reported for tobacco (*Nicotiana tabacum*) and *Medicago truncatula* (Gianinazzi-Pearson et al., 2000; Krajinski et al., 2002), but the function of these ATPases has yet to be assigned. Here, we use genetic and biochemical data to show that plasma membrane H⁺-ATPases of rice (*Oryza sativa*) and *M. truncatula* are essential to sustain mycorrhizal colonization and required for symbiotic phosphate transport. Our data suggest a critical role for these H⁺-ATPases in energizing the periarbuscular membrane for symbiotic nutrient exchange between the fungus and the plant.

RESULTS

Proton Pumping Activity Increases in Roots Colonized by Mycorrhizal Fungi

Proton-coupled phosphate transporters have been identified on both the fungal arbuscular membrane and the plant periarbuscular membrane (Harrison and van Buuren, 1995; Rausch et al., 2001; Harrison et al., 2002; Paszkowski et al., 2002), and it is thought that a proton gradient energizes the membrane for nutrient exchange (Gianinazzi-Pearson et al., 1991; Palmgren, 2001; Sondergaard et al., 2004). To validate this idea, plasma membrane vesicles were isolated from colonized roots and tested for H⁺-pumping activity, measured as a decrease of acridine orange absorbance at 495 nm. The plasma membrane proton pumping activity of rice and *M. truncatula* increased by ~40%, 6 weeks postinoculation with *Rhizophagus irregularis* (Figures 1A and 1B). Considering that H⁺-ATPases are induced upon AM colonization (Gianinazzi-Pearson et al., 2000; Krajinski et al., 2002), it seemed likely that the increased activity we observed was at least partly a result of these AM-induced pumps.

Rice contains eight potential H⁺-ATPases in the genome, with all gene families represented (Figure 2A). One of these gene family members, *Os03g0100800/LOC_Os03g01120* (from here on referred to as *Os-HA1*), is highly induced 5 weeks postinoculation with *R. irregularis* (Supplemental Figure 1), suggesting that it is a good candidate for the increased H⁺-pumping activity present in AM-colonized rice roots. This induction was validated with a time course following AM inoculation. *Os-HA1* is highly induced at later stages of the AM symbiosis, and this is in good agreement with the total level of AM colonization (Figures 2B and 2C). Arbuscules develop at later stages of the mycorrhizal association, and the time points at which *Os-HA1* shows induction, 28 and 35 d

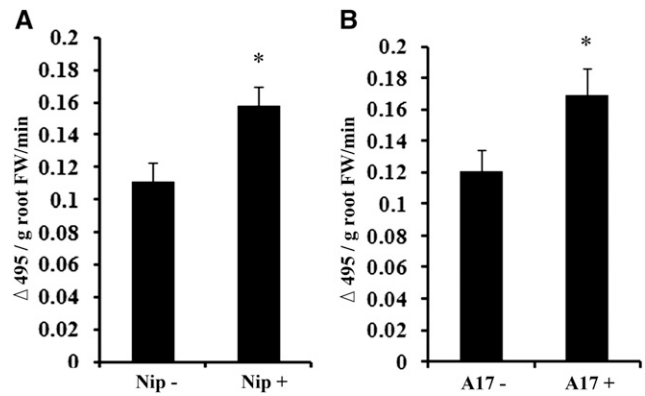


Figure 1. Plasma Membrane H⁺-Pumping Activity Increases in Mycorrhizal Roots of Rice and *M. truncatula*.

Plasma membrane vesicles were isolated from plants mock-inoculated (–) and inoculated (+) with *R. irregularis* for 6 weeks. Quenching of acridine orange was monitored as a measure for intravesicular acidification. Optical density at 495 nm was measured 0 and 5 min after addition of MgSO₄ and the relative change per minute and gram fresh weight (FW) calculated. Rice Nipponbare (Nip) (A) and *M. truncatula* A17 (B). Statistical significance (*P < 0.05) was evaluated using Student's *t* test. Error bars are SE (*n* = 3).

postinoculation, is consistent with the timing of arbuscule development. Furthermore, laser microdissection experiments showed an order of magnitude higher *Os-HA1* expression in arbuscule-containing cells compared with nonarbusculated root cortical cells (Uta Paszkowski, personal communication).

A Rice *ha1* Mutant Is Impaired in Mycorrhizal Colonization, Arbuscule Development, and Nutrient Uptake

To test whether *Os-HA1* is required for the mycorrhizal interaction, we identified plants carrying mutations in the *Os-HA1* gene from publicly available rice mutant collections (<http://signal.salk.edu/cgi-bin/RiceGE>). One line carrying a *Tos17* transposon insertion (T00086T) in exon 12 of *Os-HA1* was identified (Figure 3A). The presence of the transposon within *Os-HA1* in the mutant line (named *Os-ha1*) was confirmed by PCR and sequencing of the insertion site. To test the effect of the mutation on phosphate (Pi) uptake and growth in the presence and absence of AM symbiosis, the *Os-ha1* mutant and wild-type plants were inoculated with *R. irregularis* and grown under conditions of low phosphate supply. Shoot fresh weight did not differ significantly between inoculated and mock-inoculated plants, but shoot Pi levels increased by 25% in inoculated wild-type plants (Figures 3B and 3C). By contrast, there was no difference in shoot Pi levels between inoculated and noninoculated *Os-ha1* mutant plants (Figure 3B).

To characterize the mycorrhizal interaction in *Os-ha1*, plants were inoculated with *R. irregularis* and the degree of colonization was counted 6 weeks postinoculation. In wild-type plants, arbuscules were well developed and showed a high degree of branching, and total colonization levels reached 55% of the root length (Figures 3D and 3E). By contrast, in *Os-ha1* roots, the numbers of arbuscules were reduced to 25% of root length (Figure 3D; Supplemental Figure 2A). The defect in mycorrhizal

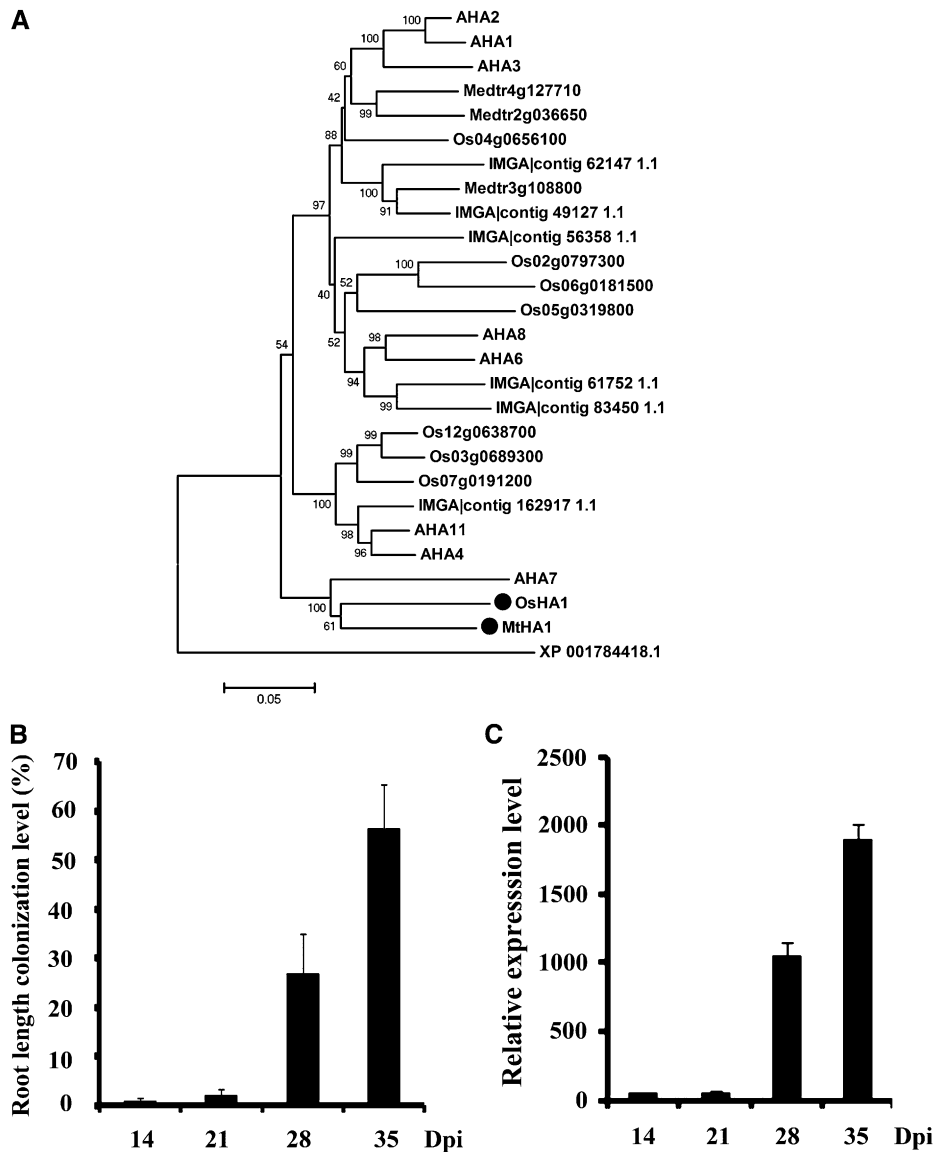


Figure 2. H⁺-ATPase Phylogenetic Tree and Expression Pattern during Mycorrhizal Colonization.

(A) Neighbor-joining phylogenetic tree of H⁺-ATPases from *Arabidopsis*, rice (*Os*), and *M. truncatula* (Medtr or IMG|contig). H⁺-ATPase from *Physcomitrella patens* was used as an outgroup. Branch support was obtained from 1000 bootstrap repetitions.

(B) Quantification of *R. irregularis* colonization in wild-type rice plants at 14, 21, 28, and 35 days postinoculation (Dpi).

(C) Time course of relative expression of *Os03g0100800* (*Os-HA1*) upon inoculation with *R. irregularis*. These experiments were repeated three times with similar results. Error bars are SE.

colonization cosegregated with the insertion within *Os-HA1* in a segregating population of 119 plants, suggesting the *Tos17* insertion in *Os-HA1* is causative of the mycorrhizal defect. Furthermore, the level of *Os-HA1* expression was greatly reduced in the *Os-ha1* mutant (Figure 3F). By contrast, the level of expression of the genes immediately upstream and downstream of *Os-HA1* did not significantly differ, suggesting that this *Tos17* insertion specifically affects *Os-HA1*. Taken together, we conclude that *Os-HA1* is required for normal arbuscule development in rice.

The phenotype of mutants may vary with the fungal species tested (Gao et al., 2001; Manjarrez et al., 2009). Therefore, we

assessed the role of *Os-HA1* for arbuscule development with *Gigaspora rosea*, which is present in a phylogenetic clade different to that of *R. irregularis* (Schüßler et al., 2001). The *Os-ha1* mutant showed reduced levels of AM colonization (Supplemental Figures 2B and 2C), implying that *Os-HA1* is essential for normal AM colonization with at least two different AM fungi.

Os-HA1 Is an Active Plasma Membrane H⁺-ATPase

To test the H⁺-ATPase activity of *Os-HA1*, we first measured plasma membrane proton pumping activity in wild-type and mutant roots.

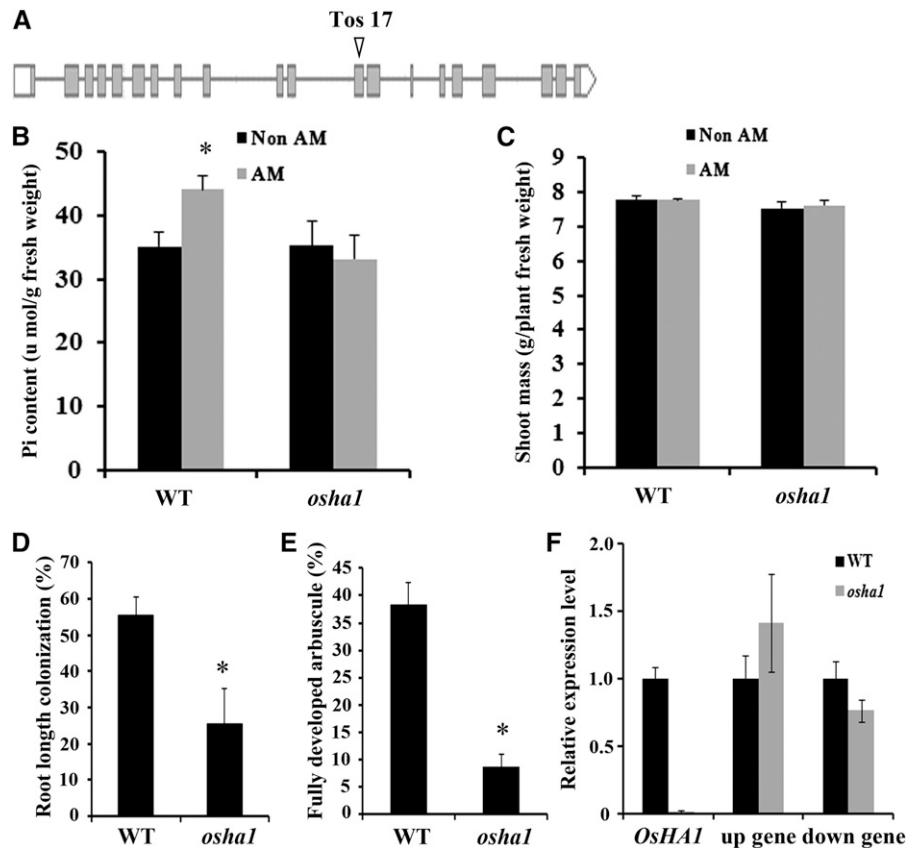


Figure 3. *Os-HA1* Is Required for Normal Arbuscule Development and Function in Rice.

(A) *Os-HA1* (*Os03g0100800/LOC_Os03g01120*) has 20 exons, and a single *Tos17* insertion was identified that has an insertion in exon 12. (B) Increase of Pi levels in wild-type shoots, but not in *Os-ha1*. $P < 0.05$ compared with the wild type without mycorrhizal inoculation (Student's *t* test). (C) No increase of shoot mass in response to mycorrhizal symbiosis 9 weeks postinoculation. (D) Quantification of *R. irregularis* colonization at 6 weeks post inoculation. (E) Quantification of arbuscule formation during *R. irregularis* colonization at 6 weeks post inoculation. (F) Expression level of *Os-HA1* (*LOC_Os03g01120*) and the genes located immediately upstream (*LOC_Os03g01110*; up gene) and downstream (*LOC_Os03g01130*; down gene) in 2-week-old wild-type and *Os-ha1* mutant root without inoculation with *R. irregularis*. The experiments were replicated two times. * $P < 0.05$ compared with the wild type (Student's *t* test) in (D) and (E). Error bars are SE ($n = 12$). This is a representative experiment that was repeated three times in (B) to (E).

The H⁺-ATPase activity of mycorrhizal colonized *Os-ha1* mutant roots 5 weeks postinoculation was 70% of that of the inoculated wild-type roots, and at a level similar to that of uninoculated wild type (Figure 4A). This suggests that a large proportion of the stimulation in H⁺-ATPase activity is based on the activity of *Os-HA1*. However, we cannot discriminate whether this difference is the result of less proton ATPase activity or fewer arbuscules in *Os-ha1*. We therefore overexpressed *Os-HA1* under the control of the cauliflower mosaic virus 35S promoter using hairy root transformation in *M. truncatula*. The plasma membrane H⁺-ATPase activity increased significantly in roots of OE-*OsHA1* lines in two separate experiments (Figure 4B), consistent with a high expression level of *Os-HA1* in transgenic plants (Supplemental Figure 3A). This supports the idea that *Os-HA1* directly functions in the H⁺-ATPase activity, and consistent with this, a fusion protein of *Os-HA1* and green fluorescent

protein (GFP) localized to the plasma membrane in *M. truncatula* roots (Supplemental Figure 3B).

M. truncatula HA1 Is Required for Appropriate AM Associations

M. truncatula Tnt1 transposon insertion lines were tested in a forward genetic screen for reduced colonization by arbuscular mycorrhizal fungi as described (Tadege et al., 2008). In mutant lines showing reduced colonization, insertion sites were identified and sequenced as described by Ratet et al. (2010). We identified a line, NF0905 (*Mt-ha1-1*), carrying an insertion in exon 15 of *Mt-HA1*, which encodes a predicted plasma membrane H⁺-ATPase (Figure 5A). Homozygous progeny arising from a backcross with the wild type (Figure 5B) showed a strongly reduced level of *Mt-HA1* transcripts compared with the wild type (Figure 5C). *Mt-HA1* is

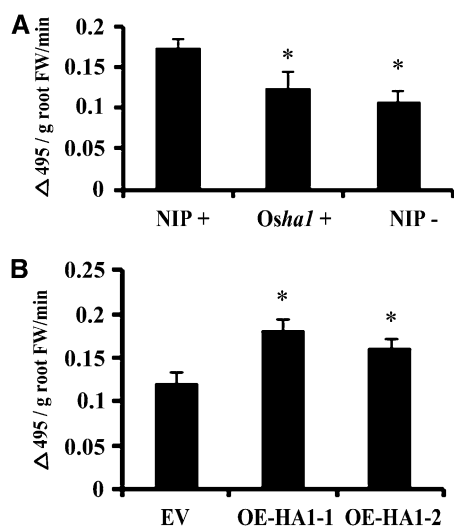


Figure 4. Os-HA1 Is Active as a Plasma Membrane H⁺-Pump.

(A) Os-HA1 is required for the increase in plasma membrane H⁺-pumping during AM symbiosis in rice. Δ495 was recorded as a measure for H⁺-pumping activity in roots as in Figure 1. Statistical significance (*P < 0.05) was evaluated using Student's *t* test. This is a representative experiment that was repeated two times. Error bars are SE.

(B) Overexpressing Os-HA1 in *M. truncatula* increased plasma membrane H⁺-pumping activity of the roots. The asterisk indicates a significant increase relative to empty vector control (P < 0.05) as evaluated using Student's *t* test. OE-HA1s are two independent *OsHA1* over-expression lines. This is a representative experiment that was repeated two times. Error bars are SE. EV, empty vector.

specifically expressed in arbuscule-containing cells (Krajinski et al., 2002), and our own data confirmed that expression of Mt-HA1 increases over time, consistent with the increase in AM colonization and arbuscule development (Figures 5E and 5F). In wild-type *M. truncatula* R108 and in the progenitor line *tnk* (carrying *Tnt1*), arbuscules were well developed and highly branched, essentially filling inner cortical cells (Supplemental Figure 4A). Total colonization levels reached 40% of the root length (Figure 5D). By contrast, colonization levels were low in backcrossed Mt-*ha1-1* (Figure 5D; Supplemental Figure 4B), and some apparent defects were present in the arbuscules (Figure 6; Supplemental Figure 4A; also see Figure 3 in the accompanying article, Krajinski et al., 2014). The morphology of hyphopodia and intercellular growth appeared comparable to that in wild-type plants. However, a marked septation of external and internal hyphae was observed in the mutant (Figure 6A), which is indicative of hyphal degeneration. We recovered a second mutant allele of Mt-*ha1* (Figure 5A) from a reverse screen of the *Tnt1* insertion population, and this allele, Mt-*ha1-3* (in line NF10330), also showed low AM colonization (Supplemental Figure 5A).

Many legume mutants defective in the AM symbiosis are also defective in the interaction with rhizobial bacteria (Parniske, 2008; Oldroyd, 2013). Therefore, we assessed whether this was also the case with Mt-*ha1-1*. Normal-looking pink nodules developed on Mt-*ha1-1* (Supplemental Figure 5B), indicating that root nodule symbiosis was fully functional.

Small and less intensely branched arbuscules as well as septation of hyphae were also reported for mutants defective in the phosphate transporter Mt-PT4 (Javot et al., 2007). Based on the hypothesis that the activity of the H⁺-ATPase is required for efficient cotransport of phosphate and protons across the periarbuscular membrane, we expected the phenotype of Mt-*ha1* mutants to mirror that of Mt-*pt4* mutants, and this is exactly what we observed. When comparing mycorrhizal colonization in Mt-*ha1* and Mt-*pt4* mutants grown side by side, along with the respective wild-type genotypes, colonization levels of R108 and A17 increased steadily over a period of 5 weeks, whereas very little colonization was seen for the two mutants (Figure 6B). Moreover, the distribution of arbuscule sizes found in the Mt-*ha1-1* mutant closely resembled that reported for Mt-*pt4* mutants at 5 weeks postinoculation (Javot et al., 2007; Figure 6C). These data are compatible with the idea that Mt-HA1 affects mycorrhizal colonization through Mt-PT4 mediating nutrient exchange between plant and fungus. Consistent with this, like its rice counterpart, mutation of Mt-HA1 reduced the total shoot Pi content following AM colonization (Figure 6D), but unlike in rice, we also observed a promotion of shoot growth in *M. truncatula* upon AM colonization that was absent in Mt-*ha1-1* (Figure 6E).

Impaired Arbuscule Development in Mt-*ha1* Mutants Persists in the Presence of Nurse Plants

The phenotype of reduced colonization and impaired arbuscule formation could be explained by a lack of carbon supply to the fungus when the Pi supply by the fungus is inadequate (Fitter, 2006). Therefore, we tested to what extent carbon supply in the presence of nurse plants could complement the Mt-*ha1* mutant phenotype. Mt-*ha1-1* seedlings were grown in the presence of colonized leek (*Allium porrum*) plants. The percentage of the root length in which hyphae and vesicles were found was similar in Mt-*ha1-1* mutants and in the wild type at 4 weeks post-inoculation (Figure 7). By contrast, when the size of arbuscules was quantified, Mt-*ha1-1* showed greatly reduced levels of fully developed arbuscules (Figure 7). This was paralleled by an increase in the number of smaller arbuscules in the mutant compared with wild-type roots (Figure 7). This phenotype is reminiscent of what was reported previously in Mt-*pt4* mutants (Javot et al., 2011) and in mutants defective in a half ABC transporter (Zhang et al., 2010; Gutjahr et al., 2012). These results demonstrate that the defect caused by mutation in Mt-HA1 cannot be overcome by supplying carbon to the AM fungus from an alternative host plant.

HA1 Energizes the Plasma Membrane and Facilitates Nutrient Uptake

The plasma membrane potential is thought to play an important role in driving nutrient uptake into the cell. Our data showed that an H⁺-ATPase is required for uptake of inorganic phosphate through the AM symbiosis in rice and in *M. truncatula*. If HA1 is an active proton pump, we would expect that it is involved in generating an H⁺ electrochemical gradient to support the uptake of Pi via Mt-PT4/Os-PT11. To assess this hypothesis, we

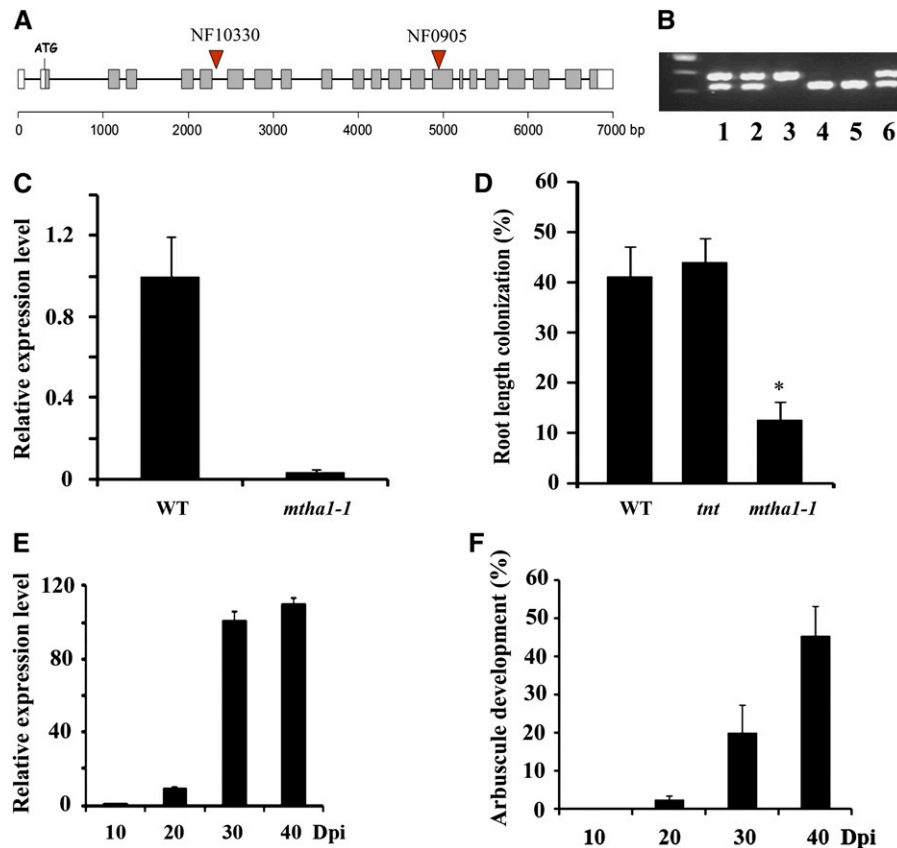


Figure 5. Mt-*HA1* Is Required for the Mycorrhizal Symbiosis in *M. truncatula*.

(A) Mt-*HA1* has 22 exons; *Tnt1* insertions in exon 15 in line NF0905 and intron 6 in line NF10330.

(B) PCR genotyping of the F2 of a backcross of NF0905 with wild-type R108. A product size of 398 bp indicates homozygous insertion in Mt-*HA1* (lane 3), a product size of 336 bp indicates homozygous wild type, and the two products in the same reaction indicates heterozygous plants.

(C) Expression level of Mt-*HA1* in 1-week-old wild-type and Mt-*ha1-1* mutant roots without inoculation with *R. irregularis*.

(D) Reduced colonization of Mt-*ha1-1* roots 4 weeks postinoculation with *R. irregularis*. Total colonization measured (arbuscules, hyphae, and vesicles). Error bars indicate SE of 8 to 10 plants.

(E) Mt-*HA1* is induced upon colonization with *R. irregularis* at 10, 20, 30, and 40 d postinoculation (Dpi), measured by quantitative RT-PCR. Error bars are SE.

(F) Quantification of arbuscule formation during colonization with *R. irregularis* at 10, 20, 30, and 40 d postinoculation. Error bars are SE ($n = 12$ plants). This is a representative experiment that was repeated three times in (C) to (F).

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measured the plasma membrane potential of root epidermal cells in the *M. truncatula* OE-*OsHA1* lines. In the *OsHA1*-overexpressing roots, the plasma membrane potential was more negative than in control plants transformed with the empty vector (Figures 8A and 8B). To assess whether the increased negative potential of the plasma membrane leads to more efficient nutrient uptake, shoot Pi levels were measured in control plants and OE-*OsHA1* plants in which the level of *OsHA1* expression was high (Supplemental Figure 6). Pi levels increased significantly, by ~50%, in two batches of independently generated composite plants grown in the presence of low concentrations of phosphate (Figure 8C). These results suggest that HA1 does indeed energize the membrane to facilitate nutrient transport, including uptake of Pi, most likely through the action of Mt-PT4/*Os-PT11* in the arbuscule and presumably other

phosphate transporters when ectopically expressed in the absence of AM fungi.

DISCUSSION

Plasma membrane H⁺-ATPases have been shown to fulfill essential functions in plants through the energization of the plasma membrane (Sze et al., 1999; Sondergaard et al., 2004; Haruta et al., 2010). Here, we demonstrate that a specific H⁺-ATPase from rice and *M. truncatula* is required for a fully functional AM symbiosis. Among the eight putative H⁺-ATPase genes in rice, the transcript level of a single member of the gene family (*Os-HA1*) was strongly upregulated at the stage of arbuscule development. The expression of *Os-HA1* in colonized roots is associated with increased H⁺-pumping activity in isolated membrane vesicles;

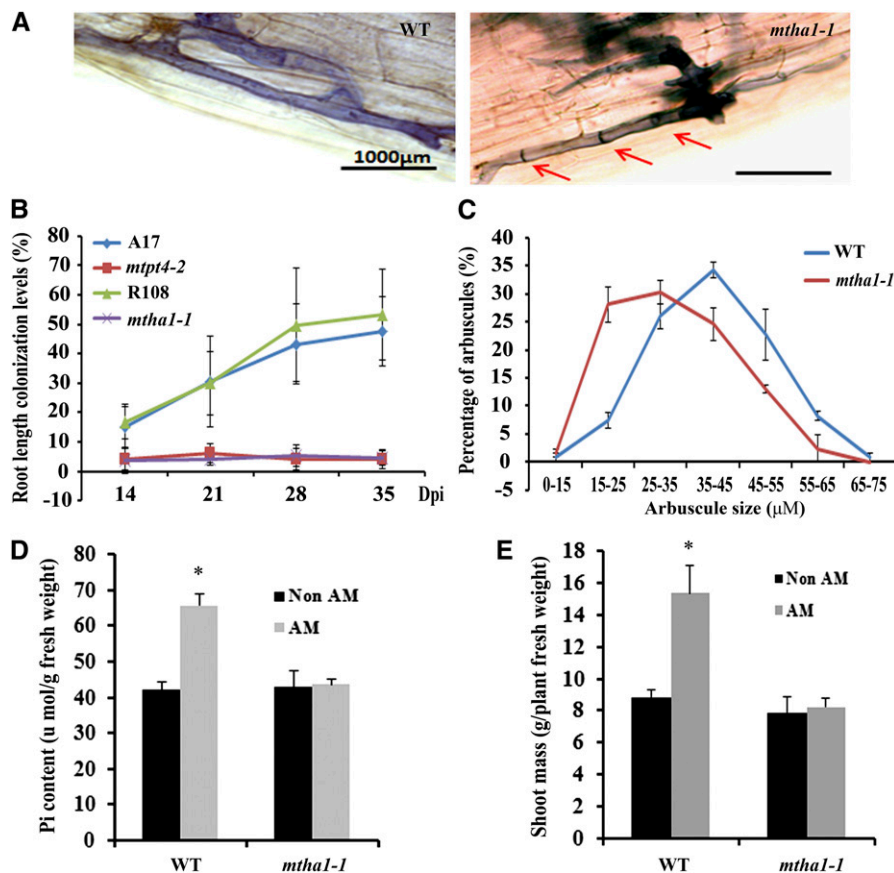


Figure 6. Mt-HA1 and Mt-PT4 Are Required for the Mycorrhizal Symbiosis in *M. truncatula*.

(A) Many external and internal hyphae show septation (indicated by red arrows) in the *Mt-ha1-1* mutant, whereas R108 (WT) did not. This phenotype is representative of 13 plants tested 14 d postinoculation.

(B) Total root length colonization of *Mt-ha1-1* was similar to that of the *Mt-pt4-2* mutant. Error bars are SE. Dpi, days postinoculation.

(C) Quantification of ink-stained roots revealed smaller and less intensely branched arbuscules in the *Mt-ha1-1* mutant (25 arbuscules per plant were counted). Error bars are SE.

(D) A significant increase of Pi levels in wild-type shoots, but not in *Mt-ha1-1* 9 weeks postinoculation. Error bars indicate SE of 12 plants.

(E) A significant increase of shoot biomass in the wild type, but not in *Mt-ha1-1* 9 weeks postinoculation. Error bars indicate SE of 12 plants. The experiments in (D) and (E) were repeated three times.

furthermore, this proton pumping activity depends on the integrity of the *Os-HA1* gene. Conversely, overexpression of *Os-HA1* in *M. truncatula* roots leads to increased H⁺-pumping activity. Together, these data provide strong evidence for HA1 being an active plasma membrane H⁺-ATPase. The fact that mutation of *HA1* in rice and *M. truncatula* reduces AM colonization and symbiotic Pi uptake highlights a likely role for this H⁺-ATPase in the energization of the periarbuscular membrane. Similar results for *M. truncatula* were obtained independently by Krajinski et al. (2014) (accompanying article).

Previously, cytochemical approaches suggested that there was H⁺-ATPase activity in mycorrhizal roots and that it was localized to the periarbuscular membrane and fungal membranes (Gianinazzi-Pearson et al., 1991). Furthermore, accumulation of acidotropic dyes in the periarbuscular space indicated that the pH in this space is significantly lower than that of the vacuole (Guttenberger, 2000). Putative H⁺-ATPase-encoding genes are

induced in mycorrhizal roots (Murphy et al., 1997; Gianinazzi-Pearson et al., 2000; Ferrol et al., 2002; Krajinski et al., 2002). Gianinazzi-Pearson et al. (2000) reported that in tobacco, two H⁺-ATPase isoforms are expressed in arbuscule-containing cells and that the encoded proteins are localized at the periarbuscular membrane. While the tobacco isoforms are also expressed in the root meristem, expression of *Mt-HA1* was reported to be specific for arbuscule-containing cells (Krajinski et al., 2002). More sensitive measurements using laser microdissection revealed expression of *Mt-HA1* in cortical cells with arbuscules, but also in nearby uninfected cells, but not in cortical cells from uninfected roots (Hogekamp et al., 2011; Gaude et al., 2012). A similar pattern of expression was described for *Mt-PT4* (Gaude et al., 2012).

The *Mt-PT4* protein had been specifically localized to arbuscule branches and is absent from arbuscule trunks and the plasma membrane of arbusculated cells (Harrison et al., 2002; Pumplin and Harrison, 2009; Pumplin et al., 2012). Similar results were

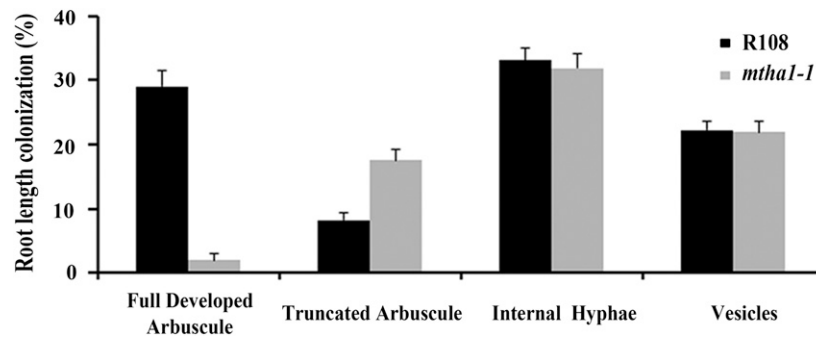


Figure 7. Small Arbuscules in *Mt-ha1* Mutants Persist in the Presence of Nurse Plants.

Internal root length colonization of *Mt-ha1-1* mutant (gray bars) and wild-type R108 (black bars) in the presence of leek nurse plants. Graphs are representative of three experiments. Error bars show standard errors of mean values of 19 to 21 plants.

reported for the rice phosphate transporter *Os-PT11* (Kobae and Hata, 2010). Whether the location of H⁺-ATPase protein is the same as that of the phosphate transporter remains to be shown. *Mt-PT4* and *Mt-HA1* are coexpressed and thus may have linked functions. Indeed, compromising *HA1* led to reduced acquisition of symbiotic phosphate by the mutant plants. However, since the colonization and especially the abundance of arbuscules were

drastically reduced in the mutants, it is not possible to determine whether the diminished phosphate uptake is cause or consequence of decreased arbuscule formation. However, a knockout mutant of *Mt-PT4* displayed the same overall phenotype as the *Mt-ha1-1* mutant, namely, a reduced level of colonization and a dramatically decreased number of fully developed arbuscules (Javot et al., 2007). Therefore, it is much more likely that the lack

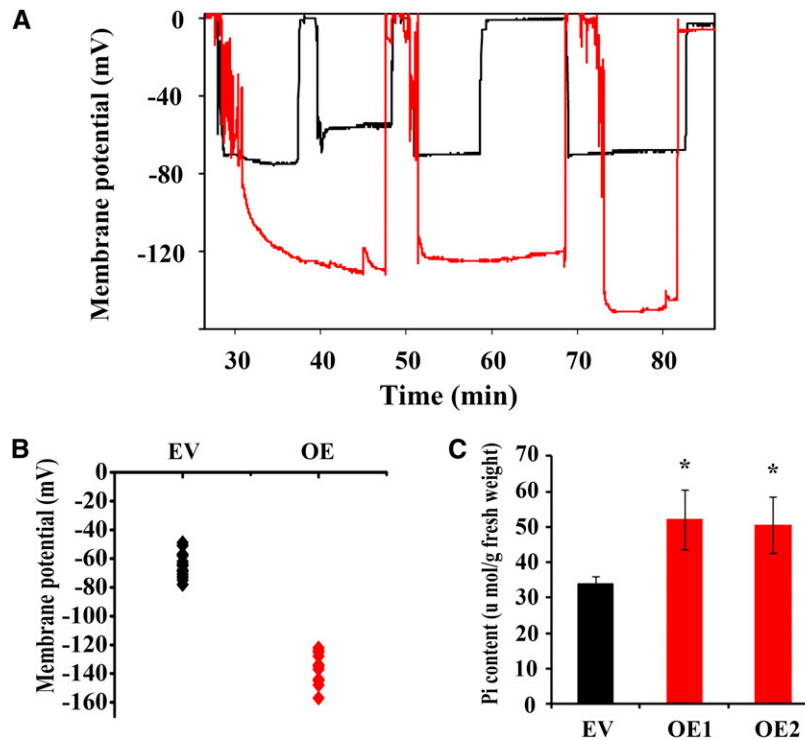


Figure 8. Overexpression of *Os-HA1* in *M. truncatula* Increases Plasma Membrane Potential and Phosphate Uptake.

(A) and (B) The plasma membrane potential is more negative in the *Os-HA1*-overexpressing plants than in control plants. Red indicates overexpressing plant; black indicates control plant. Values of zero indicate that the electrode tip was outside of the cells.

(C) A significant increase in shoot Pi levels (35%) in *Os-HA1*-OE compared with control plant under low Pi supply. This is a representative experiment that was repeated two times. EV, empty vector control. OEs are two independent *Os-HA1* overexpression lines. Error bars are SE. Asterisks indicate a significant increase relative to control plants by Student's *t* test ($P < 0.05$).

of H⁺-ATPase activity prevents the phosphate transporter from functioning efficiently, leading to more rapid senescence of arbuscules, as reported by Javot et al. (2007). Consistent with their data, we also found a range of arbuscule sizes whereby the average size was significantly reduced in the *Mt-ha1-1* mutant (Figure 6C). Furthermore, overexpression of *Os-HA1* led to increased Pi uptake, further supporting a role for HA1 in providing the energy for phosphate uptake.

Wild-type *M. truncatula* plants showed an almost 2-fold increase in shoot fresh weight compared with noninoculated control plants after 9 weeks of growth with *R. irregularis* (Figure 6E). This mycorrhiza-derived growth benefit was completely abolished in the *Mt-ha1-1* mutant. In the absence of AM fungi, the wild type and *Mt-ha1-1* differ neither in phosphate uptake nor in growth, indicating a symbiosis-specific role of Mt-HA1. This symbiotic Mt-HA1 function is not compensated for by other members of the H⁺-ATPase family, which is in agreement with the reported gene expression patterns (Krajinski et al., 2002; Hogekamp et al., 2011).

Kiers et al. (2011) showed that the plant allocates more carbon to a mycorrhizal fungus when the P supply by the fungus is high. Conversely, the fungus supplies more P to the plant when the carbon allocation is high. Strikingly, the same principle was found to operate when the transport of nitrogen was investigated. Carbon flow through arbuscules to the fungus leads to a stimulation of nitrogen transport by the fungus (Fellbaum et al., 2012). Javot et al. (2011) showed that arbuscule degeneration in *Mt-pt4* mutants does not take place under conditions of low nitrogen availability. Therefore, one can speculate that arbuscule growth and longevity is secured by appropriate carbon supply as long as at least one limiting nutrient is supplied to the plant via this interface. We have not tested our H⁺-ATPase mutant plants under conditions of low nitrogen supply, but it is possible that HA1 can energize the periarbuscular membrane for uptake of other nutrients in addition to phosphate. Guether et al. (2009) characterized a plant ammonium transporter expressed in arbusculated cells and found that this transporter binds NH₄⁺, but transfers uncharged NH₃ to the plant cell cytosol. They speculated that through the release of protons this nonelectrogenic transfer might reinforce the H⁺ gradient across the periarbuscular membrane. In terms of the H⁺-ATPase mutants, we could postulate that arbuscule development would be similar to the wild type under conditions of low nitrogen availability, analogous to what Javot et al. (2011) reported for the *Mt-pt4* mutant. Moreover, an increased flux of ammonium through the arbuscules might then partially compensate for the lack of H⁺ pumping activity.

The discovery of a fungal plasma membrane H⁺-ATPase gene specifically expressed in arbuscule-containing cells suggests that acidification of the periarbuscular space can also occur by proton pumping through the fungal membrane (Balestrini et al., 2007). Our results suggest that the mycorrhiza-associated proton pumping activity in isolated rice membrane vesicles is largely dependent on the presence of *Os-HA1* (Figure 4A). Therefore, the fungal H⁺-ATPase cannot compensate for the defect in the *Os-ha1* mutant. Hence, acidification of the periarbuscular space by the fungus is not sufficient in itself to provide the proton-motive force across the periarbuscular membrane for phosphate uptake into the plant. Since the activity of the plasma membrane ATPase is tightly

regulated by means of autoinhibition (Duby and Boutry, 2009; Ekberg et al., 2010; Fuglsang et al., 2011), it is possible that fungal proton pumping activity was low under our experimental conditions.

Our data show that in rice and *M. truncatula*, a single plasma membrane H⁺-ATPase is required to provide the electrochemical energy to drive the uptake of nutrients across the periarbuscular membrane. It is likely that on the fungal side, arbuscule-specific proton pumps in turn energize the fungal membrane for the uptake of monosaccharides (Balestrini et al., 2007; Helber et al., 2011). The lack of Pi supply to the proton pump mutants reduces the abundance of fully developed arbuscules. Functional arbuscules cannot be produced even when the mutant plants are colonized by fungal hyphae arising from functional nurse plants. Consequently, arbuscule growth and maintenance must be based on chemical cues delivered within the root cortex.

The AM symbiosis has received increasing attention for its potential exploitation in the nutrition of crop plants (Smith and Smith, 2011a, 2011b) and especially in sustainable agriculture (Fitter et al., 2011). The function of the arbuscular plasma membrane H⁺-ATPase in energizing nutrient transfer may well be employed in crop improvement. It has already been demonstrated that increased expression of a vacuolar H⁺-pyrophosphatase leads to enhanced plant growth, which appears to be in part due to the subsequent upregulation of plasma membrane H⁺-ATPase (Li et al., 2005; Yang et al., 2007; Gaxiola et al., 2012). Improved plant growth was observed under normal conditions as well as under nutrient limitation and abiotic stress, such as salt or drought stress (Gaxiola et al., 2012). Our demonstration of the functional importance of the arbuscular H⁺-ATPase leads us to speculate that this will likewise be an excellent target for plant breeding with the scope to improve crop plant productivity via symbiotic nutrient uptake.

METHODS

Plant Growth and Mycorrhizal Inoculum

Rice genotype used was *Oryza sativa* ssp *japonica* cv Nipponbare. For functional analysis of *Os-HA1*, a single *Tos17* insertion mutant, T00086T/H0310, was obtained from a transposon-mutagenized population in the Nipponbare background. Homozygous mutant lines were identified and the exact position of the insertion was determined by sequencing using primers H0310-LP, H0310-RP, and Tos17-BP (Supplemental Table 1). *Medicago truncatula* genotypes A17 and R108 were used. *Tnt1* insertions were in the R108 background (Tadege et al., 2008). The insertion of *Tnt1* in NF0905 (mutant allele *ha1-1*) was identified through the transposon display PCR protocol (Ratet et al., 2010). The genotype of backcrossed plants was tested using primers MthA1-1F, MthA1-1R, and LTR51 (Supplemental Table 1). Mutant allele *ha1-3* was identified in insertion line NF10330 by a reverse genetic screen as described (Cheng et al., 2011) using primers MthA1-2F, MthA1-2R, and Tnt1F (Supplemental Table 1).

Rice plants were inoculated with *Rhizophagus irregularis* (previously named *Glomus intraradices*) (Krüger et al., 2012) or *Gigaspora rosea*, as described previously (Gutjahr et al., 2008). For germination, seeds were imbibed in water at 28°C. Seedlings were placed in a 1:1 mixture of Terragreen (Oil-Dri Company) and sand and inoculated with 200 spores per plant, harvested from the *R. irregularis*/carrot (*Daucus carota*) coculture. The plants were grown in the glasshouse at 28°C with 12 h light and 24°C with 12-h-dark cycles. *R. irregularis* was used in most experiments and was maintained on plates cocultivated with carrot hairy root cultures (St-Arnaud

et al., 1996). Colonized roots were treated with 10% KOH for 15 min at 95°C, followed by 6 min incubation in ink at 95°C as described (Vierheilig et al., 1998). Root length colonization was quantified using the grid line intersect method (Giovannetti and Mosse, 1980) using a Nikon Eclipse 800 light microscope.

M. truncatula seeds were scarified on sand paper, surface sterilized using 10% bleach, and imbibed on water agar plates at 4°C. The seeds were germinated on the water agar plates by transferring them to room temperature in the dark and then planted in pots for mycorrhizal analysis or immediately treated with *Agrobacterium rhizogenes* for hairy root transformation (Boisson-Denier et al., 2001). Plants were grown at 22°C with 16-h-light/8-h-dark cycles. To test mycorrhizal colonization, roots were treated with 10% KOH for 6 min at 95°C, followed by 3 min in ink at 95°C.

For nurse pot experiments, 6-week-old leek plants colonized with *R. irregularis* were used. To produce colonized leek plants, leek seedlings were planted in nine parts of a mixture of autoclaved sand and Terragreen (equal volumes) and one part (by volume) of leek root pieces colonized by *R. irregularis* spores. The plants were allowed to grow for ~6 weeks and then individual colonized leek plants were used as nurse plants. The leeks were potted in the center of a P6 (11 cm diameter) round pot surrounded by four to five *M. truncatula* plants.

Phylogenetic Analysis

Rice protein sequences showing similarity to H⁺-ATPase were identified with BLASTP. All eight rice (*Os*), *Arabidopsis thaliana* H⁺-ATPases, and highly conserved H⁺-ATPases in *M. truncatula* were aligned and gaps or missing data were removed (Supplemental Data Set 1). H⁺-ATPase in *Physcomitrella patens* was used as an outgroup. Phylogenetic trees were constructed using maximum likelihood with MEGA version 4.3.1 (Tamura et al., 2007). Options used were the maximum likelihood statistical method, 1000 bootstrap replications, and nearest-neighbor-interchange trees.

Overexpression and H⁺-ATPase Subcellular Localization

Rice H⁺-ATPase cDNA was amplified using the TOPO compatible primers OsHA1-F and OsHA1-R (Supplemental Table 1), cloned using the pENTR/D-TOPO cloning kit and subsequently transferred into the pK7WG2D and pK7WGF2 vectors (Karimi et al., 2002) by LR Gateway cloning. The Gateway LR Clonase II enzyme was purchased from Invitrogen. Both constructs were introduced into *A. rhizogenes* strain Arqua1 for transformation. Three weeks after transformation, nontransformed roots not showing expression of the GFP marker were removed and the plants were used for plasma membrane potential measurement or subcellular localization studies.

RNA Extraction and Quantitative Real-Time PCR Analysis

Total RNA was isolated using the plant RNeasy kit (Qiagen) from given tissues and incubated with DNaseI (if necessary), then checked for contaminating DNA using H3L or *CYCLOPHILIN2* primers (Supplemental Table 1). Total RNA (1 to 5 µg) was used for cDNA synthesis with the Superscript III system (Invitrogen). Real-time PCR was performed with SYBR green (Sigma-Aldrich) using gene-specific primers (Supplemental Table 1). *H3L* amplification was used for normalization in *M. truncatula* (Ariel et al., 2012) and *CYCLOPHILIN2* for normalization in rice (Gutjahr et al., 2008). Three biological replicates were performed.

Plasma Membrane H⁺-ATPase Activity Assays and Measurements of Total Pi

ATP-dependent proton transport across membrane vesicles was tested using acridine orange quenching at 495 nm (Palmgren, 1990; Larsson et al., 1994). Samples (10 g) of *M. truncatula* or rice roots were used and vesicles isolated and tested as described by Liu et al. (2009). Each experiment was repeated two times with independent plasma membrane

isolations. *M. truncatula* and rice leaf Pi content was measured using the Malachite Green Phosphate Assay Kit (POMG-25H; Cayman Chemical Company). Four-week-old composite plants were transferred into soil, and Pi content in leaves was determined 3 weeks later.

Electrophysiological Measurements of *M. truncatula* Root Cell Membrane Potential

The root cell membrane potential measurements were made using borosilicate glass microelectrodes with 5 to 10 MΩ resistances as described previously for barley (*Hordeum vulgare*; Fan et al. 2006). The membrane potential was measured in a simplified nutrient solution containing 0.5 mM CaCl₂, 0.05 mM KCl, and 5 mM MES adjusted to pH 6.0 with NaOH. Roots were maintained in this solution for at least 1 h before testing. Regions of the root expressing GFP were selected for measuring the membrane potential in *Os-HA1*-overexpressing lines 4 weeks after inoculation with *A. rhizogenes*. The roots were selected on plates, the underside of the plate was labeled using a permanent marker, and this region was selected for measurements after transferring the roots to a Plexiglass chamber (Fan et al., 2006). A successful membrane potential measurement of an epidermal cell was judged by recording a stable voltage value for at least 5 min, after which the tip was deliberately withdrawn and the baseline voltage returned to 0 mV.

Accession Numbers

Sequence data from this article can be found in the GenBank/EMBL databases under the following accession numbers: Os-HA1 (Os03g0100800), Os12g0638700, Os03g0689300, Os07g0191200, Os04g0656100, Os05g0319800, Os02g0797300, Os06g0181500, Mt-HA1 (IMGA|contig_74284_1.1), Medtr4g127710, Medtr2g036650, Medtr3g108800, IMGA|contig_162917_1.1, IMGA|contig_56358_1.1, IMGA|contig_49127_1.1, IMGA|contig_62147_1.1, IMGA|contig_61752_1.1, IMGA|contig_83450_1.1, AHA1 (At2g18960), AHA2 (At4g30190), AHA3 (At5g57350), AHA4 (At3g47950), AHA6 (At2g07560), AHA7 (At3g60330), AHA8 (At3g42640), AHA11 (At5g62670), and outgroup (XP_001784418.1)

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure 1. Relative Expression of the Rice H⁺-ATPase Gene Family Members 5 Weeks Postinoculation.

Supplemental Figure 2. Ink-Stained Roots of Rice Colonized with *R. irregularis* and Quantification of *Gigaspora rosea* Colonization at 6 Weeks Postinoculation.

Supplemental Figure 3. Relative Expression of *Os-HA1* in Transgenic *M. truncatula* Plants and *OsHA1*-GFP Localization.

Supplemental Figure 4. Quantification of Internal Hyphae, Arbuscules, and Vesicles of *R. irregularis* in *Mt-ha1* and Wild-Type R108.

Supplemental Figure 5. Symbiotic Phenotype of *Mt-ha1* Mutant.

Supplemental Figure 6. Relative Expression of *Os-HA1* in Transgenic *M. truncatula* Plants That Were Used for Measurement of Phosphate Uptake.

Supplemental Table 1. List of PCR Primers.

Supplemental Data Set 1. Text File of the Alignment Used for the H⁺-ATPase Phylogenetic Analysis Shown in Figure 2A.

ACKNOWLEDGMENTS

We thank the Rice Genome Resource Center (Japan) for providing the rice *Tos17* mutant lines. We thank Thomas B. Irving for critically reading

the article. This work was supported by the “1000-talents Plan” from China for young researchers, NKLPMPG, and the Strategic Priority Research Program of the Chinese Academy of Sciences (XDA08010401) to E.W., by the European Research Council as “SYMBIOSIS” to G.E.D.O. by the Biotechnology and Biological Science Research Council as BB/J004553/1 and BB/E001408/1, and by the Higher Education Commission, Pakistan.

AUTHOR CONTRIBUTIONS

E.W., N.Y., and M.S. conceived and designed the experiments. E.W., N.Y., S.A.B., C.L., A.J.M., D.C., X.Z., and M.S. performed most of the experiments. P.R., M.T., K.S.M., J.A.D., J.D.M., and G.E.D.O. contributed new analytic/computational/etc. tools. E.W. and M.S. analyzed data and wrote the article.

Received November 7, 2013; revised March 25, 2014; accepted April 9, 2014; published April 29, 2014.

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**A H⁺-ATPase That Energizes Nutrient Uptake during Mycorrhizal Symbioses in Rice and
*Medicago truncatula***

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Plant Cell 2014;26;1818-1830; originally published online April 29, 2014;
DOI 10.1105/tpc.113.120527

This information is current as of October 27, 2020

Supplemental Data	/content/suppl/2014/04/17/tpc.113.120527.DC1.html
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