The H+-ATPase HA1 of *Medicago truncatula* Is Essential for Phosphate Transport and Plant Growth during Arbuscular Mycorrhizal Symbiosis

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INTRODUCTION

Arbuscular mycorrhizal (AM) symbiosis between obligate biotrophic fungi of the phylum Glomeromycota and the majority of land plants is based upon bidirectional nutrient transfer between host plants and AM fungi (Smith and Smith, 2012). AM symbiosis is ancient and is believed to have facilitated colonization of land by aquatic plants ~450 million years ago (Redecker et al., 2000). The widespread occurrence of AM symbioses today (Smith and Read, 2008) indicates that they continue to play key roles in terrestrial ecology. Growth and spore development of AM fungi depends on successful colonization of roots to access plant carbohydrates and convert them into fatty acids and other compounds (Solaïma et al., 1999; Trépanier et al., 2005). In return, AM fungi take up minerals, especially Pi, from the soil and deliver them to their host plants (Marschner and Dell, 1994). Pi availability in the soil is known to regulate AM fungal colonization of the root, and in low-P soils, AM fungi can provide most of the P needed by plants (Bucher, 2007; Smith et al., 2011; Yang and Paszkowski, 2011). AM fungi can also transfer soil N and S to plants under some conditions (Leigh et al., 2009; Casieri et al., 2012; Koegel et al., 2013; Sieh et al., 2013).

Exchange of phosphate and carbohydrates between AM fungi and colonized plant cells is mediated by specific transporters on the fungal membrane and the surrounding plant periarbuscular membrane (PAM) (Smith and Smith, 1990). Specific plant phosphate transporters (PTs) are confined to the PAM, indicating that arbuscules are the main site of Pt transfer to the plant (Harrison et al., 2002). By contrast, localization of a fungal monosaccharide transporter to arbuscules and intraradical hyphae indicates that fungal uptake of plant sugar may not be restricted to arbuscules (Helber et al., 2001). Downregulation of either the plant phosphate transporter or the fungal monosaccharide transporter resulted in reductions not only of arbuscule abundance, but also of overall hyphal spread in the cortex of the root (Javot et al., 2007; Helber et al., 2011).

The PAM-located PT is a phosphate/H+ cotransporter. H+-ATPase activity has been observed at the PAM (Marx et al., 1982), which presumably generates the proton gradient required for phosphate transport from the periarbuscular space into the plant cytoplasm via the PAM-PT. An H+-ATPase gene, *HA1*, has been identified in *Medicago truncatula* that is expressed specifically in arbuscule-containing cells of mycorrhizal roots (Krajinski et al., 2002). To test the hypothesis that *HA1* is required to energize phosphate transport to the plant, we isolated an *ha1*-2 mutant of
*M. truncatula* and compared its symbiotic phosphate uptake and growth characteristics to those of the wild-type.

**RESULTS**

**Identification of an Insertion Mutant for HA1**

An ha1-2 mutant was identified among 8000 long terminal repeat retrotransposon Tnt1-insertion lines of *M. truncatula*, via a PCR-based screen (see Methods). Sequencing of the mutant allele revealed a Tnt1 insertion in exon 8 of HA1 (Figure 1). Homozygous ha1-2 progeny were isolated from a self-pollinated heterozygous ha1-2/HA1-2 individual. Homozygous HA1-2 progeny of the same parent were also isolated and used in subsequent experiments as wild-type controls. The effect of the Tnt1 insertion on HA1 transcription was determined by RT-PCR. Using HA1-specific primers flanking the Tnt1 insertion site, a 1123-bp cDNA fragment was amplified from wild-type plants (Figure 1). Surprisingly, a shorter amplicon was produced from cDNA of homozygous ha1-2 mutant lines (Figure 1). Subsequent sequencing of the PCR product from the ha1-2 mutant revealed the complete absence of exon 8. Therefore, insertion of Tnt1 resulted in splicing out of exon 8 in the ha1-2 mutant. Exon 8 consists of 207 bp encoding 69 amino acids of transmembrane domains 3 and 4 that are essential for H+-ATPase activity of related proteins (Palmgren, 2001; Morth et al., 2011) (Supplemental Figure 1). Therefore, the ha1-2 mutant can be regarded as a complete loss-of-function mutant. As expected due to the restricted expression of HA1 in mycorrhizal roots, non-mycorrhizal ha1-2 mutants did not show any phenotypes.

**ha1-2 Mutants Are Colonized by R. irregularis but Do Not Show a Positive Growth Response and Exhibit Altered Arbuscule Morphology**

Four weeks after inoculation with *R. irregularis*, more than 90% the root system of wild-type plants were colonized by the AM fungus (Figure 2). The roots of ha1-2 mutant plants did not show significant differences with regard to colonization frequency and hyphae and arbuscule numbers (Figure 2). Fungal colonization resulted in a large

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**Figure 1.** Insertion of a Tnt1 Transposon in the HA1 Gene Leads to Skipping of Exon 8 in the Mutant ha1-2.

(A) Schematic representation of the HA1 exon-intron structure. White boxes indicate exons 1 to 22. The position of the Tnt1 transposon in exon 8 is indicated.

(B) Three different primer combinations (a, b, and c) were used in RT-PCR with cDNA of mycorrhizal wild-type (w) or ha1-2 mutant (m), and amplification products were separated by gel electrophoresis together with a size marker (sm). Amplicon sizes are indicated. If primers were spanning exon 8 (combination b and c), amplicons were ~200 bp smaller when using cDNA of the mutant mha1-2. Subsequent sequencing of all PCR products revealed that exon 8 is missing in ha1-2 transcripts.

(C) Schematic representation of the HA1 cDNA. The 207 bp of exon 8, primer binding sites, and amplicon lengths are indicated.

[See online article for color version of this figure.]
and significant increase in shoot fresh weight of wild-type plants but not of ha1-2 mutants grown under low Pi (20 μM phosphate) conditions (Figure 2).

Roots of mycorrhizal wild-type plants exhibited typical arbuscules with fine hyphal branches, under both low- and high-Pi conditions (Figure 3). By contrast, only truncated arbuscules were observed in roots of the ha1-2 mutant under both conditions (Figure 3). Consistent with containing smaller, truncated arbuscules, levels of R. irregularis rRNA were 20-fold lower in roots of the mutant than of the wild-type (fungal RNA in Supplemental Figure 2).

**Decreased Expression of Mycorrhiza-Inducible Genes in Colonized ha1-2 Mutant Roots**

Expression of eight plant genes known to be induced in mycorrhizal roots and accumulation of R. irregularis rRNA were analyzed in roots of the ha1-2 mutant. Transcripts for all of these plant genes and fungal rRNA were significantly less abundant in the ha1-2 mutant than in the wild type; this included transcripts of PT4 (Javot et al., 2007) and other genes identified previously to be highly induced in mycorrhizal roots (Wulf et al., 2003; Pumplin et al., 2010; Hogekamp et al., 2011; Gaude et al., 2012b; Devers et al., 2013; Supplemental Figure 2).

**Artificial MicroRNA-Mediated HA1 Silencing also Leads to Truncated Arbuscules**

To confirm that the aberrant arbuscule morphology in ha1-2 mutant plants was due to loss of HA1 function, we employed the artificial microRNA (amiR) technique to reduce HA1 expression in M. truncatula roots. Expression of HA1-amiRNA was driven by the arbuscule-specific Mt-PT4 promoter (Harrison et al., 2002), and
transgenic roots were identified by coexpression of the fluorescent protein DsRED. Following fungal inoculation, control roots expressing DsRED alone produced fully developed, highly branched arbuscules (Supplemental Figure 3). By contrast, no such arbuscules were detected in roots transformed with the HA1-amiR construct and in which HA1 transcript levels were <20% of control levels. *R. irregularis* rRNA accumulation and Mt-PT4 transcript levels were also significantly decreased in HA1-amiR roots. However, transcript levels of two other H+-ATPase genes expressed in *M. truncatula* roots (Krajinski et al., 2002) were not affected in the HA1-amiR roots.

**HA1 Is Not Essential for the Root Nodule Symbiosis**

Analysis of nodulated roots and nodules of ha1-2 mutant and wild-type plants revealed no induction of HA1 transcript during root nodule symbiosis (Supplemental Figure 4).

To investigate if HA1 is essential for efficient root nodule symbiosis, we inoculated ha1-2 and wild-type plants with *Sinorhizobium meliloti*. Four weeks after inoculation, wild-type and ha1-2 plants showed increased shoot fresh weight as a consequence of symbiotic nitrogen fixation and established root nodule symbiosis (Supplemental Figure 5). The two plant lines showed...
uptake of $^{33}$P (Figure 4), indicative of $^{33}$P uptake via the mycorrhizal pathway. Roots had passed the air gap and colonized the hyphal compartment. At this time point, $^{33}$PO$_4^{-}$ fungal hyphae had passed the air gap and colonized the hyphal compartment. Half of the plants were inoculated wherein the plant and the hyphal compartment were separated by nylon meshes and an air gap. The data shown are average values of four independent replicates; error bars represent standard deviations. Different letters indicate statistical differences.

Similar nodule numbers and levels of Mt-N24 (Godiard et al., 2007) expression. These data indicate that HA1 is not necessary for efficient root nodule symbiosis.

**HA1 Is Required for the Uptake of Phosphate via the Mycorrhizal Pathway**

To test whether HA1 is required for the uptake of phosphate via the mycorrhizal pathway, we set up compartmented systems wherein the plant and the hyphal compartment were separated by nylon meshes and an air gap. Half of the plants were inoculated with *R. irregularis*. Twelve weeks after inoculation, extraradicular fungal hyphae had passed the air gap and colonized the hyphal compartment. At this time point, $^{33}$PO$_4^{-}$ was added to the hyphal compartment and plants were harvested at 15 weeks after inoculation. No differences in the frequency of mycorrhizal colonization were observed between wild-type plants and ha1-2 mutants (62.5 ± 3.1 and 59.5 ± 5.6, respectively). Moreover, hyphal length density (HLD) was not significantly different between mutants and wild-type plants; the HLD of *R. irregularis* was 4.8 cm g$^{-1}$ and 4.2 cm g$^{-1}$ soil dry weight when associated with the wild-type and mutant plants, respectively. Nonmycorrhizal control plants did not show a significant uptake of $^{33}$P. By contrast, $^{33}$P accumulated in both shoots and roots of mycorrhizal wild-type plants, indicative of $^{33}$P uptake via the mycorrhizal pathway. Roots and shoots of mycorrhizal ha1-2 mutants did not show significant uptake of $^{33}$P (Figure 4), confirming that mycorrhizal ha1-2 mutants were unable to take up phosphate via a mycorrhizal pathway. Therefore, we can assume that HA1 is essential for mycorrhizal phosphate uptake in *M. truncatula*.

**HA1 Is Required for Production of an Acidic Compartment in Arbuscule-Containing Cells**

HA1 is the only *M. truncatula* H$^+$-ATPase-encoding gene that is strongly induced in arbuscule-containing cells. Strong expression of an H$^+$-ATPase-encoding gene in arbuscule-containing cells presumably results in an electrochemical proton gradient and thus in acidification of apoplastic compartments. Therefore, a functional knockout should result in reduced acidification of apoplastic spaces in arbuscule-containing cells. To test this, we applied the pH-sensitive Lysosensor DND-189 dye to stain mycorrhizal roots of wild-type and ha1-2 mutants (Figure 5). This acidotropic probe accumulates in acidic compartments, where protonation at pH 5.2 leads to a green fluorescence. Staining of mycorrhizal wild-type roots revealed strong fluorescence in the arbuscule-containing cells of the inner cortical cell layer (Figure 5A). Subsequent application of carboxylicanil-d-m-chlorophenylhydrazon (CCCP), which acts as protonophor, led to a strong reduction of the fluorescence confirming that the observed fluorescence after Lysosensor DND-189 staining results from acidification in the corresponding compartments (Figure 5B). By contrast, fluorescence was barely detectable in mycorrhizal ha1-2 mutant roots, pointing to diminished acidification around the abnormal arbuscules (Figure 5C). Laser scanning microscopy confirmed that arbuscule-containing cells of wild-type roots showed strong fluorescence, whereas arbuscule-containing cells of ha1-2 mutant roots did not (Figures 5D and 5E). This indicates HA1 is required for the production of an acidic compartment in arbuscule-containing cells.

**Figure 4. ha1-2 Mutants Are Unable to Take up Phosphate via the Mycorrhizal Pathway**

ha1-2 mutants and wild-type plants were inoculated with *R. irregularis* and grown in compartmented systems. $^{33}$P was added to the hyphal compartment 12 weeks after inoculation, and plants were harvested 15 weeks after inoculation. The data shown are average values of four independent replicates; error bars represent standard deviations. Different letters indicate statistical differences.

**Figure 5. HA1 Function Results in Production of an Acidic Compartment in Arbuscule-Containing Cells**

Mycorrhizal roots of wild-type ([A], [B], and [D]) and ha1-2 mutant ([C] and [E]) plants were stained with the acidotropic dye Lysosensor DND-189. Accumulation of the dye in acidic compartments results in green fluorescence and labels arbuscule-containing cortex cells of the wild type ([A] and [D]), but not of the mutant ([C] and [E]). Treatment of the section shown in (A) with CCCP (B) led to a clear reduction of fluorescence. (A) to (C) are micrographs taken using an epifluorescence microscope. (D) and (E) are micrographs obtained by laser scanning microscopy. WGA indicates fungal structures labeled by wheat germ agglutinin conjugated to tetramethylrhodamine resulting in red fluorescence. Bars = 100 μm in (A) to (C) and 10 μm in (D) and (E).
DISCUSSION

Here, we have shown that the H+-ATPase HA1 of *M. truncatula*, which is strongly induced in arbuscule-containing cells, is required for arbuscule development. Disruption of the gene in *ha1-2* mutants leads to truncated arbuscules and decreased acidification of apoplastic spaces in arbuscule-containing cells. The *ha1-2* mutants also show strongly impaired uptake of phosphate by the mycorrhizal uptake pathway. From this, we conclude that HA1 is essential for P transport via the mycorrhizal uptake pathway.

Phosphorus is often the main limiting factor for plant growth. One major advantage of the AM symbiosis for plants is the more effective uptake of phosphate via the mycorrhizal pathway (Smith et al., 2011), which often results in increased growth of mycorrhizal plants under low-P conditions. AM symbiosis increased the growth of wild-type *M. truncatula* plants by more than threefold (shoots) under low-P conditions, whereas AM symbiosis had no effect on the growth of *ha1-2* mutant plants. Uptake experiments in compartmented systems showed that *ha1-2* mutants were unable to take up phosphate via the mycorrhizal pathway. The uptake of phosphate from the periarbuscular space presumably requires a proton gradient generated by HA1. The acidic nature of the periarbuscular space in functional arbuscule-containing cells has been shown previously (Guttenberger, 2000). We found that arbuscule-containing cells of *ha1-2* mutants showed an increased pH in the apoplast as a consequence of lacking HA1 proton pumping activity. Therefore, it can be concluded that HA1 is essential for phosphorus transfer from AM fungi to the plant. A model explaining the role of HA1 in phosphate uptake into plants is shown in Figure 6.

Reduced phosphate transport across the PAM as a result of reduced H+-pumping across this membrane in the *ha1-2* mutant probably also explains the abnormal arbuscule development observed in the mutant (Figure 3). Truncated arbuscules were similarly observed in rice mutants defective for Os-HA1, which also encodes a H+-ATPase induced during AM symbiosis (Wang et al., 2014). Moreover, two other *M. truncatula ha1* mutant alleles have been identified (Wang et al., 2014), confirming that the phenotype described herein results from a mutation in the *HA1* gene. Mutation in PT4 of *M. truncatula*, which encodes a phosphate transporter located in the PAM, also resulted in a phenotype with degenerated arbuscules (Javot et al., 2007). Moreover, a similar phenotype was described in rice (*Oryza sativa*) defective for the Pt11 phosphate transporter (Yang et al., 2012). Thus, there appears to be a phosphate-sensitive checkpoint in the host plant cell that aborts the resource-intensive arbuscule development (Gaude et al., 2012a) at an early stage if phosphate is not transferred from the fungus to the plant.

Similar arbuscule morphology was reported recently for *stunted arbuscule* (str) mutants, which are defective in two half-

![Figure 6. Schematic Model of the Role of HA1 in Phosphate Uptake from the Periarbuscular Space into Plant Cells.](image-url)

Phosphate is transferred from the fungal cytoplasm across the fungal membrane in arbuscule-containing cells by a yet unidentified transport mechanism and released to the periarbuscular space. Specific PT proteins (blue) are located in the periarbuscular membrane and mediate the transport of phosphate across this membrane. Plant PTs are phosphate/proton symporters. Thus, a proton gradient is essential for phosphate uptake into arbuscule-containing cells. In *M. truncatula*, this proton gradient is generated by a H+-ATPase protein (HA1; orange).
ABC transporters (Zhang et al., 2010; Gutjahr et al., 2012). However, the str mutants showed drastically reduced fungal colonization of roots, in contrast to the ha1-2 mutant in which the degree of colonization was normal (Figure 2). The reduced fungal colonization of str mutants might be explained by lack of transport of unknown signal molecule(s), the substrate(s) of the ABC transporters (Zhang et al., 2010). If this is the case, then HA1 is not required for such signaling. HA1 is clearly also not required for nutrient supply to the invading fungus, which shows normal hyphal growth in the roots of the ha1-2 mutant (Figures 2 and 3). This is in contrast to M. truncatula pt4 mutants, in which fungal growth is severely impaired (Javot et al., 2007). It is assumed that functional PT4 proteins are required to signal the presence of a beneficial AM fungus and to allow fungal growth (Javot et al. 2007). R. irregularis–colonized ha1-2 mutants show significantly reduced PT4 expression, which, however, might be sufficient to sustain these signaling events and thus sustain fungal growth.

The normal level of fungal colonization in ha1-2 mutant roots indicates that colonization is not dependent on mature arbuscules, which are absent in the mutant. This assumption is supported by the finding that plants defective for a vesicle-associated membrane protein required for PAM synthesis show truncated arbuscules with very few branches but normal levels of intraradical colonization (Ivanov et al., 2012). AM fungi receive much of the carbon from their plant host in the form of sugars, and at least one fungal hexose transporter has been found on the intraradical fungal hyphae in the plant host in the form of sugars, and at least one fungal hexose transporter has been found on the intraradical fungal hyphae in addition to the arbuscule (Helber et al., 2011). Here, our results with the ha1-2 mutant indicate that carbon transport across the PAM in mature arbuscule-containing cells is not required for hyphal growth. No differences in the frequency of mycorrhizal colonization were observed between wild-type plants and ha1-2 mutants, and hyphal length density was also not significantly different between the mutants and wild-type plants. Since the majority of fungal carbon is derived from the host plants, our observations suggest that carbon transport from the plant to the fungus is not dependent on prior phosphate transport from the AM fungus. However, carbon transport across the PAM in mature arbuscule-containing cells might still be important for fungal metabolism within the arbuscule.

The assumption that fungal growth and especially arbuscule development are not strictly dependent on phosphate transfer to the host plant is also supported by pt4 mutants, which show normal arbuscules when grown under nitrogen-replete conditions (Javot et al., 2011). In addition, strong asymmetry in carbon investment by the plant and the phosphate provided by the AM fungus has been observed in common mycorrhizal networks (Walder et al., 2012). This and the phenotype observed here of unaltered fungal growth and impaired mycorrhizal phosphate uptake challenges the prevailing view that the plant regulates fungal colonization according to the amount of phosphate it receives from the fungus.

METHODS

Isolation of an ha1-2 Mutant

To identify a Medicago truncatula mutant carrying a Tnt1 insertion in HA1, we screened ~8000 plants with Tnt1 insertions (Pislaru et al., 2012). Using two pairs of nested primers (Supplemental Table 1), we identified a M. truncatula mutant plant with a Tnt1 insertion in exon 8 of HA1.

Plant Growth and Inoculation with Rhizophagus irregularis

M. truncatula seed germination was performed as described (Branscheid et al., 2010). Seedlings were transplanted into pots containing a mixture of expanded clay and silica sand. For inoculation with R. irregularis, an inoculum was mixed with the growth substrate (1:10 v/v). The inoculum was obtained by growing Allium schoenoprasum with R. irregularis as described previously (Morsø et al., 2009). Unless otherwise indicated, all plants were grown in a greenhouse at 24°C with a 16-h-light/8-h-dark cycle. Plants were fertilized with half-strength Hoagland solution (Hoagland and Arnon, 1950) containing either 20 µM or 1 mM phosphate, twice per week.

DNA and RNA Extraction

RNA of roots and leaves was extracted using the Invisorb Spin Plant Mini kit (Invitek). Genomic DNA was isolated using the DNeasy Plant Mini kit (Qiagen).

Quantitative RT-PCR and RT-PCR

Quantitative RT-PCR was performed as described recently (Branscheid et al., 2010). Oligonucleotide sequences of all primers are listed in Supplemental Table 1. The amplification efficiencies (E) were calculated using the LinRegPCR program (Ramakers et al., 2003). At least three biological replicates were performed for each quantitative RT-PCR analysis.

Staining of Fungal Structures

Roots were stained with wheat germ agglutinin coupled to AlexaFlour488 according to Gaude et al. (2012b). Images were collected on a Leica TCS-SP5 confocal microscope (Leica Microsystems) using a 63× water immersion objective with a numerical aperture of 1.2, zoom 1.6. AlexaFlour488 was excited at 488 nm, and emitted light was collected from 505 to 582 nm. Optical sections were acquired at 0.3- to 0.5-µm intervals. Images were processed using ImageJ software (Wayne Rasband, National Institutes of Health).

Phosphate Uptake Experiments

The phosphate uptake experiments were set up using compartmented microcosms (Koegel et al., 2013), wherein one plant and one hyphal compartment were connected, but separated by two 21-µm nylon meshes and an air gap in between. The air gap was created by placing two 5-mm plastic meshes between the two 21-µm nylon meshes and an air gap in between. The air gap was created by placing two 5-mm plastic meshes between the two 21-µm nylon meshes. The two compartments were filled with sterile sand (quartz sand from Alsace, 0.125 to 0.25 mm; Kaltenhouse) and zeolithe (2:1 w/w). M. truncatula seedlings (the wild type and ha1-2 mutants) were inoculated with a 2-g (~100 spores) inoculum of R. irregularis BEG-75 or with 2 g of sterilized (120°C, 20 min) inoculum as a nonmycorrhizal control. In the center of the hyphal compartment, a 21-µm nylon mesh bag of 15 mL was inserted and kept empty until introduction of the 33P-spiked substrate 12 weeks after inoculation. Then, the nylon mesh bag was filled with 13 g sand labeled with 750 kBq 33P-spiked sand without inducing mass flow. The microcosms were irrigated with distilled water twice a week. In addition, the compartments were amended weekly with 8 mL of Long Ashton nutrient solution. Plants were grown under controlled conditions (16 h light at 28°C and 8 h dark at 15°C, constant relative aerial humidity of 65%). Plants were harvested 15 weeks after inoculation. The root colonization was estimated by a modified line intersection method (McGonigle et al., 2006). Shoot and root samples were dried for 24 h at 105°C and weighed separately. Dried shoots and roots were ground at 30 Hz in a mixer mill (MM2224; Retsch). Phosphorus was extracted by acid digestion (Murphy and Riley, 1962), and 33P contents were measured using a Packard 2000 liquid scintillation counter.
(Hewlett-Packard). Mean comparisons were performed by independent paired t tests for 3P uptake and for root colonization.

HLD

HLD was measured separately for the root and hyphal compartments and was determined by the modified grid-line intersection method (Jakobsen et al., 1992) using 10 g of the growth substrate. After sieving successively through a 400- and a 32-μm mesh, the material was collected and transferred into 50 mL of distilled water and homogenized for 10 s at full speed in a blender. The suspension was transferred into a beaker, diluted to 500 mL, and stirred for 1 min before five subsamples were taken every 10 s and loaded onto the filtration apparatus (MF-Membrane filter 1.2 μm; Millipore).

Staining of Acidic Compartments

Roots of mycorrhizal wild-type and ha1-2 plants were sectioned into 200- to 300-μm-thick sections by hand. Sections were transferred into a solution consisting of 3 μM Lysosensor DND-189 (Invitrogen) and 15 μg/mL wheat germ agglutinin conjugated with tetramethylrhodamine for localization of fungal structures in 10 mM MOPS/KOH buffer (pH 8.0) and incubated in the dark at room temperature overnight. As a control, stained root sections were incubated with 50 μM CCCP in 10 mM MOPS/KOH buffer for 20 min. Sections were analyzed either by epifluorescence microscopy using a multi-zoom microscope AZ-100 (Nikon) equipped with the proper filter cube (EX460/500/505/BA510) or by confocal microscopy using an LSM700 (Zeiss). For the latter, the excitation wavelengths 488 and 555 nm were used, and fluorescence light was collected in the range of 493 to 544 nm and 560 to 630 nm for Lysosensor DND-189 and WGA-tetramethylrhodamine, respectively. All settings (laser power, pinhole diameter, and detector gain) remained constant for all pictures.

amiR Silencing

An HA1-amiR sequence (5′–3′: TTGTTAAGTACATGAAGCCC) was designed using the WMD3 microRNA designer tool (Ossowski et al., 2008), inserted into the mtr-mir159b backbone by an overlapping PCR strategy including parts of the pBluescript II SK cloning site and subcloned into pCR2.1-TOPO (Invitrogen). For amiR expression in Roots of mycorrhizal wild-type and plants were sectioned into 200- to 300-μm-thick sections by hand. Sections were transferred into a solution consisting of 3 μM Lysosensor DND-189 (Invitrogen) and 15 μg/mL wheat germ agglutinin conjugated with tetramethylrhodamine for localization of fungal structures in 10 mM MOPS/KOH buffer (pH 8.0) and incubated in the dark at room temperature overnight. As a control, stained root sections were incubated with 50 μM CCCP in 10 mM MOPS/KOH buffer for 20 min. Sections were analyzed either by epifluorescence microscopy using a multi-zoom microscope AZ-100 (Nikon) equipped with the proper filter cube (EX460/500/505/BA510) or by confocal microscopy using an LSM700 (Zeiss). For the latter, the excitation wavelengths 488 and 555 nm were used, and fluorescence light was collected in the range of 493 to 544 nm and 560 to 630 nm for Lysosensor DND-189 and WGA-tetramethylrhodamine, respectively. All settings (laser power, pinhole diameter, and detector gain) remained constant for all pictures.

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Statistics

To test for differences between plant genotypes and treatments, data were analyzed by ANOVA followed by Tukey HSD test or by Student’st test for pairwise comparisons using the Sigmaplot software package (Systat).

Accession Numbers

Sequence data from this article can be found in the GenBank/EMBL databases under accession number AJ132891.1 (HA1).

Supplemental Data

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

Supplemental Figure 1. Loss of Exon 8 Leads to Loss of Transmembrane Domains 3 and 4.

Supplemental Figure 2. Mycorrhizal ha1 Mutant Plants Show Decreased Expression of Mycorrhiza-Induced Genes.

Supplemental Figure 3. Artificial MicroRNA (amiR)-Mediated Knockdown of HA1.

Supplemental Figure 4. HA1 Is Not Induced during Root Nodule Symbiosis.

Supplemental Figure 5. The ha1-2 Mutant Does Not Show a Nodule Phenotype.

Supplemental Table 1. Primer Sequences.

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AUTHOR CONTRIBUTIONS

F.K., P.-E.C., B.H., P.F., and M.U. designed research and wrote the article. F.K., P.-E.C., D.Z., H.Z., I.K., D.S., and B.H. performed research. M.B. and N.G. contributed to phosphate uptake studies.

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The H+-ATPase HA1 of *Medicago truncatula* Is Essential for Phosphate Transport and Plant Growth during Arbuscular Mycorrhizal Symbiosis

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