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Aquaporin-mediated long-distance polyphosphate translocation directed towards the host in arbuscular mycorrhizal symbiosis: application of virus-induced gene silencing

Yusuke Kikuchi¹, Nowaki Hijikata¹, Ryo Ohtomo², Yoshihiro Handa³, Masayoshi Kawaguchi³, Katsuharu Saito⁴, Chikara Masuta¹, and Tatsuhiko Ezawa^{1*}

¹Graduate School of Agriculture, Hokkaido University, Sapporo 060-8589 Japan, ²National Agriculture and Food Research Organization, Hokkaido Agricultural Research Center, Sapporo 062-8555 Japan, ³Division of Symbiotic Systems, National Institute for Basic Biology, Okazaki 444-8585 Japan, and ⁴Faculty of Agriculture, Shinshu University, Minamiminowa 399-4598 Japan.

***Author for correspondence:**

Tatsuhiko Ezawa

Graduate school of Agriculture, Hokkaido University, Sapporo, Hokkaido 060-8589 Japan (tell & fax +81-11-857-9732; e-mail tatsu@res.agr.hokudai.ac.jp).

Summary

- Arbuscular mycorrhizal fungi translocate polyphosphate through hyphae over a long distance to deliver to the host. More than three decades ago, suppression of host transpiration was found to decelerate phosphate delivery of the fungal symbiont, leading us to hypothesize that transpiration provides a primary driving force for polyphosphate translocation, probably via creating hyphal water flow in which fungal aquaporin(s) may be involved.
- The impact of transpiration suppression on polyphosphate translocation through hyphae of *Rhizophagus clarus* was evaluated. An aquaporin gene expressed in intraradical mycelia was characterized and knocked down by virus-induced gene silencing to investigate the involvement of the gene in polyphosphate translocation.
- *R. clarus aquaporin 3 (RcAQP3)* that was most highly expressed in intraradical mycelia encodes an aquaglyceroporin responsible for water transport across the plasma membrane. Knockdown of *RcAQP3* as well as the suppression of host transpiration decelerated polyphosphate translocation in proportion to the levels of knockdown and suppression, respectively.
- These results provide the first insight into the mechanism underlying long-distance polyphosphate translocation in mycorrhizal associations at the molecular level, in which host transpiration and the fungal aquaporin play key roles. A hypothetical model of the translocation is proposed for further elucidation of the mechanism.

Keywords

arbuscular mycorrhiza; aquaglyceroporin; aquaporin; cucumber mosaic virus; polyphosphate translocation; *Rhizophagus clarus*, transpiration; virus-induced gene silencing

Introduction

Arbuscular mycorrhizal (AM) fungi form symbiotic associations with most land plants: the fungi take up and deliver inorganic phosphate (Pi) to the host through hyphal networks constructed both in the soil and roots (Smith & Read, 2008). Pi is poorly mobile in the soil, but the fungi accumulate polyphosphate (polyP) in the vacuoles (Viereck *et al.*, 2004; Kuga *et al.*, 2008) and translocate it to the host more rapidly than Pi diffusion in the soil (Smith *et al.*, 2011). Despite the significance of the processes of polyP translocation, the molecular mechanism underlying has been largely unexplored.

In early studies, polyP translocation in AM fungi was interpreted by the energy-dependent processes, e.g., vesicle transport driven by cytoplasmic streaming in random directions (Cox *et al.*, 1980; Cooper & Tinker, 1981), which is likely to facilitate carbon (lipid) translocation towards hyphal tips (Bago *et al.*, 2002). The discovery of motile tubular vacuoles in filamentous fungi (Shepherd *et al.*, 1993; Rees *et al.*, 1994; Uetake *et al.*, 2002), however, raised an alternative mechanism; solute in the vacuoles diffuses bidirectionally (i.e. in random direction) along source-sink gradients (Darrah *et al.*, 2006). On the other hand, AM fungi associating with an autotrophic plant seem to translocate polyP more rapidly towards the host than those associating with the hairy root culture (Hijikata *et al.*, 2010), suggesting that there is a mechanism for 'directional' polyP translocation towards the roots, although no such mechanism has been proposed so far.

There is increasing evidence that mycorrhizal fungi transport water towards the host (Egerton-Warburton *et al.*, 2007; Li *et al.*, 2013; Xu *et al.*, 2015). Although the

physiological mechanism has yet to be elucidated, it is likely that water moves along water potential gradients between the root and fungal cells, in which transpiration would primarily create the gradients (Plamboeck *et al.*, 2007). More than three decades ago, Cooper & Tinker (1981) demonstrated that suppression of host transpiration reduced Pi delivery from an AM fungus, leading us to hypothesize that transpiration primarily drives polyP translocation towards the roots. Cooper & Tinker (1981), however, considered that the suppression of transpiration inhibited photosynthesis and thus carbon supply to the fungus, which could potentially inhibit the energy-dependent processes. Therefore, the involvement of transpiration in polyP translocation should be examined with attention to fungal energy status.

In the water transport processes, aquaporins play a key role. Fungal aquaporins are classified into three groups: orthodox aquaporins, aquaglyceroporins, and X intrinsic proteins (Nehls & Dietz, 2014). In the ectomycorrhizal fungus *Laccaria bicolor*, one orthodox aquaporin and five aquaglyceroporins have been identified in the genome, and two aquaglyceroporins showed highest water transport activity across the plasma membrane among them (Dietz *et al.*, 2011). In the AM fungus *R. irregularis* the aquaglyceroporin AQPF2 also showed higher water transport activity than the other two orthodox aquaporins (Aroca *et al.*, 2009; Li *et al.*, 2013). These observations suggest that in fungi aquaglyceroporins play a main role in water transport across the plasma membrane.

In modern biology gene knockdown/knockout by genetic transformation are essential techniques to study loss-of-function phenotypes of genes. These techniques, however, could not be applied to AM fungi due to the nature of coenocytic obligate biotrophs.

Recently, two effector genes of the obligate biotrophic pathogen *Blumeria graminis* were successfully knocked down by the introduction of RNA interference (RNAi)-construct into the host cell by several methods, including virus-induced gene silencing (VIGS) (Nowara *et al.*, 2010). The present study addresses the hypothesis that there is a mechanism for directional translocation of polyP towards the roots, in which transpiration and fungal aquaporins are involved. To test this hypothesis, VIGS was applied for knockdown of a fungal gene.

Materials and Methods

Culture conditions and experimental system

The dual mesh bag two-compartment culture system was employed (Hijikata *et al.*, 2010); the root-hyphal compartment (RHC) and hyphal compartment (HC) were separated by a cone-shaped 37 μ m dual nylon mesh bag, and in between the inner and outer mesh bags autoclaved subsoil with a high-Pi absorption coefficient was layered as a Pi-diffusion barrier (Supporting Information Figure S1). Pregerminated seeds of *Lotus japonicus* L. MG-20 or wild-type *Nicotiana benthamiana* Domin were sown to the RHC (four seedlings per pot), inoculated with *R. clarus* strain HR1 (MAFF 520076) at 500 spores pot⁻¹, and grown for seven weeks as described in Kikuchi *et al.* (2014). At the beginning of the eighth week, a 1 mM KH₂PO₄ solution at pH 4.8 (approx. 15 mL) was applied to the HC and washed out with deionized water one h after application, and then mycorrhizal roots in the RHC and extraradical mycelia in the HC were harvested at prescribed time points, weighed, frozen in liquid nitrogen, and stored at -80°C as described in Kikuchi *et al.* (2014).

PolyP and protein concentrations in the frozen materials were determined by the polyphosphate kinase-luciferase and Bradford methods, respectively, as described in Ezawa *et al.* (2004). Rates of polyP translocation were calculated based on net increases in polyP from zero to 12 or 24 h after Pi application and expressed per unit time per unit protein. To evaluate energy status of the fungus (Besserer *et al.*, 2008), ATP concentration in the mycelia was determined also by the luciferase method in which polyphosphate kinase reaction was omitted.

Suppression of host transpiration

Transpiration of *L. japonicus* was suppressed to different extents by the following three methods: foliar application of abscisic acid (ABA), dark treatment, and shoot removal. For the ABA application, either 0.5 mM ABA in 50 mM MES buffer at pH 5.8 or the MES buffer (control) was applied to the above-ground part with a sprayer 1 h after Pi application, during which the surface of the medium was covered with a plastic film to prevent the solution from dropping into the medium. For the dark treatment, fluorescent light in the growth chamber was turned off 2 h before Pi application until the end of experiment. For the shoot removal, shoots of the seedlings were cut at 10 mm above the ground, and the remaining shoot was sealed immediately with Parafilm prior to Pi application. Transpiration of the plants was measured by the gravimetric method (Aroca *et al.*, 2007) and expressed on the basis of either leaf areas (only for the ABA and dark treatments) or root fresh weight (for all three treatments).

Gene identification

A gene repertoire of *R. clarus* HR1 was obtained by RNA-Seq, and open reading frames (ORFs) of 50 or longer amino acid residues were predicted as described in Supporting Information Methods S1. Fungal aquaporins (*RcAQP1 – 3*) and α -tubulin (*RcTUB α*) genes were identified in the ORFs, and their nucleotide sequences have been deposited in DDBJ under accession numbers LC015358, LC015359, LC015360, and LC015361, respectively. Expression levels of *RcTUB α* were used for standardization of expression levels of fungal aquaporin genes, as well as for a biomass marker of the fungus.

Pi transporter genes of *N. benthamiana* were searched from the database, aligned with the known mycorrhiza-specific Pi transporters (Harrison *et al.*, 2002; Paszkowski *et al.*, 2002; Nagy *et al.*, 2005), and their expression levels were compared between the mycorrhizal and non-mycorrhizal plants (Supporting Information Methods S1 and Figure S2) to select a gene that is specifically and highly expressed in the mycorrhizal roots as an indicator for functional colonization of the fungus (Floss *et al.*, 2013).

Functional characterization and virus-induced gene silencing of *RcAQP3*

Water transport activity of the aquaporin *RcAQP3* was assessed by heterologous expression in yeast cell. The gene was cloned into a yeast expression vector and transformed, and sensitivity of the transformant protoplast cells to a hypo-osmotic shock was examined according to Pettersson *et al.* (2005). Detailed methods are described in Supporting Information Methods S2, and the primers used for gene amplification are listed in Table S1.

A partial sequence of the gene was amplified and cloned into the CMV2-A1 vector developed from RNA2 of *Cucumber mosaic virus* Y strain (CMV-Y) (Otagaki *et al.*, 2006) and designated as a knockdown construct. The same vector into which a partial sequence of *Cauliflower mosaic virus* 35S promoter was cloned (Kanazawa *et al.*, 2011) was employed as a control construct. These constructs, as well as RNA1 and RNA3 of CMV-Y, were transcribed *in vitro*, combined, replicated *in vivo* in *N. benthamiana* seedlings, and then viral inocula were prepared by grinding an infected leaf (Supporting Information Methods S3). For VIGS experiments, *N. benthamiana* was employed as a host, because the systemic infection occurs in *N. benthamiana*, but not in *L. japonicus*. Five-week-old *N. benthamiana* seedlings grown in association with *R. clarus* HR1 in the dual mesh bag culture system were dusted with carborundum (Nacalai Tesque, Kyoto), and rub-inoculated either with the knockdown or control virus. The mycorrhizal roots and extraradical mycelia were harvested from the RHC and HC, respectively, 1 – 3 weeks after virus inoculation, frozen in liquid nitrogen, and stored at -80°C. Quantitative reverse transcription-PCR for evaluation of gene expression levels and stem-loop PCR for detection of small interference RNA (siRNA) were performed as described in Supporting Information Methods S4.

Results

Suppression of host transpiration decelerates polyphosphate translocation

The foliar application of abscisic acid (ABA), dark treatment, and shoot removal reduced total transpiration for 12 h by 44.3%, 71.0%, and 96.1%, respectively

(Supporting Information Figure S3). Without these treatments, application of 1 mM Pi solution to the HC triggered rapid accumulation of polyP in extraradical mycelia in the HC, followed by gradual increases in polyP in the mycorrhizal roots (intraradical mycelia) in the RHC (Supporting Information Figure S4), indicating that polyP accumulated in the extraradical mycelia was translocated to intraradical mycelia several hours after Pi application (Hijikata *et al.*, 2010). The dark and shoot removal treatments increased the polyP levels in the HC and decreased those in the RHC, but the effect of ABA was not obvious, likely reflecting the suppression levels of transpiration in these treatments. Correlation analysis between the rates of transpiration and polyP translocation to the RHC was conducted using a combined dataset of all three experiments; the translocation rates were positively correlated with the transpiration rates ($r = 0.65$, $P < 0.01$) (Figure 1), indicating that the suppression of transpiration decelerated polyP translocation towards the roots. The ATP levels in extraradical mycelia in the HC were not altered by the suppression of transpiration (Supporting Information Figure S4).

Identification and characterization of fungal aquaporin genes

Three putative aquaporin genes, *RcAQP1*, *RcAQP2*, and *RcAQP3*, with full-ORF sequences were found in 19,493 gene models predicted by the RNA-Seq. Phylogenetic analysis (Figure 2a) indicated that *RcAQP1* and *RcAQP2* are most similar to the *R. irregularis* orthodox aquaporins *RiAQP1* and *RiAQP2*, respectively, that showed low or no water transport activity (Aroca *et al.*, 2009; Li *et al.*, 2013). *RcAQP3* was highly similar to the aquaglyceroporins of *R. irregularis* (*RiAQP3*) and *L. bicolor* (Lacbi1:247946) of which water transport activity across the plasma membrane was

confirmed (Dietz *et al.*, 2011; Li *et al.*, 2013). *RcAQP3* was most highly expressed both in extraradical and intraradical hyphae among the three both in *L. japonicus* and *N. benthamiana* (Figure 2b). Accordingly, we considered that *RcAQP3* would play a major role in water transport in the fungus and thus chose the gene for further analysis.

RcAQP3 encodes a putative polypeptide of 316 amino acids with the NPA motifs in the loops B and E (Supporting Information Figure S5), which are typical features of aquaporins (Pettersson *et al.*, 2005). Water transport activity of *RcAQP3* across the plasma membrane was confirmed by heterologous expression in the yeast *Saccharomyces cerevisiae*; protoplasts of the cell transformed with a vector carrying *RcAQP3* burst more rapidly than those of the control transformant in response to a hypo-osmotic shock (Figure 2c).

Impact of *RcAQP3* knockdown on polyphosphate translocation towards the host

Preliminary experiments showed that *RcAQP3* expression in the mycorrhizal roots was successfully knocked down from two to three weeks after viral inoculation, which did not affect the expression levels of *RcAQP2* that is moderately expressed in intraradical hyphae (Supporting Information Figure S6). One of the siRNAs originated from the *RcAQP3* insert was detected in the roots inoculated with the knockdown virus by stem-loop PCR, confirming that the reduction in *RcAQP3* expression was induced by the siRNAs. Accordingly, we decided to conduct the main experiments three weeks after viral inoculation.

In the main experiments, *RcAQP3* expression was reduced to 32 – 35% of the control

levels, and these levels were maintained before and 24 h after Pi application (Figure 3a). Pi application to the HC triggered massive accumulation of polyP in the HC both in the knockdown and control treatments, but in the knockdown treatment little polyP was translocated to the RHC even 24 h after Pi application (Figure 3b). In fact, there was a strong positive correlation between the rates of polyP translocation and *RcAQP3* expression ($r = 0.90$, $P < 0.001$) (Figure 3c).

The knockdown of *RcAQP3* significantly reduced shoot dry weight and phosphorus (P) content by 27.5% and 25.4%, respectively, but did not affect transpiration (Supporting Information Figure S7). Shoot dry weight was significantly correlated with P content ($r = 0.84$, $P < 0.01$), suggesting that plant growth was limited by Pi availability under the experimental conditions. The *RcAQP3* knockdown did not alter expression levels of the indicator genes for fungal biomass (*RcTUB α*) and functional colonization (mycorrhiza-specific Pi transporter gene *NbS00058434g0003*) (Supporting Information Figure S8). Fresh weight and ATP content of extraradical mycelia in the HC were also not different between the knockdown and control treatments. All these observations indicated that the knockdown of *RcAQP3* significantly reduced Pi uptake of the host, but had a minimum impact on fungal biomass, functional colonization, and energy status.

Discussion

The present study provides the first insight into the mechanism of polyP translocation at the molecular level via applying VIGS. The fungal aquaglyceroporin *RcAQP3* is highly expressed in intraradical mycelia and responsible for water transport across the

plasma membrane, and the knockdown of the gene, as well as the suppression of transpiration, decelerated polyP translocation towards the roots without altering the fungal physiological status. These results provide evidence that there is a mechanism for directional polyP translocation towards the roots, in which host transpiration and the fungal aquaporin play key roles.

The feasibility of gene silencing in AM fungi was first demonstrated by host-induced gene silencing (HIGS), in which the hairy root culture transformed with the RNAi construct was employed (Helber *et al.*, 2011). Both in VIGS and HIGS, siRNAs generated in the host tissue would be transferred to the fungal cell, probably through the exocytic/endocytic exchange mechanisms at the plant–fungal interface, and trigger gene silencing (Nowara *et al.*, 2010). One benefit of VIGS is that selection of stable genetic transformants is unnecessary, and thus the technique enables rapid screening of candidate genes (Sahu *et al.*, 2012). In addition, gene silencing can be induced at any developmental stage of target organisms in VIGS, which allows us to study genes that are expressed in mature to late developmental stages of the targets.

We propose a new role of the fungal aquaporin in solute transport. To interpret the roles of plant transpiration and the fungal aquaporin in the processes, we constructed a model based on the findings, clarifying issues that remain to be addressed in the future (Figure 4). In this model, transpiration provides a primary driving force for polyP (and probably also Pi) translocation via creating hyphal water flow through the mycorrhiza-inducible plant aquaporin in the periarbuscular membrane (Giovannetti *et al.*, 2012). RcAQP3 is localized in the fungal plasma membrane, probably enriched in arbuscules as observed in *RiAQPF2* (the ortholog of *RcAQP3*) that is up-regulated in

arbuscules (Li *et al.*, 2013), and mediates the water flow across the membrane. This model raises the following two questions: i) whether there is water flow not only through the cytosol but also through the vacuolar lumen in which polyP is accumulated and ii) how polyP is transported across gaps between vacuoles, which are discussed in the following sections.

So far, little information about water permeability of tonoplast has been obtained in fungi. One possibility is that RcAQP3 acts as a versatile aquaporin that facilitates water transport not only across the plasma membrane but also across the tonoplast, because RiAQP2, the aquaglyceropoin encoded by the ortholog of *RcAQP3*, was localized in intracellular membranes as well as in the plasma membrane (Li *et al.*, 2013). Elucidation of the mechanism of transvacuolar water flow in AM fungi is necessary.

Dynamic connections between tubular vacuoles, e.g., extension/fission and subsequent fusion of two vacuoles, would facilitate a pathway for solute transport across gaps between the vacuoles (Shepherd *et al.*, 1993). In addition, intervacuolar transport may also occur via the cytosol. In *R. clarus* HR1, not only polyP polymerase genes but also the genes encoding vacuolar polyP hydrolase and vacuolar Pi exporter were up-regulated during polyP accumulation (Kikuchi *et al.*, 2014). This suggests that polyP/Pi are turning over between the vacuoles and cytosol i.e. Pi generated by hydrolysis of polyP could be released into the cytosol from one vacuole and polymerized in the next vacuole, facilitating a pathway for polyP transport. Knockdown experiments on the hydrolase and exporter genes are currently being undertaken to examine the idea.

The finding that host transpiration is involved in polyP translocation in the fungi has important implications for understanding the regulatory mechanisms of nutrient exchange between the host and fungal symbionts. It has been proposed that plant carbon source strength largely regulates fungal Pi delivery (Kiers *et al.*, 2011; Fellbaum *et al.*, 2014), whereas Walder *et al.* (2012) suggested that not only carbon source strength but also other unknown factors are involved in the Pi delivery. The present study highlights the significance of plant transpiration in the efficiency of fungal Pi delivery, which would provide a new interpretation of the regulatory mechanisms.

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Author contributions

Y.K., C.M., and T.E. planned and designed the research, Y.K. and N.H. performed the main experiments, R.O. measured polyP, Y.K., Y.H., M.K., and K.S. performed RNA-Seq, Y.K. and T.E. analyzed the data and wrote the manuscript.

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Figures

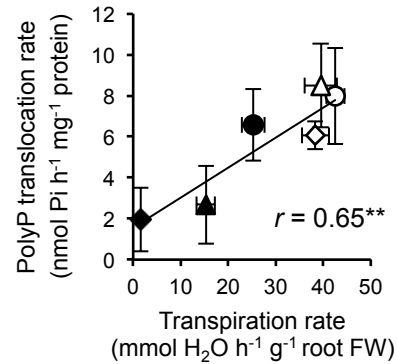


Figure 1. Correlation between the rates of *Lotus japonicus* transpiration and polyphosphate (polyP) translocation through mycelia of *Rhizophagus clarus* HR1 to the root-hyphal compartment. The rates of net increase in polyP in the root-hyphal compartment from zero to 12 h after phosphate application in the ABA application (closed circles), dark treatment (closed triangles), and shoot removal (closed diamonds) experiments (calculated from Figure S3) are plotted against the transpiration rates (recalculated from Figure S2 on the basis of root FW). The open symbols represent the data of corresponding control treatments. All raw data were used for the correlation analysis, but only the average values of individual treatments were presented. Vertical and horizontal bars are $\pm$ SE. **, $P < 0.01$.

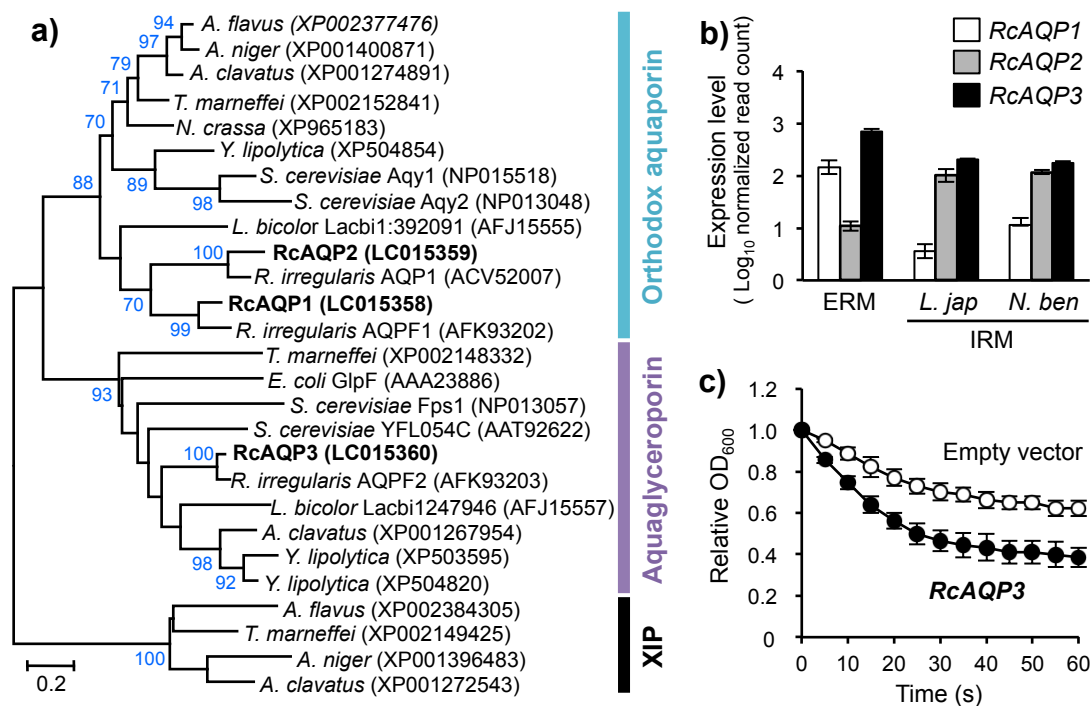


Figure 2. Characterization of three aquaporin genes (*RcAQP1* – 3) of *Rhizophagus clarus* HR1. **(a)** Neighbor-joining tree constructed based on the deduced amino acid sequences of fungal aquaporins using MEGA 6.06. Bootstrap values more than 70% are indicated at the nodes (1000 replicates). Fungal aquaporins are classified into three groups: orthodox aquaporin, aquaglyceroporin, and X intrinsic protein (XIP). Accession numbers are given in parentheses. **(b)** Expression levels of *AQP1* – 3 of *R. clarus* HR1 in extraradical (ERM) and intraradical mycelia (IRM) in the mycorrhizal roots of *Lotus japonicus* and *Nicotiana benthamiana*. The expression data in extraradical hyphae were obtained in the fungus associated with *L. japonicus*. The raw read counts in the fungus obtained by the RNA-Seq analysis were normalized in units of mapped Reads Per Kilobase of coding sequence per Million reads (RPKM). Vertical bars are $\pm$ SE ($n = 3$). **(c)** Water transport activity of *RcAQP3* across plasma membrane as assessed by heterologous expression in yeast cells. *Saccharomyces cerevisiae*

481 protoplasts transformed with the yeast expression vector pYES2 containing *RcAQP3*
482 (closed circles) burst more rapidly than those harboring an empty vector (open circles)
483 in response to a hypo-osmotic shock. Protoplast burst was monitored by decreases in
484 optical density at 600 nm (OD_{600}). Vertical bars are $\pm$ SE ($n = 3$).
485

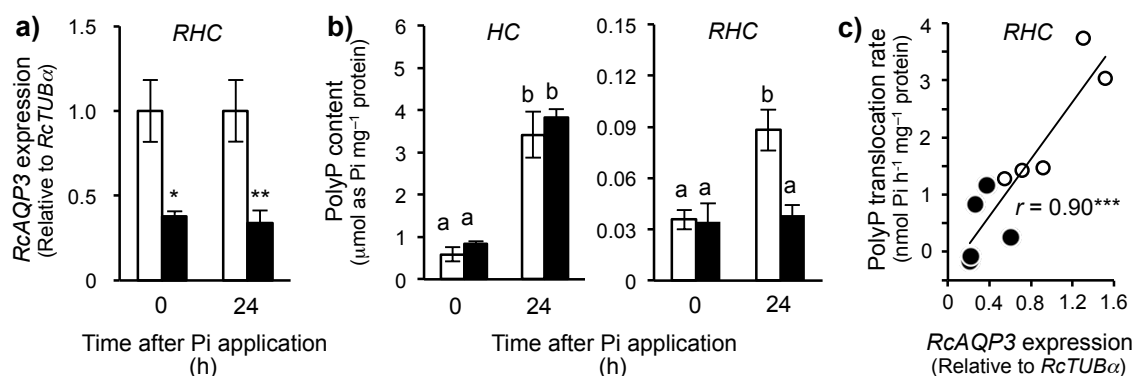


Figure 3. Effect of *RcAQP3* knockdown on polyphosphate (polyP) translocation in *Rhizophagus clarus* HR1. *Nicotiana benthamiana* colonized with *R. clarus* HR1 was rub-inoculated with the recombinant *Cucumber mosaic virus* containing a partial sequence of either *RcAQP3* for knockdown (closed bars and circles) or *Cauliflower mosaic virus* 35S promoter for control (open bars and circles), and the levels of *RcAQP3* expression and polyP were measured three weeks after virus inoculation. (a) The expression levels in the mycorrhizal roots in the root-hyphal compartment (RHC) at time zero and 24 h after phosphate application: *, $P < 0.05$; **, $P < 0.01$ (Student's *t*-test). Vertical bars are $\pm$ SE ($n = 5$). (b) PolyP content in extraradical mycelia in the hyphal compartment (HC) and in the mycorrhizal roots in the RHC at time zero and 24 h after phosphate application. Different letters indicate significant differences at $P < 0.05$ (Tukey–Kramer test). Vertical bars are $\pm$ SE ($n = 5$). (c) Correlation between *RcAQP3* expression and the rates of polyP translocation to the RHC. The rates of net increase in polyP from zero to 24 h after Pi application in the RHC were plotted against the expression levels. Asterisks indicate significance of correlation at $P < 0.001$.

Supporting Information

Methods S1. RNA-Seq, gene prediction, and digital gene expression analysis

Germinated seeds of *L. japonicus* or *N. benthamiana* were sown in the RHC in the single mesh bag culture system in which the P-diffusion barrier was omitted, inoculated with or without *R. clarus* HR1, and grown under the same conditions for 4 weeks as described in Culture conditions and experimental system in Materials and Methods. Total RNA was extracted from the roots collected from the RHC with RNeasy Plant Mini Kit (Qiagen, Tokyo, Japan) and digested with RNase-free DNase I (Qiagen). Sequencing libraries were constructed using 500 ng of total RNA with TruSeq RNA Sample Prep kit (Illumina, Tokyo, Japan) according to the manufacturer's instructions, and paired-end sequencing were performed with Illumina HiSeq2000. Raw sequence data were deposited in DDBJ Sequence Read Archive under accession number DRA002842 (mycorrhizal roots of *L. japonicus*) and DRA002839 (non-mycorrhizal and mycorrhizal roots of *N. benthamiana*).

High-quality reads obtained from the mycorrhizal roots of *L. japonicus* of which > 90% bases showed Phred quality scores > 20 were mapped to the *L. japonicus* genome assembly build 2.5 (<http://www.kazusa.or.jp/lotus/>) using TopHat program with default parameters (Trapnell *et al.*, 2009) to exclude those originated from the host, and unmapped reads were combined with those of extraradical mycelia of *R. clarus* HR1 (DRA001877) obtained by Kikuchi *et al.* (2014) and subjected to *de novo* assembly with Trinity program (Grabherr *et al.*, 2011). The assembled cDNA contigs were queried against the predicted gene models (Gloin1_all_transcripts_20120510.nt.fasta)

and expressed sequence tags (EST) (Gloin1_ESTs_20120510_Combest_RNA_and_EST_contigs.fasta) of *R. irregularis* DAOM 181602 in the DOE Joint Genome Institute (<http://genome.jgi.doe.gov/Gloin1/Gloin1.home.html>) as well as against the GenBank nucleotide collection with the BLASTN algorithm at an e-value cutoff 10^{-5} , and contigs that showed higher similarity i.e. lower e-value and higher bit score to the sequences in the *R. irregularis* genome database than those in GenBank were defined to be of *R. clarus* HR1 origin. ORFs of 50 or longer amino acid residues were predicted and extracted from the contigs by using the utility program of Trinity (transcripts_to_best_scoring_ORFs.pl) and clustered using CD-HIT-EST with a 95% sequence identity cutoff, and then the longest ORFs were selected from each cluster as representatives to minimize duplication of putative splice variants, polymorphisms, and fragmented (frameshifted) ORFs in the predicted gene set (Li & Godzik, 2006). Fungal aquaporins (*RcAQPI* – 3) and α -tubulin (*RcTUB α*) genes were identified in the ORF sequences through BLASTP searches against the *S. cerevisiae* protein database (orf_trans.fasta) in the *Saccharomyces* Genome Database (<http://www.yeastgenome.org/>).

Putative phosphate (Pi) transporter genes of *N. benthamiana* were identified from the predicted ORF sequences provided by the Sol Genomics Network (Niben.genome.v0.4.4.proteins.fasta) at the Boyce Thompson Institute (<http://solgenomics.net>) through BLASTP searches against the Reference Sequence database of plant proteins at National Center for Biotechnology Information. Phylogenetic analysis and construction of neighbor-joining tree was implemented using MEGA 6.06 (Tamura *et al.*, 2013).

Expression levels of transcripts were estimated based on the number of reads that were uniquely mapped to the corresponding ORF sequences using Burrows-Wheeler Aligner's Smith-Waterman algorithm as described in detail in Kikuchi *et al.* (2014).

Methods S2. Functional characterization of the fungal aquaporin gene *RcAQP3*

Total RNA extracted from the *L. japonicus* roots colonized with *R. clarus* HR1 was reverse-transcribed, and the full ORF sequence of *RcAQP3* was PCR amplified using the primer pair of *RcAQP3*-full to which the Kozak sequence (AAAAAAATGTCT) was introduced at the translation initiation site (Supporting Information Table S1) and cloned into pT7Blue T-vector (Novagen, Madison, WI). The construct was transformed into *E. coli* JM 109-competent cell (Takara, Shiga, Japan), and the plasmid vector was purified and digested with the restriction enzymes *EcoRI* and *XbaI*. The restriction fragment was subcloned into the *EcoRI-XbaI* restriction site of the yeast expression vector pYES2 (Invitrogen, Waltham, MA) and transformed into *S. cerevisiae* INVSc1 using *S. cerevisiae* Direct Transformation Kit (Wako Pure Chemicals, Osaka, Japan) according to the manufacturer's instructions. The empty pYES2 vector was also transformed into the yeast to obtain a control strain. Water transport activity of the yeast was evaluated according to Pettersson *et al.* (2005). The transformed cells were grown in 1 ml of the minimal media with 2% raffinose and 2% galactose and without uracil to an optical density at 600 nm (OD₆₀₀) of 1.2, washed once with sterile water and then with 1 M sorbitol, suspended in 1 ml SCE buffer (1 M sorbitol, 0.1 M sodium citrate, 10 mM EDTA, 0.2 mM β-mercaptoethanol, pH 6.8), digested with 2 000 U ml⁻¹ lyticase (Sigma-Aldrich, Tokyo, Japan) at 30°C for 4 h with

shaking, washed twice with STC buffer (1 M sorbitol, 10 mM Tris-HCl, 10 mM CaCl₂, pH 7.5), and resuspended in 1 ml STC buffer. Then the resultant protoplasts were subjected to a hypo-osmotic shock by diluting the suspension with 4-fold volume of 0.5 M sorbitol in the STC buffer (final sorbitol concentration was 0.6 M), and OD₆₀₀ was monitored immediately after dilution at 5-s intervals for 60 s.

Methods S3. Vector construction and inoculum preparation for virus-induced gene silencing

Total RNA (500 ng) was extracted from the *N. benthamiana* roots colonized with *R. clarus* HR1 and reverse-transcribed with a mixture of random and oligo dT primers using PrimeScript RT Master Mix Kit (Takara, Shiga, Japan), and a partial sequence (108-bp) of *RcAQP3* was amplified using the primer pair of *RcAQP3*-VIGS to which *Mlu*I and *Stu*I restriction sites were introduced to the forward and reverse primers, respectively (Supporting Information Table S1). The amplicon was cloned into pT7Blue T-vector, digested with *Stu*I and *Mlu*I restriction enzymes, and subcloned into the restriction sites of CMV2-A1 vector derived from RNA2 of CMV-Y (Otagaki *et al.*, 2006). Similarly, a partial sequence (346 bp) of *Cauliflower mosaic virus* (CaMV) 35S promoter was amplified with the primer pair of CaMV-35S from genomic DNA of *N. benthamiana* line 16c to which the CaMV-35S promoter sequence had been introduced (Ruiz *et al.*, 1998) and cloned into CMV2-A1 vector (Kanazawa *et al.*, 2011) for the control virus. The CMV2-A1 constructs, as well as RNAs 1 and 3 of CMV-Y that are cloned in plasmid vectors separately (Suzuki *et al.*, 1991), were linearized and transcribed *in vitro* in a mixture of 1 µg of the linearized vector, 25 U T7 RNA polymerase (Takara), 20 U ribonuclease inhibitor, 5 mM DTT, 1 mM ATP, 1 mM CTP,

1 mM UTP, 0.1 mM GTP, 1 mM m⁷G(5')PPP(5')G (Invitrogen) as a cap analogue in 40 mM Tris-HCl (pH 8.0), 8 mM MgCl₂, and 2 mM spermidine-HCl at 37°C for 60 min.

Prior to the experiments, the viral inocula were first replicated as follows. Leaves of 5-week-old *N. benthamiana* were dusted with carborundum (Nacalai Tesque, Kyoto, Japan), rub-inoculated with a mixture of the transcribed viral RNAs, grown for one week, and viral inocula were prepared by grinding an infected leaf (about 100 mg) in a mortar and pestle with 1 ml of 10 mM sodium *N,N*-diethyldithiocarbamate trihydrate in 100 mM sodium phosphate buffer pH 7.1.

Methods S4. Quantitative reverse transcription-PCR and stem-loop PCR

For quantitative reverse transcription-PCR, total RNA (500 ng) was reverse-transcribed with a mixture of random and oligo dT primers using PrimeScript RT Master Mix Kit (Takara, Shiga, Japan) and amplified using SYBR Premix Ex Taq II (Takara) with LightCycler (Roche diagnostics, Tokyo Japan) according to the manufacturer's instructions with the following thermal cycle program: 95°C for 30 s and 40 cycles of 95°C for 5 s and 60°C for 20 s. The levels of transcripts were estimated by the relative standard curve method. The expression levels of the fungal aquaporin genes were standardized on the basis of *RcTUBα* expression, whereas those of *RcTUBα* and *NbS00058434g0003* were standardized on the basis of *N. benthamiana* 60S ribosomal protein gene (*NbL23*) expression (Liu *et al.*, 2012). All primers used for these experiments are listed in Supporting Information Table S1.

Stem-loop PCR was employed for detecting one of siRNAs (ATCGCTCAAACCGTACTTAGCA) generated from the *RcAQP3* insert of the knockdown construct according to Varkonyi-Gasic *et al.* (2007). Total RNA was extracted from the roots of *N. benthamiana* in the knockdown and control treatments, reverse-transcribed with the primer *RcAQP3*-SLPCR_RT (Supporting Information Table S1), amplified with the primer pair of *RcAQP3*-SLPCR, visualized on polyacrylamide gel electrophoresis, cloned into a plasmid vector, and sequenced by the dideoxy method. U6 snRNA was also amplified as internal standard (Feng *et al.*, 2013).

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Figures

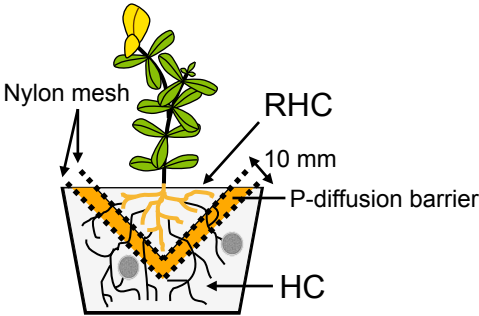


Figure S1. Dual mesh bag two-compartment culture system. The root-hyphal (RHC) and hyphal (HC) compartments were separated by a cone-shaped dual 37 µm nylon mesh bag. The medium in the RHC and HC was autoclaved river sand, and autoclaved subsoil with a high-P absorption coefficient was layered in between the inner and outer mesh bags as a P-diffusion barrier (10 mm in width).

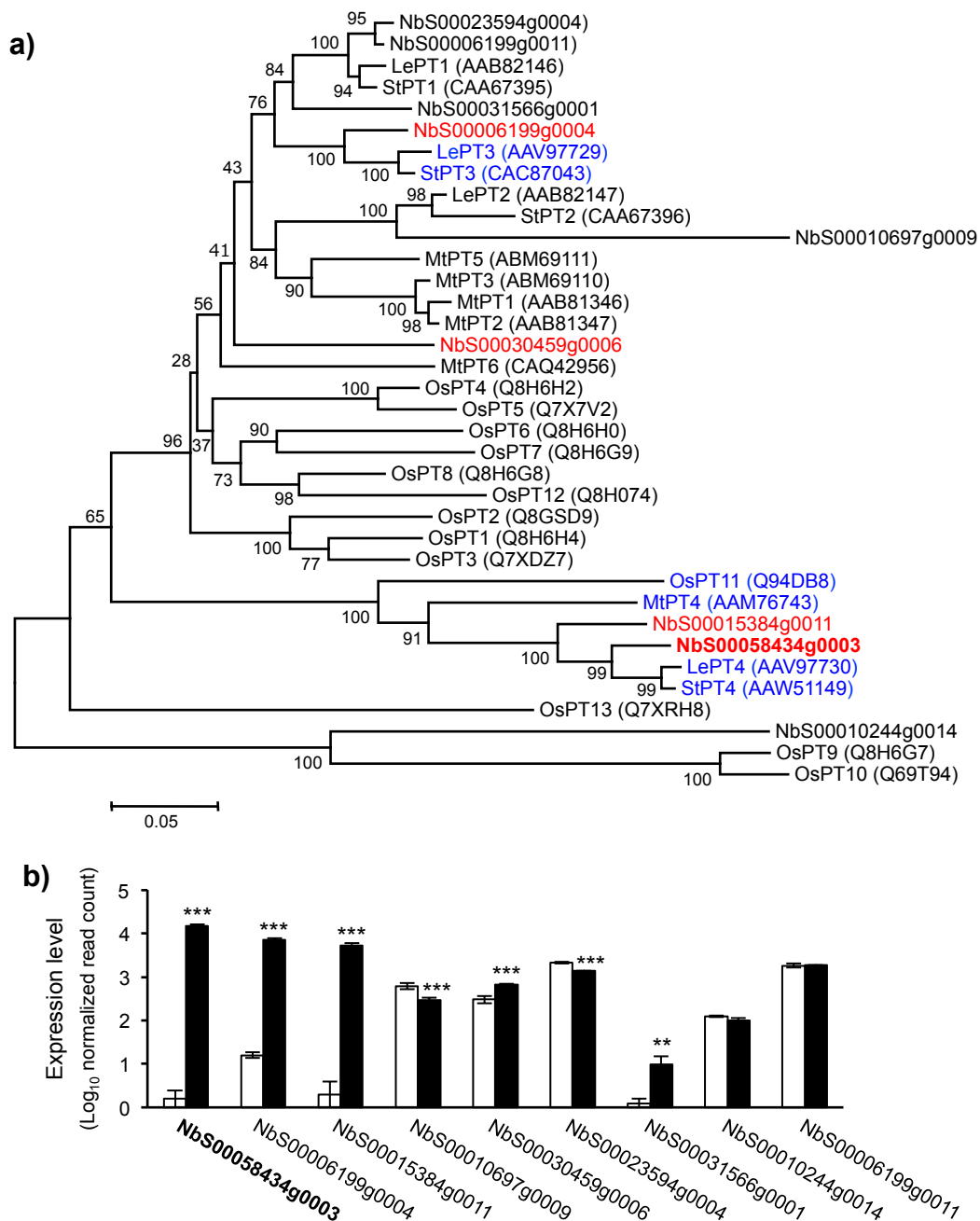


Figure S2. Identification of mycorrhiza-specific phosphate transporter genes of *N. benthamiana*. **(a)** Phylogenetic analysis of plant phosphate transporter genes based on the deduced amino acid sequences. The tree was constructed by neighbor-joining method, and percent bootstrap values (1000 replicates) are indicated at the nodes. *N. benthamiana* genes that were up-regulated more than 100-fold in response to mycorrhizal colonization are written in red letters. The mycorrhiza-specific phosphate

transporter genes that have previously been described in literature are written in blue letters. Accession numbers are given in parentheses. **(b)** Expression profiles of putative phosphate transporter genes in the presence (closed bars) and absence (open bars) of *R. clarus* HR1. Total RNA was extracted from the roots 4 weeks after sowing and subjected to RNA-Seq. Statistical testing for differential expression between the treatments was performed using the edgeR package in R coupled with iDEGES/edgeR normalization. Asterisks indicate significant differences: **, false discovery rate (FDR) < 0.01; ***, FDR < 0.001. Vertical bars are $\pm$ SE ($n = 3$). *NbS00058434g0003* was employed as an indicator gene for functional colonization of *R. clarus* HR1 is written in bold letters.

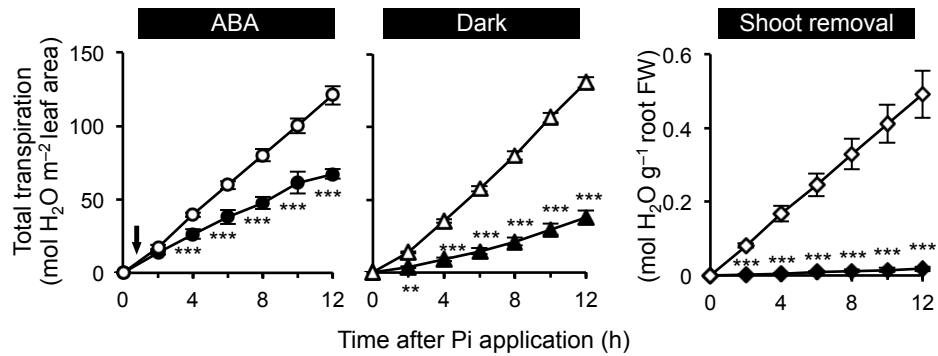


Figure S3. Effect of abscisic acid (ABA) application, dark treatment, and shoot removal on transpiration in 8-week-old *L. japonicus* colonized with *R. clarus* HR1. Transpiration was suppressed by one of the following three treatments before or after 1 mM phosphate (Pi) application to the hyphal compartment (at time zero): ABA, either 0.5 mM ABA in 50 mM MES buffer (closed circles) or 50 mM MES buffer (open circles) was applied to the above-ground parts with a sprayer 1 h after Pi application (arrow); Dark, the plants were incubated either under the dark (closed triangles) or light (open triangles) conditions 2 h prior to Pi application; Shoot removal, the shoots were either removed (closed diamonds) or not (open diamonds) at time zero. Vertical bars are $\pm$ SE ($n = 3$). Asterisks indicate significant differences between the treatments at the same time point: **, $P < 0.01$; ***, $P < 0.001$ (Student's *t*-test).

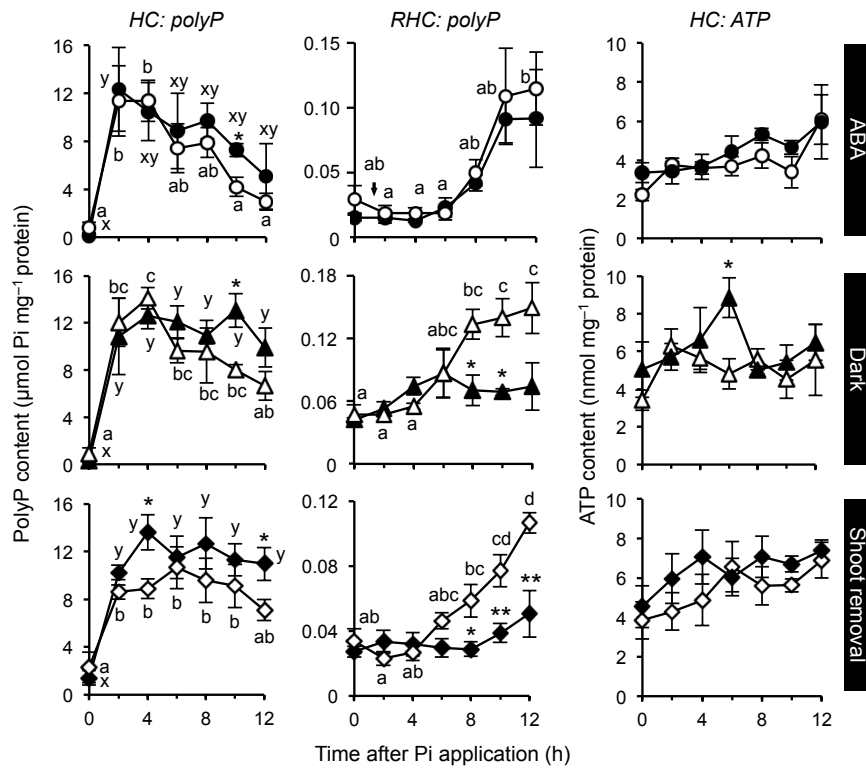


Figure S4. Effect of suppression of *L. japonicus* transpiration by abscisic acid (ABA), dark treatment, and shoot removal on polyphosphate (polyP) and ATP dynamics in *R. clarus* HR1: polyP dynamics in extraradical mycelia in the hyphal compartment (HC: polyP), those in the mycorrhizal roots (intraradical mycelia) in the root-hyphal compartment (RHC: polyP), and ATP dynamics in extraradical mycelia in the hyphal compartment (HC: ATP). Transpiration of 8-week-old seedlings was suppressed either by foliar application of 0.5 mM ABA, dark treatment, or shoot removal, and polyP contents were measured after 1 mM phosphate (Pi) application at 2-h intervals. The closed and open symbols represent the transpiration-suppressed and control treatments, respectively. Vertical bars are $\pm$ SE ($n = 3$ for the ABA and dark treatments, and $n = 5$ for the shoot-removal treatment). Asterisks indicate significant differences between the treatments at the same time point: *, $P < 0.05$; **, $P < 0.01$ (Student's *t*-test). Different letters indicate significant differences among the time points within the same treatments at $P < 0.05$ (Tukey–Kramer test).

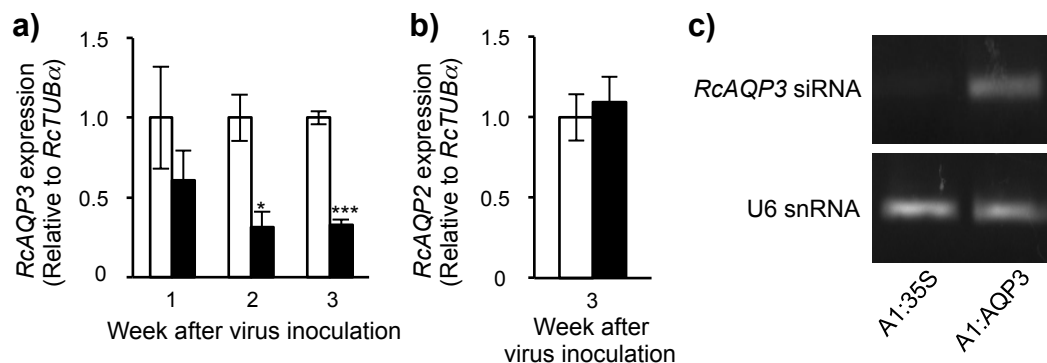


Figure S6. Knockdown of *RcAQP3* in intraradical mycelia of *R. clarus* HR1 by virus-induced gene silencing. **(a)** Duration and effectiveness of knockdown of *RcAQP3*. *N. benthamiana* colonized with *R. clarus* HR1 was inoculated with the recombinant CMV carrying either A1:AQP3 (closed bars) or A1:35S (open bars) 5 weeks after sowing. Total RNA was extracted from the roots at 1, 2, and 3 weeks after virus inoculation, and the expression levels were measured by qRT-PCR and indicated on the basis of *RcTUB α* expression. Vertical bars are $\pm$ SE ($n = 3$). Asterisks indicate significant differences between the knockdown and control treatments: *, $P < 0.05$; ***, $P < 0.001$ (Student's *t*-test). **(b)** Effect of *RcAQP3* knockdown on *RcAQP2* expression. Total RNA was extracted from the roots 3 weeks after virus inoculation, and the expression levels were measured by qRT-PCR and indicated on the basis of *RcTUB α* expression. Vertical bars are $\pm$ SE ($n = 3$) **(c)** Stem-loop PCR targeting a siRNA (ATCGCTCAAACCGTACTTAGCA). Total RNA was extracted from the roots 3 weeks after virus inoculation, and the siRNA originated from the RcAQP3 insert of A1:AQP3 construct and U6 snRNA of *N. benthamiana* (internal control) was amplified and electrophoresed on polyacrylamide gel.

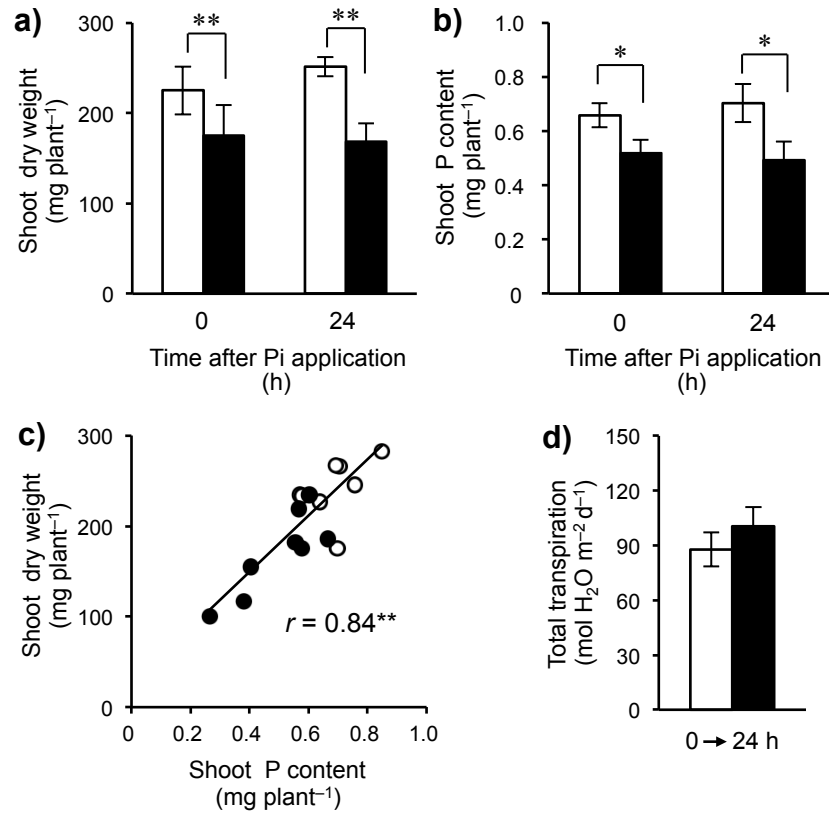


Figure S7. Impact of *RcAQP3* knockdown on the physiological status of the host plant. *N. benthamiana* colonized with *R. clarus* HR1 was inoculated with the recombinant CMV carrying either the knockdown (closed bars) or control (open bars) construct 5 weeks after sowing and harvested 3 weeks after virus inoculation: (a) shoot dry weight, (b) shoot P content, (c) correlation between shoot dry weight and P content, and (d) total transpiration. Shoot dry weight and P content were measure at time zero ($n = 3$) and 24 h after phosphate (Pi) application ($n = 5$), while total transpiration represents water loss from time zero to 24 h after Pi application ($n = 5$). Vertical bars are $\pm$ SE. Asterisks indicate levels of significance: *, $P < 0.05$; **, $P < 0.01$. Total transpiration is not significantly different between the knockdown and control treatments (Student's *t*-test).

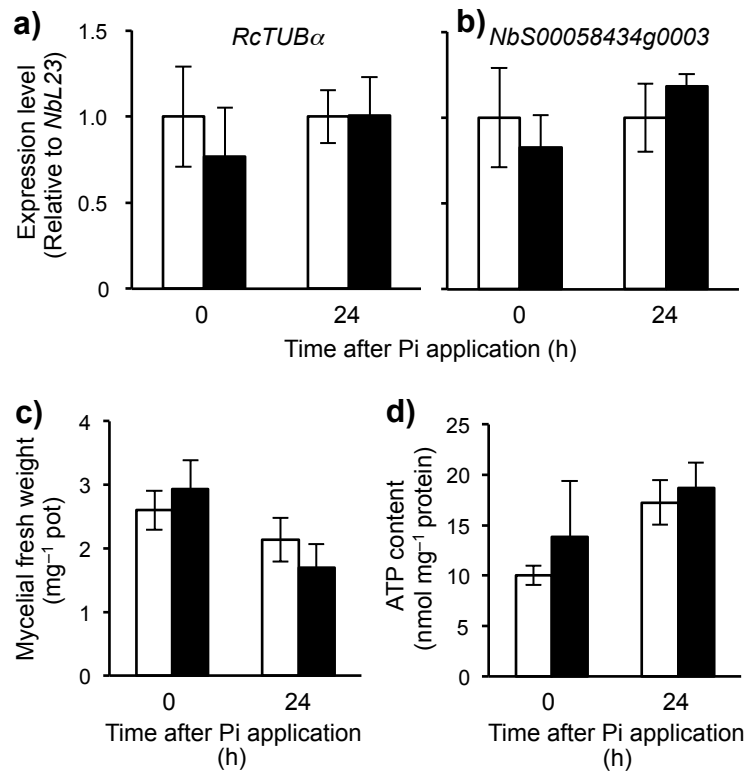


Figure S8. Impact of *RcAQP3* knockdown on the colonization and physiological status of *R. clarus* HR1. *N. benthamiana* colonized with *R. clarus* HR1 was inoculated with the recombinant CMV carrying either the knockdown (closed bars) or control (open bars) construct 5 weeks after sowing, and expression levels of the fungal tubulin gene *RcTUB α* and *N. benthamiana* mycorrhiza-specific phosphate (Pi) transporter gene *NbS00058434g0003* in the roots (a), fresh weight of extraradical mycelia (b), and ATP content of the mycelia (c) were determined at time zero ($n = 3$) and 24 h after Pi application ($n = 5$). Two-way ANOVA indicated that there were no significant differences between the treatments in all parameters. Vertical bars are $\pm$ SE.

Table

Table S1. Primers used in this study.

Primer pair name		Sequence (5' → 3')
<i>RcAQP3</i> -full	Forward	<u>AAAAAAATGTCT</u> ¹ GATGAAAGTGGACCAATTAACAAG
	Reverse	CTAAGCTACCGACCTATGCTCATG
<i>RcAQP3</i> -VIGS	Forward	CGC <u>ACGCGT</u> ² GGTGTAGGAAGTATCGCTCAAAC
	Reverse	CG <u>AGGCCT</u> ³ ACTAACTGCGACTCCCAAGG
CaMV-35S	Forward	A <u>AGGCCT</u> ³ ATTGAGACTTTTCAACAAAGGG
	Reverse	CG <u>ACGCGT</u> ² TCCTCTCCAAATGAAATGAAC
<i>RcAQP3</i> -qPCR	Forward	CAGGAGGAACAAGAGGAACAAC
	Reverse	CGATACTTCCTACACCAAATGAAAC
<i>RcAQP2</i> -qPCR	Forward	ATACCAGCTTTTGGGAATAGGAACC
	Reverse	CGCTACAATAGCTGGACCAAGAG
<i>RcTUBα</i> -qPCR	Forward	GCTTGTTGTTTGTGTATCGTG
	Reverse	TGAATGGTACGTTTGGTCTTG
<i>RcAQP3</i> -SLPCR_RT	Reverse	GTTGGCTCTGGTGCAGGGTCCGAGGTATTCGCACCAG AGCCAAC TGCTAA
<i>RcAQP3</i> -SLPCR	Forward	CGTATCGCTCAAACCGATC
	Reverse	GTGCAGGGTCCGAGGTATTC
U6 snRNA-PCR	Forward	CATGGCCCCTGCGCAAGGAT
	Reverse	CATTTCTCGATTTATGCGTG
<i>NbL23</i> -qPCR	Forward	AAGGATGCCGTGAAGAAGATGT
	Reverse	GCATCGTAGTCAGGAGTCAACC
<i>NbS00058434g0003</i> -qPCR	Forward	CGGCTGGTTAGGAGACAAAG
	Reverse	ATACCCAAAGGACAAGCCAG

¹Consensus Kozak sequence for efficient translation initiation in yeast cells.

²*Mlu*I restriction enzyme site.

³*Stu*I restriction enzyme site.