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Original Article

Laccaria bicolor aquaporin LbAQP1 is required for Hartig net development in trembling aspen (*Populus tremuloides*)

Alfonso Navarro-Ródenas[1], Hao Xu[1], Minna Kemppainen[2], Alejandro G. Pardo[2] & Janusz J. Zwiazek[1]

[1]*Department of Renewable Resources, University of Alberta, Edmonton, AB T6G 2E3, Canada*[2]*Laboratorio de Micología Molecular, Departamento de Ciencia y Tecnología, Universidad Nacional de Quilmes and Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), Bernal, Provincia de Buenos Aires B1876BXD, Argentina*

ABSTRACT

The development of ectomycorrhizal associations is crucial for growth of many forest trees. However, the signals that are exchanged between the fungus and the host plant during the colonization process are still poorly understood. In this study, we have identified the relationship between expression patterns of *Laccaria bicolor* aquaporin *LbAQP1* and the development of ectomycorrhizal structures in trembling aspen (*Populus tremuloides*) seedlings. The peak expression of *LbAQP1* was 700-fold higher in the hyphae within the root than in the free-living mycelium after 24 h of direct interaction with the roots. Moreover, in *LbAQP1* knock-down strains, a non-mycorrhizal phenotype was developed without the Hartig net and the expression of the mycorrhizal effector protein *MiSSP7* quickly declined after an initial peak on day 5 of interaction of the fungal hyphae with the roots. The increase in the expression of *LbAQP1* required a direct contact of the fungus with the root and it modulated the expression of *MiSSP7*. We have also determined that LbAQP1 facilitated NO, H_2O_2 and CO_2 transport when heterologously expressed in yeast. The report demonstrates that the *L. bicolor* aquaporin LbAQP1 acts as a molecular signalling channel, which is fundamental for the development of Hartig net in root tips of *P. tremuloides*.

Key-words: carbon dioxide; ectomycorrhizae; fungal aquaporins; hydrogen peroxide; MiSSP7; nitric oxide.

INTRODUCTION

While only around 3% of seed-bearing plants establish ectomycorrhizal (ECM) symbiosis with fungi, ECM is the most common form of symbiosis for the majority of boreal and temperate forest trees (Smith & Read 2008). The main benefits of ECM fungi to trees include the improvement of nutrient and water uptake as well as increased abiotic stress tolerance and pathogen resistance (Smith & Read 2008; Lehto & Zwiazek 2011). For many trees, the development of ECM associations appears to be crucial for seedling establishment and tree growth (Veneault-Fourrey *et al.* 2013). However, some of the key questions concerning ECM colonization of roots remain unanswered including those concerning the signals that are exchanged between the fungus and the host plant during the colonization process.

It has recently been demonstrated that *Mycorrhiza-induced Small Secreted Protein 7* (*MiSSP7*), the most highly symbiosis-up-regulated gene from the ECM basidiomycete *Laccaria bicolor* (Maire) P.D. Orton, encodes an effector protein indispensable to the establishment of mutualism (Plett *et al.* 2011). This effector is secreted by the fungus after receiving diffusible signals from plant roots, and is targeted to the plant nucleus where it alters the transcriptome of the plant cell (Plett *et al.* 2011). It has been also proposed that MiSSP7 protein may play an important role in controlling plant hormonal pathways to foster mutualistic associations (Plett *et al.* 2014a). *MiSSP7* presents characteristic expression pattern where the expression increases quickly during the presymbiotic stage, reaches a peak in the early stage of the symbiosis, and decreases slowly while the mycorrhiza is developing (Plett *et al.* 2011). *L. bicolor MiSSP7* knock-down strains were also demonstrated to be able to form a typical mantle but not the Hartig net (Plett *et al.* 2011). Jasmonic acid (JA) and ethylene (ET) were shown to limit Hartig net development through a combination of ET/JA transcriptional crosstalk and root treatments that induce genes controlled by ET and JA can prevent the formation of mycorrhiza (Plett *et al.* 2014a). In accordance with these findings, MiSSP7 from *L. bicolor* has recently been demonstrated to interact with the host plant JA signalling repressors in *Populus* and promote symbiosis exactly by blocking JA action (Plett *et al.* 2014b).

In the same way that the fungal partner plays an active role in establishing a mycorrhizal association, it is plausible that the host plant is also actively participating in a crosstalk between signalling pathways. In fact, the presence of root-diffusible molecules secreted into the rhizosphere can attract the ECM mycelia (Horan & Chilvers 1990). Flavonoids, such as rutin or quercitin as well as cytokinins can modify hyphal morphology at very low concentration (Lagrange *et al.* 2001; Martin *et al.* 2001). Exogenous quercitin and rutin treatments have also been demonstrated to induce the expression of *MiSSP7* in the absence of the tree host (Plett & Martin 2012). Therefore, it is plausible that the ECM host plants may also produce variable effector molecules which are capable of modulating fungal responses to establishment of symbiotic structures and functions through transcriptomic regulation.

Correspondence: J. J. Zwiazek. e-mail: jzwiazek@ualberta.ca

Aquaporins (AQPs) are water channel proteins that facilitate and regulate passive movement of water and other small molecules along a water potential gradient. These proteins belong to the large major intrinsic protein (MIP) family of transmembrane proteins and are present in all kingdoms (Zardoya 2005). In general, AQP proteins consist of six transmembrane α-helices. Loops B and E, which contain the AQP signature NPA-motif (asparagine, proline, alanine), form a seventh half-transmembrane helix and the selective pore. In fungi, MIPs are subdivided into four clusters (I–IV) based on the identity of member sequences. Clusters I to IV correspond to the orthodox AQPs, aquaglyceroporins, facultative AQPs, and XIPs, respectively (Xu *et al*. 2013).

The mechanisms behind the water and nutrient exchange and the precise nature of exchanged molecules in ECM associations remain uncovered. Increasing scientific interest has recently been directed towards fungal AQP functions as they have been hypothesized to act as nitrogen efflux carriers in the symbiotic interface (Nehls & Dietz 2014). In addition to water transport (Marjanović *et al*. 2005; Lee *et al*. 2010), some eukaryotic AQPs appear to play an important role in facilitating the transmembrane passage of small molecules that could act as molecular signalling such as CO_2, H_2O_2, and NO (Wu & Beitz 2007; Puppo *et al*. 2013). This has led us to the hypothesis that some of the AQPs may be involved in molecular signalling processes that are important not only for the function but also for the establishment of ECM association. In the present study, we used a heterologous system in yeast to examine the permeability of various fungal aquaporins to CO_2, H_2O_2 and NO and we identified LbAQP1 as the aquaporin that could potentially be involved in the signalling processes during ectomycorrhizal development. Therefore, we generated LbAQP1 knock-down strains of *L. bicolor* and compared the development of ectomycorrhizal structures in roots of aspen (*Populus tremuloides*) following their inoculation with this strain and the wild-type *L. bicolor*. We also examined the expression levels of seven *L. bicolor* aquaporins and the mycorrhizal effector protein *MiSSP7* at different times following fungal interaction with roots to test the hypotheses that (1) the expression of AQPs shows symbiosis-regulated patterns which reflect different states of mycorrhizal development; and (2) altered expression of the fungal AQPs would lead to a severely altered mycorrhizal phenotype.

MATERIALS AND METHODS

Plant and fungal materials and growth conditions

Vegetative mycelium of *L. bicolor* strain UAMH 8232 was maintained at 25 °C on modified Melin-Norkrans (MMN) medium (Marx 1969). *L. bicolor* strain UAMH 8232 has six AQP genes (*LbAQP1*, *LbAQP3*, *LbAQP5*, *LbAQP6*, *LbAQP7-1* and *LbAQP7-2*; Xu *et al*. 2013). The *LbAQP1* (NCBI accession number: *JQ585592*) knock-down strains and a mock strain were maintained at 25 °C on MMN supplemented with hygromycin (0.3 mg mL^{-1}). Transformation of *L. bicolor* UAMH 8232 was performed using the RNAi/*Agrobacterium*-mediated method (Kemppainen *et al*. 2005). The inverted repeated sequence arms in the ihpRNA expression cassette corresponded to 575 bp PCR amplicons produced with *LbAQP1* cDNA plasmid clone as template (primers listed in Supporting Information Table S1). The sequence arms were cloned into *Sna*BI/*Hind*III and *Stu*I/*Sph*I sites in pSILBAγ. The RNAi expression cassette was further cloned as full-length *Sac*I-linearized vector into *Sac*I site in pHg to generate the final pHg/pSILBAγ/*LbAQP1* transformation/RNAi construct (Kemppainen & Pardo 2010). The vector was introduced into the *Agrobacterium tumefaciens* strain AGL1 by electroporation. The *L. bicolor* UAMH8232 wild-type strain was transformed with pHg/pSILBAγ/*LbAQP1* via *Agrobacterium* according to Kemppainen *et al*. (2005) with the following modifications: the fungal colonies were pre-grown on cellophane membranes for 3 d, co-cultivation with *Agrobacterium* lasted for 3 d and elimination of *Agrobacterium* during transformant selection was carried out with 0.2 mg mL^{-1} of ceftriaxone in the growth medium. To generate the mock transformant strains, *L. bicolor* UAMH8232 wild type was *Agro*-transformed with pHg/pSILBAγ. This mock vector carried a full-length, *Sac*I-linearized, empty pSILBAγ cloning vector in T-DNA of pHg. Ten independent strains of *LbAQP1* RNAi transformants and 12 strains of mock transformants were generated.

Six *L. bicolor* transformants with the *LbAQP1* RNAi construct and four mock transformants were screened for transgene expression by SYBR Green qRT-PCR assay. Relative transcript abundance of *LbAQP1* and reference genes was quantified in *L. bicolor* mycelia grown on solid MMN medium at 20 °C for 3 weeks (Xu *et al*. 2015). Transcript abundance of *LbAQP1* was normalized to the geometric mean of that of the reference genes *α-tubulin* and *translation elongation factor EF2* in each strain (primers listed in Supporting Information Table S1). Relative transcript abundance of *LbAQP1* in transgenic strain was compared with that in wild type to show the fold change of *LbAQP1* as the effect of RNA interference (WT; $n = 3 \pm$ SE). In the RNAi strains, L3-4 and L3-9 showed the lowest levels of *LbAQP1* transcript abundance, with values about 30% and 40% of that of WT, respectively (Supporting Information Fig. S1). In the mock strains, transcript abundance of *LbAQP1* in the strain mock 2 was not significant different from that of WT (Supporting Information Fig. S1).

Southern blot analysis was used to determine transgene copy number (Xu *et al*. 2015). Ten μg of gDNA extracted using the DNeasy Plant Maxi Kit (Qiagen, Valencia, CA, USA) was digested using *Sac*I or *Bam*HI (FastDigest; Fermentas, Vilnius, Lithuania). An 870 bp PCR product was amplified using the 1026 bp *hygromycin phosphotransferase gene* (*hph*) in the binary vector as the template and gene-specific primers *hph* For and *hph* Rev (Supporting Information Table S1), and used as both positive control and probe. The digested gDNA was purified and denatured before loading for electrophoresis using 0.8% agarose gel in 0.5× TBE buffer at 80 V for four hours. DNA was transferred onto H^+-bond membrane (GE Healthcare, Buckinghamshire, UK)

using a vacuum blotter (BioRad, Richmond, CA, USA). Probe hybridization, stringent wash and detection reaction were conducted according to the manufacturer's protocol (Amersham AlkPhos Direct Labeling and Detection System with CDP-*Star*; GE Healthcare, Buckinghamshire, UK), as previously described (Kemppainen *et al*. 2008). Chemiluminescence generated via an alkaline phosphatase reaction was detected by a CCD sensor using a 30 min exposure time (BioRad ChemiDoc, Richmond, CA, USA). It was confirmed that mock 2, L3-4 and L3-9 strains each harboured a random single insertion of the transgenic cassette, indicated by a single clear hybridization band in lanes 3, 10 and 11, respectively (Supporting Information Fig. S2). Based on the screening outcomes of qRT-PCR and Southern blot analysis, mock 2 was selected as the mock control, and L3-4 and L3-9 were selected as the knock-down strains for further study.

Phenotype and dry mass of the mycelia of different strains were examined after growing on MMN medium at either 20 °C or 5 °C for 3 weeks. No clear difference in phenotypes was observed between WT, mock and the two RNAi strains at either 20 °C (Supporting Information Fig. S3a) or 5 °C (Supporting Information Fig. S3b). The dry mass of all of the strains was significantly higher at 20 °C than at 5 °C, but the difference between the strains was not significant at either 20 °C or 5 °C (Supporting Information Fig. S3c). Therefore, the examined strains were used in the following plant–fungus system.

For the different experiments, aspen seeds were surface-sterilized (Cseke *et al*. 2007) and sown on WPM (Woody Plant Medium) medium (Lloyd & McCown 1980) with 15 g L^{-1} of agar in Petri dishes in dark for 4 d. Once the aspen plantlets have developed true leaves, they were transplanted into Magenta boxes with sand moistened with no-sugar WPM medium and sealed with the Micropore tape (3M, St. Paul, MN, USA) to encourage the photoautotrophic metabolism. Four-week-old aspen plantlets in Magenta boxes were transferred to 14 × 14 cm square Petri dishes half-covered with a cellophane membrane, containing agar solidified no-sugar WPM medium. Aspen plantlets were vertically arranged and the lower part of the dish was covered with aluminum foil. The plantlets were grown for 3 weeks under 18 h photoperiod at 22 °C and photosynthetic photon flux density of 400 μmol m^{-2} s^{-1}.

Co-culturing in a sandwich culture system

Free-living mycelium of *L. bicolor* was grown for 15 d on cellophane membrane (previously treated with 1 mM EDTA for 1 h at 100 °C, washed with Milli-Q-water, and autoclaved) in agar plates containing sugar-reduced WPM (5 g L^{-1} glucose). For co-cultures, a mycelium-covered cellophane membrane was placed with the fungus side down (direct interaction) or fungus side up (indirect interaction) on the roots. Petri dishes were sealed with Parafilm, on lower and lateral sides, and on the upper side Micropore tape (3M) was used to ensure high gas permeability. The plates were arranged vertically, and the lower part of the dish was covered with aluminum foil to prevent light from reaching the fungus and roots. The sandwich cultures were kept under the above described conditions during the experiment.

Experiment 1: time course tracing of direct and indirect interaction

Aspen roots were co-cultured in the presence of wild-type *L. bicolor* mycelium with direct or indirect interaction. Mycelium-covered cellophane membrane was placed on 14 × 14 cm dishes with no-sugar WPM medium. The root system of each plant was considered as an experimental unit. After 1, 5, 10 and 20 d, samples from three different root systems were collected. In the case of indirect interaction, the mycelium covering the cellophane just above one root system was sampled and frozen in liquid N_2. In the case of direct interaction, cellophane membrane was removed carefully and mycelium covering it just above one root system was sampled and frozen in liquid N_2. This mycelium was considered as extraradical mycelium. Three putative macroscopically visible mycorrhizal tips from each plant were collected for microscopy and roots and adhered mycelium were sampled and frozen in liquid N_2. Additionally, at the same times, samples of free-living mycelium grown on cellophane and moved to no-sugar WPM medium, were collected and frozen in liquid N_2.

Experiment 2: time course tracing of *LbAQP1* knock-down strains

The time course of fungal AQP expression and mycorrhization was analysed in two independent *L. bicolor* *LbAQP1* knock-down strains (L3-4 and L3-9) and one mock strain with direct interaction on the roots of aspen plantlet. Mycelium-covered cellophane membrane was placed on 14 × 14 cm dishes with no-sugar WPM medium. The root system of each plant was considered as an experimental unit. After 1, 5, 10 and 20 d, samples from three different root systems were collected. Cellophane membrane was removed carefully and mycelium covering it just above one root system was sampled and frozen in liquid N_2. This mycelium was considered as extraradical mycelium. Three putative macroscopically visible mycorrhizal tips from each of the three plants per treatment at each time were collected for microscopy. Additionally, at the same times, samples of free-living mycelium were collected and frozen in liquid N_2.

Microscopy

Mycorrhizal colonization was microscopically examined in three root tips from each of the three plants per treatment at each time. The root tips were fixed in FAA (formalin-acetic acid-alcohol) for 1 h under vacuum at room temperature and kept overnight at 4 °C. The samples were dehydrated in ethanol, embedded in paraffin and thin transverse sections from approximately 2 mm above the root apex were prepared with a microtome (model RM2125 RTS; Leica, Solms, Germany). The sections were stained with toluidine blue. The

stained samples were observed under ZEISS AXIO compound light microscope (Carl Zeiss, Jena, Germany) with the MacroFire Digital Camera (Optronics, Goleta, CA, USA).

CO_2, NO and H_2O_2 transport in yeast

For CO_2 transport assays, the complete ORF of *L. bicolor LbAQP1* (NCBI accession number *JQ585592*), *LbAQP3* (*JQ585593*), *LbAQP5* (*JQ585594*), *LbAQP6* (*JQ585595*), *LbAQP7-1* (*JQ585596*), *LbAQP7-2* (*JQ585597*) were inserted into yeast expression vector pAG426GAL-ccdB (http://www.addgene.org/yeast-gateway/), respectively, using the Gateway technology (Invitrogen, Carlsbad, CA, USA). The constructs were verified by sequencing. *S. cerevisiae* strain INVSc1 (MATa his3D1 leu2 trp1-289 ura3-52; Invitrogen), containing the pAG425GAL-ccdB vector (http://www.addgene.org/yeast-gateway/) with the *carbonic anhydrase gene* (NCBI accession number U55838.1) of *P. tremuloides*, was transformed following the protocol of small-scale yeast transformation recommended by Invitrogen. Selection was based on ura3 and leu2 complementation. Transformed yeasts were cultured in glucose containing synthetic complete medium without Ura/Leu for 20 h (1.2 *g*, 30 °C). Cultures were diluted to an optical density of 0.6 at 600 nm, and heterologous protein expression was induced by changing the carbon source of the medium from glucose to galactose and growing for 16 h (1.2 *g*, 30 °C). CO_2 uptake was observed by intracellular acidification, monitoring the decrease of fluorescence in complete yeast cells loaded with fluoresceine-bisacetate (Otto *et al*. 2010). Cells, suspended in 75 mM NaCl, 25 mM HEPES-NaOH, pH 6, were rapidly mixed in a stopped-flow spectrometer (model SX18MV-R; Applied Photophysics, Surrey, UK) with an equal volume of buffer containing 75 mM $NaHCO_3$, HEPES-NaOH, pH 6. The time course was fitted to a single exponential decay function over the initial 30 ms, in order to calculate the rate constant using the Biokine software. CO_2 permeability (P_{CO2}) was calculated as described by Yang *et al*. (2000). The cell diameter was 5.7 ± 0.5 μM. Yeasts expressing the *Arabidopsis thaliana β-glucuronidase gene* and *P. tremuloides carbonic anhydrase gene* were used as controls. The P_{CO2} values were obtained for at least six independent induction experiments with an average of 20 measurements per experiment.

For NO and H_2O_2 transport assays, yeast strain *S. cerevisiae* INVSc1 (MATa his3D1 leu2 trp1-289 ura3-52; Invitrogen) containing the empty pAG425GAL-ccdB vector was transformed with the vector pAG426GAL-ccdB expressing *L. bicolor LbAQP1*, *LbAQP6*, *Nicotiana tabacum NtAQP1* (AJ001416), *Homo sapiens HsAQP1* (P29972) or the empty vector pAG426GAL-ccdB as the mock control, respectively. Transformed yeasts were cultured in glucose containing synthetic complete medium without Ura/Leu for 20 h (1.2 *g*, 30 °C). Cultures were diluted to $OD_{600} = 0.6$, and heterologous protein expression was induced by changing the carbon source of the medium from glucose to galactose and growing in dark for 24 h (1.2 *g*, 30 °C). DAF-FM DA (4-amino-5-methylamino-2′,7′-difluorofluorescein diacetate; Sigma-Aldrich, St. Louis, MO, USA) and CM-H_2DCFDA [5-(&-6)-chloromethyl-2′,7′-dichlorodihydrofluorescein diacetate, acetylester mixed isomers] (Life Technologies; Carlsbad, CA, USA) were used for detecting NO (Ryu *et al*. 2013) and H_2O_2 (Bienert *et al*. 2007), respectively. Greater increase in fluorescence over time indicates higher intracellular concentration of the examined molecules being transported. Non-dye mock yeasts were examined to show the background inflorescence.

For NO transport assay, yeasts were washed with 1× PBS buffer and re-suspended to reach $OD_{600} = 1.4$, and then were dyed with 10 μM DAF-FM DA in 1× PBS buffer for 2 h in dark at 30 °C and 1.2 *g* (Ryu *et al*. 2013). Yeasts were washed twice with 1× PBS buffer to remove excessive dye, and re-suspended in 1× PBS buffer. SNAP (S-nitroso-N-acetylpenicillamine, Sigma-Aldrich; Zhang *et al*. 2003) and cPTIO (carboxy-PTIO potassium salt, Sigma-Aldrich) (Goldstein *et al*. 2003) were used as NO donor and NO scavenger, respectively. In SNAP treatment, 100 μM of SNAP was used. In SNAP+cPTIO treatment, 100 μM SNAP and 200 μM cPTIO were used. In PBS treatment, neither NO donor nor NO scavenger was applied. Fluorescence generated from each 200 μL reaction per well of a 96-well microplate was recorded using a microplate reader (Fluostar Optima, BMG Labtech, Ortenberg, Germany) with excitation wavelength of 485 nm and emission wavelength of 520 nm at 27 s intervals for 45 min ($n = 6$).

For H_2O_2 transport assay, yeasts were dyed for 24 h in dark (1.2 *g*, 30 °C) in galactose synthetic medium with 1 μM of CM-H_2DCFDA, and then were washed with 20 mM HEPES buffer (pH = 7) for five times and re-suspended in HEPES to reach $OD_{600} = 1.4$ (Bienert *et al*. 2007). In H_2O_2 treatment, 100 μM H_2O_2 was used as H_2O_2 source. In H_2O_2+$AgNO_3$ treatment, yeasts were incubated in 15 μM of the aquaporin inhibitor $AgNO_3$ for one hour in dark before H_2O_2 was added (Bienert *et al*. 2007). In HEPES treatment, neither H_2O_2 nor $AgNO_3$ was applied. Fluorescence generated from each 200 μL reaction per well of a 96-well microplate was recorded as above described for 30 min ($n = 6$).

Synthesis of first strand cDNA and qRT-PCR

RNA was isolated from aspen and from the fungal mycelia using spin columns (RNeasy Plant Mini Kit, Qiagen) according to the manufacturer's instruction with the addition of polyethylene glycol 8000 20 mg mL^{-1} in RLT buffer. The concentration of RNA was quantified by measuring the absorbance at 260 nm. The QuantiTect Reverse Transcription kit (Qiagen) was used for cDNA synthesis with integrated removal of genomic DNA contamination according to the manufacturer's instruction from 1 μg of total RNA, using RT primer mix (Qiagen). Gene expression of *LbAQP1*, *LbAQP3*, *LbAQP5*, *LbAQP6*, *LbAQP7-1*, *LbAQP7-2* and *MiSSP7* (JGI protein ID 298595; http://genome.jgi-psf.org/Lacbi2/Lacbi2.home.html) was examined by real-time PCR using the 384 wells 7900HT System (Applied Biosystems). *MiSSP7* was measured since it is a strong mutualism effector that controls the establishment of the symbiotic relationship and can be considered as a marker for mycorrhizal

development because of its expression in the presence of direct or indirect root interaction (Plett *et al*. 2011). The primer sets used to amplify the genes in the synthesized cDNA are shown in Supporting Information Table S1. The primers were designed with the online software Primer 3 v 0.4.0 according to specific criteria, which included a predicted melting temperature of 60 ± 2 °C, a primer length of 18 to 24 nucleotides, a product size of 80 to 150 bp and a GC content of 45 to 60%. Each 10 μL reaction contained 1 μL of a 1:10 dilution of the cDNA, 50 nM of each primer and 5 μL of QPCR Mastermix. The 2X QPCR Mastermix (*Dynamite*) used in this study is a proprietary mix developed and distributed by the Molecular Biology Service Unit (MBSU), in Department of Biological Science at the University of Alberta (Edmonton, Alberta, Canada). It contains Tris (pH 8.3), KCl, $MgCl_2$, glycerol, Tween 20, DMSO, dNTPs, ROX as a normalizing dye, SYBR Green (Molecular Probes) as the detection dye, and an antibody inhibited Taq polymerase. The PCR program consisted of a 2 min incubation at 95 °C to activate the antibody inhibited Taq polymerase, followed by 35 cycles of 45 s at 95 °C and 60 s at 62 °C, at which temperature the fluorescence signal was measured. The specificity of the PCR amplification procedure was checked with a heat dissociation protocol (from 70 to 100 °C), after the final cycle of the PCR. The efficiency of the primer set was evaluated by performing real-time PCR on a series of dilutions of cDNA. The results obtained for the different treatments were normalized with geometric mean of two reference genes; *Elongation Factor 3* (JGI protein ID: 293350) and *Metalloprotease* (JGI protein ID: 245383). These reference genes were chosen because of stability of expression during the mycorrhization process (Plett *et al*. 2011). Primers used to amplify them are shown in Supporting Information Table S1. Real-time PCR experiments were carried out in three plantlets, with the Ct determined in triplicate. The relative transcription levels were calculated by using the $2^{-\Delta\Delta Ct}$ method (Livak & Schmittgen 2001). Negative controls without cDNA were used in all PCR reactions.

Statistical analysis

All data were analysed using one-way ANOVA and Tukey's test. For gene expression analysis, statistically significant differences between the different treatments and strains (Figs 1, 2, 3 & 5), were determined for each measurement day and are presented in Supporting Information Table S2.

RESULTS

LbAQP1 expression pattern in presymbiotic state

LbAQP1, a gene encoding an orthodox fungal water channel protein, showed an expression pattern with the peak that preceded the mycorrhizal development (Fig. 1a). The peak expression of *LbAQP1* was 700-fold higher in the mycelium within the root than in the free-living mycelium, just 24 h after direct interaction. After the initial increase, the expression level sharply decreased and stayed constant for the remaining 20 d (Fig. 1a). In the extraradical mycelium surrounding the root, an expression peak of lower strength (~450-fold higher than in free-living mycelium) was identified on day 10 and this *LbAQP1* expression decreased gradually towards the 20 day endpoint of fungus–plant interaction. Finally, in the mycelium with the indirect interaction with the root, which was obtained by physically separating the two organisms with cellophane membrane, an AQP expression peak was observed as in the extraradical mycelium surrounding the root. However, while the maximum expression was reached in both cases on day 10, the expression levels in indirect root interaction were only half of the ones detected in extraradical mycelium in direct interaction. The remaining tested AQP genes did not show changes in the expression levels (Fig. 2).

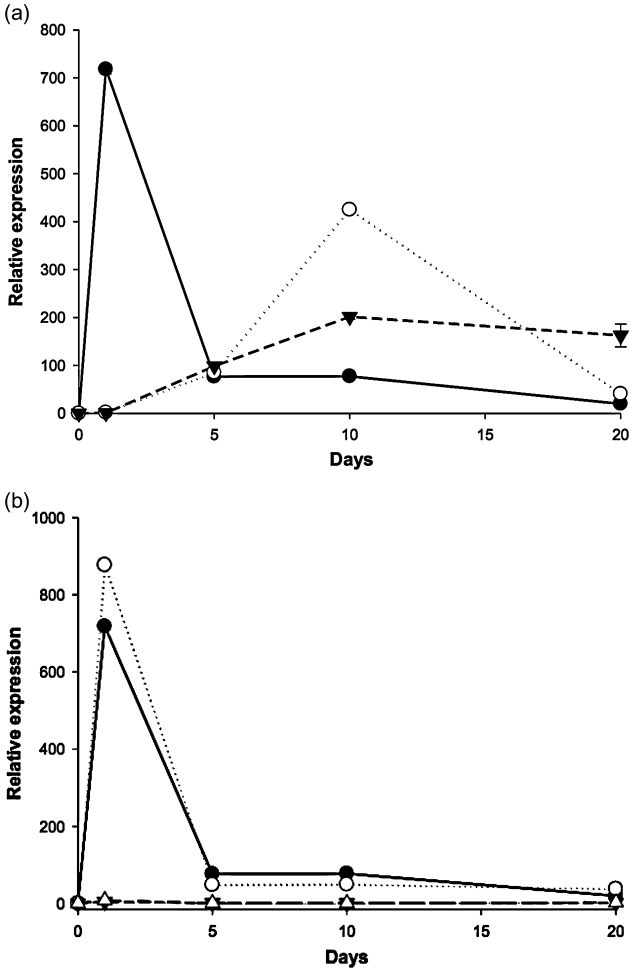

Figure 1. Relative expression of *LbAQP1* gene ($2^{\Delta Ct(\text{root-mycelium})-\Delta Ct(\text{free-mycelium})}$) determined by quantitative real-time PCR in wild-type mycelium within the *Populus tremuloides* root (●), extraradical mycelium (○) and the mycelium with indirect root contact (▼) after 1, 5, 10 and 20 d of root interaction (a). Relative expression of *LbAQP1* in mycelium within the root in wild-type (●), knock-down strains L3-4 (▼), L3-9 (△) and mock control transformant (○) after 1, 5, 10 and 20 d of root interaction (b). Each point is the mean of three replicates ± SE.

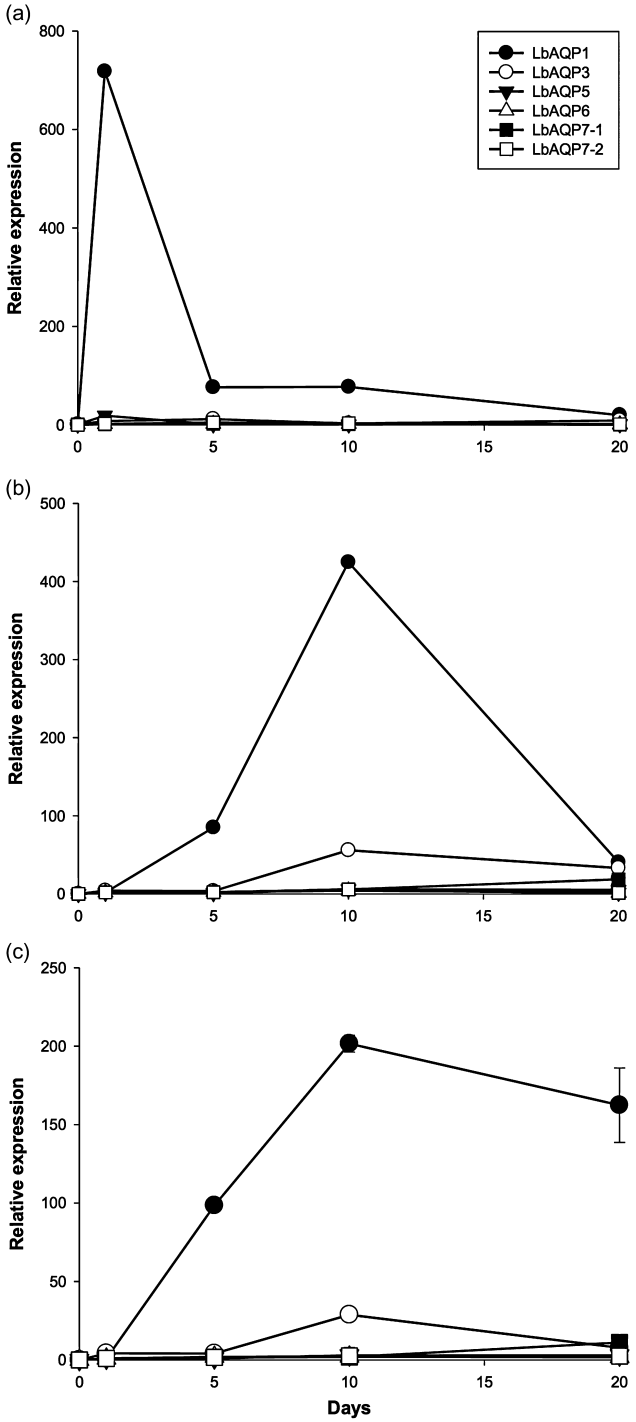

Figure 2. Relative expression of *LbAQP1*, *LbAQP3*, *LbAQP5*, *LbAQP6*, *LbAQP7-1* and *LbAQP7-2* genes ($2^{\Delta Ct(root\text{-}mycelium)-\Delta Ct(free\text{-}mycelium)}$) determined by quantitative real-time PCR in wild-type mycelium within the root (a), in extraradical mycelium (b) and mycelium with indirect contact (c) after 1, 5, 10 and 20 d of root interaction. Each point is the mean of three replicates ± SE.

MiSSP7 regulation and Hartig net development

The time course of mycorrhizal structure development was examined in *LbAQP1* knock-down strains and compared with the wild-type and mock control transformant strain. Together with the *AQP* expression, the *MiSSP7* expression was measured. The *LbAQP1* expression in the mycelium within the root in knock-down strains was maintained at around zero during the whole time course of the assay indicating efficient target gene RNA silencing while in the mock control strain, *LbAQP1* expression pattern and strength were comparable with the wild-type fungus (Fig 1b). Expression of other *AQP*s was not altered in *LbAQP1*-silenced strain (Fig. 3). In wild-type and mock strains, after 1 d of direct interaction, mycelia were attached to the root surface (Supporting Information Fig. S4a,b). After 5 d, the fungal mantle was present surrounding the roots (Supporting Information Fig. S4e,f) and after 10 d, Hartig net was present, and it was restricted only to the epidermal layer (Fig. 4a–d). In *LbAQP1* knock-down (L3-4 and L3-9) strains, the devel-

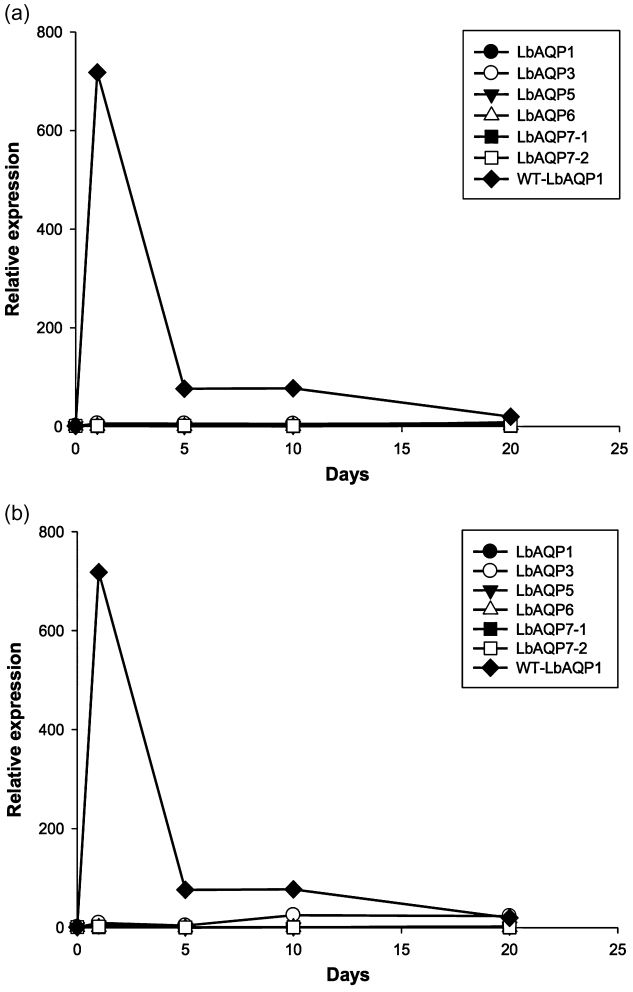

Figure 3. Relative expression of *LbAQP1*, *LbAQP3*, *LbAQP5*, *LbAQP6*, *LbAQP7-1* and *LbAQP7-2* genes ($2^{\Delta Ct(root\text{-}mycelium)-\Delta Ct(free\text{-}mycelium)}$) determined by quantitative real-time PCR in mycelium within the root of knock-down strain L3-4 (a) and knock-down strain L3-9 (b) after 1, 5, 10 and 20 d of root interaction. Together is showed the expression level of *LbAQP1* in mycelium within the root of wild type. Each point is the mean of three replicates ± SE.

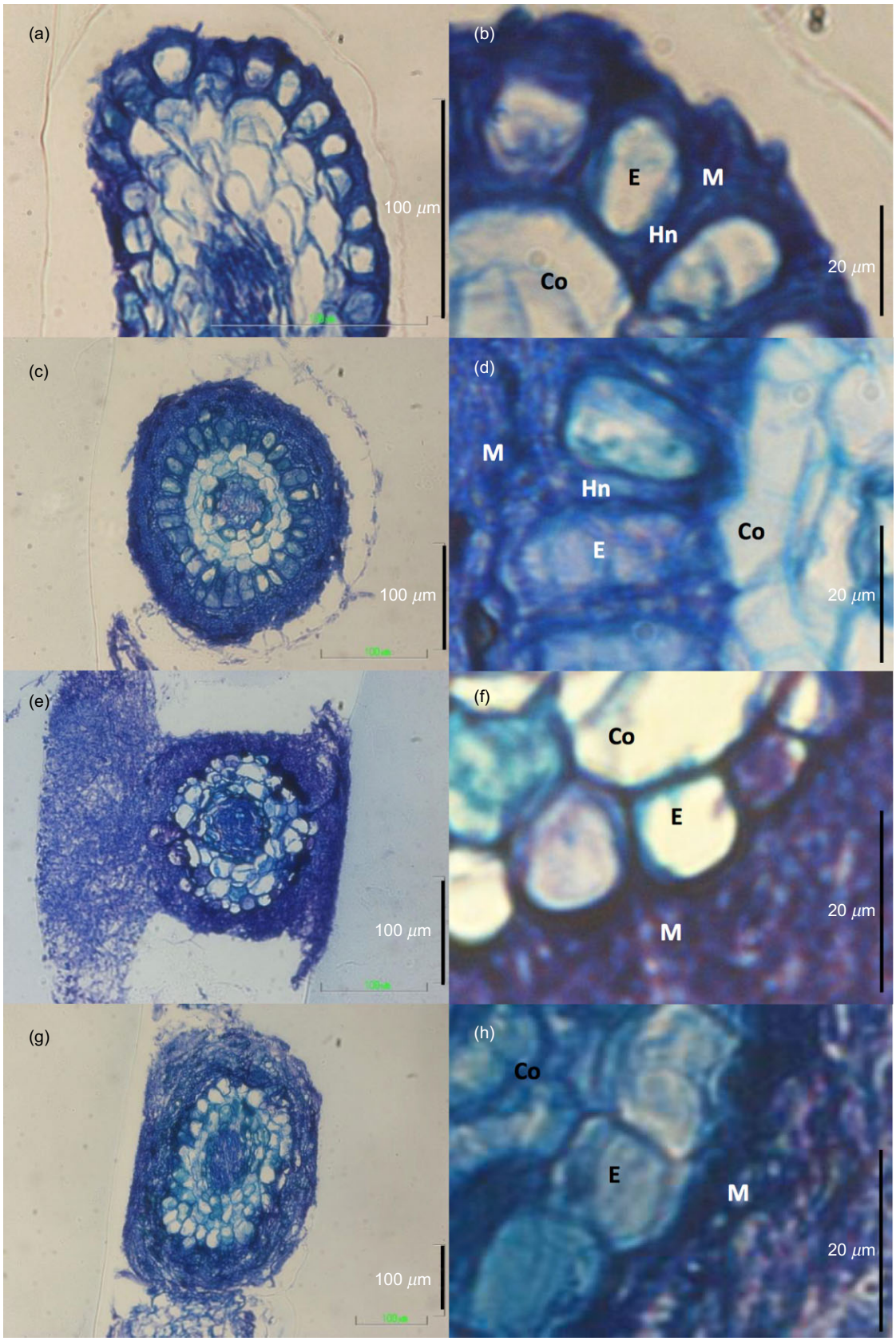

Figure 4. Cross sections of *Populus tremuloides* roots showing time course of colonization by different strains of *Laccaria bicolor* in an *in vitro* sandwich culture. (a and b) wild-type, (c and d) mock, (e and f) *LbAQP1* knock-down L3-4 and (g and h) *LbAQP1* knock-down L3-9 strains on day 10. Bars are 100 μM. Co, cortex; E, epidermis; Hn, Hartig net; M, Mantle.

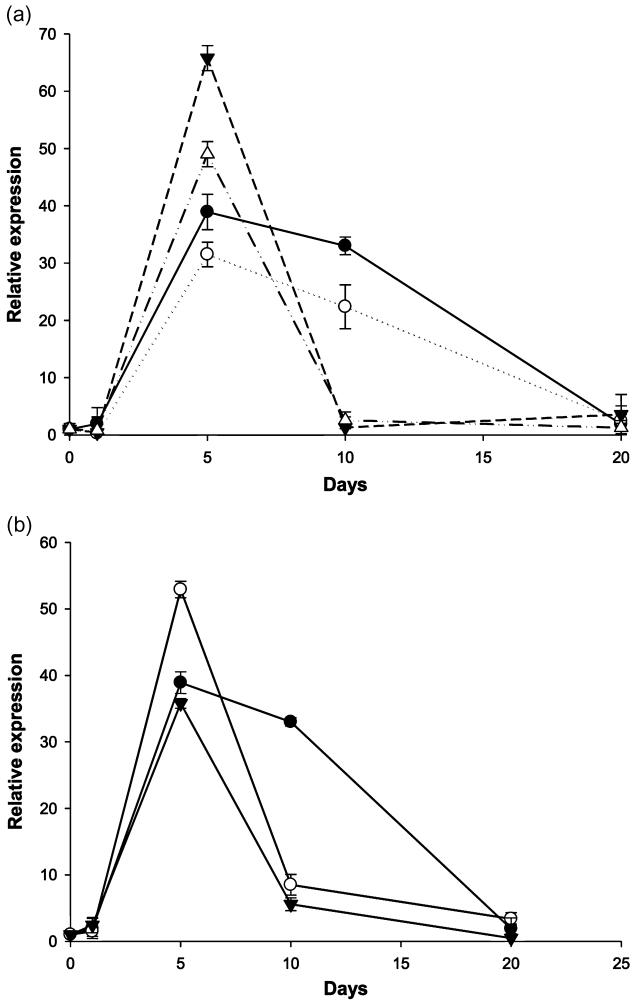

Figure 5. Relative expression of *MiSSP7* gene ($2^{\Delta Ct(\text{root-mycelium})-\Delta Ct(\text{free-mycelium})}$) determined by quantitative real-time PCR in mycelium within the root of wild-type (●), knock-down L3-4 (▼), L3-9 (△) and mock (○) strains after 1, 5, 10 and 20 d of root interaction (a). Relative expression of *MiSSP7* gene in wild-type mycelium within the root (●), extraradical mycelium (○) and the mycelium with indirect contact (▼) (b). Each point is the mean of three biological replicates ± SE.

opment of mycorrhizal structure was identical to the wild-type and mock strains on day 1 (Supporting Information Fig. S4c,d) and day 5 (Supporting Information Fig. S4g,h), but no Hartig net was observed on day 10 (Fig. 4e–h). When the time course expression of *MiSSP7* was measured in the mycelium within trembling aspen (*P. tremuloides* Michx.) roots in the wild-type, *LbAQP1* knock-down, and mock strains, a peak of *MiSSP7* expression was observed in all of the strains on day 5 (Fig. 5a). This time point coincides with the mantle formation (Supporting Information Fig. S4e–h). However, while in the wild-type and mock strains, the *MiSSP7* expression decreased slowly over time (Fig. 5a), a sharp decrease in *MiSSP7* expression occurred in *LbAQP1* knock-down strains between days 5 and 10 (Fig. 5a). Interestingly, this time course between 5 and 10 d of fungus–plant interaction overlaps exactly with Hartig net development in the experimental system that was used (Fig. 4a–d). No such structures were formed by the *LbAQP1* knock-down strains. Time-course expression of *MiSSP7* was also measured in extraradical mycelium and mycelium with indirect interaction in wild-type strain. In those mycelia *MiSSP7* reached a peak of expression on day 5, similar to mycelium within the roots, but after that time, the *MiSSP7* expression sharply decreased between days 5 and 10 similar to *LbAQP1* knock-down strains (Fig. 5b).

CO_2, NO and H_2O_2 transport

The transport of CO_2, NO and H_2O_2 through different AQPs was examined in yeast heterologous system. For CO_2, of the six examined fungal AQPs, only the yeasts expressing *LbAQP1* and *LbAQP3* showed significant increases in intracellular acidification rates in a CO_2 gradient compared with control (Fig. 6). In the yeasts expressing *LbAQP5*, *LbAQP6*, *LbAQP7-1* and *LbAQP7-2*, P_{CO2} values were similar to those measured in control (Fig. 6).

The transport of NO was enhanced in yeast cells expressing *LbAQP1*, *NtAQP1* and *HsAQP1* while there was no difference in NO transport between yeast cells expressing *LbAQP6* and the mock control (Fig. 7a). When 200 μM cPTIO was added as NO scavenger, the 485/520 nm fluorescence generated by DAF-FM DA caused by the influx of NO decreased to the levels measured in the control (PBS buffer only; Fig. 7a).

The increase in H_2O_2 transport occurred only in the yeast cells expressing *LbAQP1*and *NtAQP1* while no change compared with mock control was observed in yeasts expressing *LbAQP6* and *HsAQP1* (Fig. 7b). When the yeast cells were pre-incubated in 15 μM $AgNO_3$, which was used as an aquaporin transport inhibitor, fluorescence generated by CM-H_2DCFDA caused by the influx of H_2O_2 was similar or lower compared with the mock control (Fig. 7b).

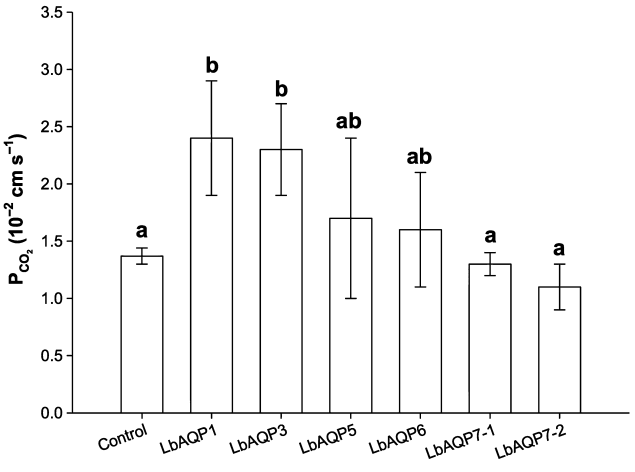

Figure 6. Effect of *LbAQP1*, *LbAQP3*, *LbAQP5*, *LbAQP6*, *LbAQP7-1*, *LbAQP7-2* or *Arabidopsis thaliana gus* (control) expression on CO_2 permeability of yeast plasma membrane. The bars show the P_{CO2} means ± SE ($n = 4$). Different letters above bars indicate statistically significant differences at $P \leq 0.05$, as determined by the analysis of variance and Tukey's test.

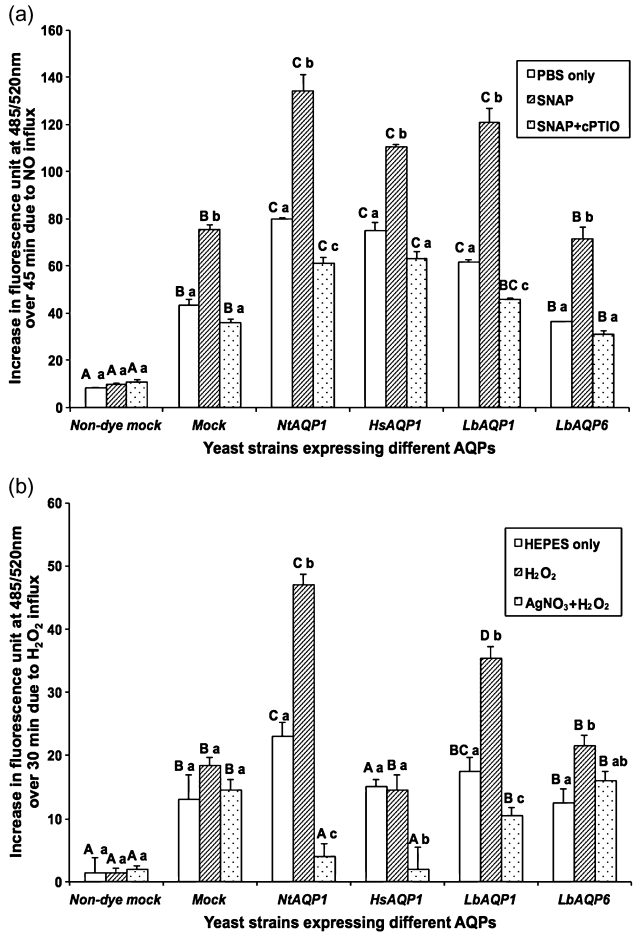

Figure 7. Effect of the expression of *NtAQP1* (*Nicotiana tabacum*), *HsAQP1* (*Homo sapiens*), *LbAQP1*, *LbAQP6* or the mock control of empty expression vector on NO (a) and H_2O_2 (b) permeability of yeast plasma membrane. The bars show mean increases in fluorescence generated by DAF-FM DA caused by the influx of NO (a) or in fluorescence generated by CM-H_2DCFDA caused by the influx of H_2O_2 (b) ($n = 4 \pm$ SE). Different uppercase letters above bars indicate statistically significant differences between yeast strains under the same treatment at $P \leq 0.05$, whereas different lowercase letters indicate statistically significant differences between treatments within each strain at $P \leq 0.05$, as determined by the analysis of variance and Tukey's test.

DISCUSSION

We demonstrated that among the six AQP genes of *L. bicolor* strain UAMH8232, one gene, *LbAQP1*, showed specific, symbiosis-regulated expression pattern. *LbAQP1* was strongly up-regulated during the early stages of mycorrhizal development reaching up to 700-fold higher expression levels in the symbiosis-engaged fungus in comparison with the free-living one. This up-regulation depended on the presence of host root system and showed an expression peak 24 h after initiation of fungus–plant interaction. Moreover, this AQP gene induction appeared to require direct cell-to-cell contact between the mycosymbiont and the host tree root epidermal cells since there was no increase of the *LbAQP1* expression levels in the extraradical mycelium or in the mycelium separated from the roots by the cellophane membrane (Fig. 1). This suggests that the contact with the epidermal cell wall is required for the gene up-regulation. After the strong initial expression peak at 24 h, *LbAQP1* was down-regulated on day 5. This timing coincided with the formation of the mantle (Supporting Information Fig. S4). The development of mantle and the initial up-regulation of *MiSSP7* were not affected by *LbAQP1* expression as demonstrated in wild-type, mock and knock-down strains (Supporting Information Figs S4 & S5). In all strains, *MiSSP7* reached a peak of expression at about day 5 following interaction with the roots. As previously demonstrated by Plett *et al.* (2011), up-regulation of *MiSSP7* was led by a diffusible molecule from the root since a peak of expression was reached on day 5 in extraradical mycelium and also in mycelium with indirect contact (Fig. 5b). The nature of this diffusible compound and mechanism behind its internalization has not yet been identified.

In the wild-type and mock strains, the *MiSSP7* expression level decreased slowly over time (Fig. 5a) and, similarly to the earlier report by Plett *et al.* (2011), the Hartig net was well developed. However, in the *LbAQP1* knock-down strains, the *MiSSP7* expression level decreased quickly between day 5 and day 10 and no Hartig net formation was observed, similarly to the phenotype of the *MiSSP7* knock-down strain (Plett *et al.* 2011). We observed that although in extraradical mycelium, mycelium with indirect contact and mycelium within the root, a peak of *MiSSP7* expression occurred at the same time, the temporal regulation pattern of the gene differed. While in root-engaged mycelia *MiSSP7* expression decreased gradually after the initial expression peak in extraradical mycelium and mycelium with indirect contact, the effector gene was rapidly down-regulated reaching its base level on day 10. Interestingly, an identical fast *MiSSP7* down-regulation took place in root-interacting *LbAQP1* knock-down strains. These strains formed mantle structures but no Hartig net. Therefore, the initial induction of *MiSSP7* expression occurred without direct root–fungus interaction but the correct *MiSSP7* regulation depended on close interaction between the root and the fungus. However, the sole contact with the root was not sufficient since in the root-engaged *LbAQP1* knock-down strains a quick *MiSSP7* expression decrease was observed. Our findings suggest the existence of a diffusible molecule which is transported from the root to the fungal cells through *LbAQP1*. Furthermore, this transport appears to be indispensable for *L. bicolor* entering into symbiotic growth mode with the plant host. Since the down-regulation of *MiSSP7* in the *LbAQP1* knock-down strains was observed after day 5 when the mantle had already been developed, and the strong *LbAQP1* expression peak occurred already after 24 h of direct fungus–plant interaction, the exchange of this diffusible molecule should take place in the mantle already at very early stages of its development and continue until the fully developed mantle is formed.

As the expression of *LbAQP1* was low during the later phases of symbiotic structure development and when the Hartig net was formed, the LbAQP1-mediated transport

appears not to be fundamental for maintenance of symbiotic structures or their functions once established. LbAQP1 appears to be involved in the signalling process only during the initial root colonization since no farther peaks of *LbAQP1* expression were observed during the 20 d of the experiment while new lateral roots were likely produced and colonized. These results could explain the reported up-regulation of black truffle (*Tuber melanosporum*) *TmeAQP1* (GSTUMT00003976001; in cluster I, Xu *et al*. 2013) in the fungal mantle compared with the Hartig net (Hacquard *et al*. 2013). Moreover, the fast decrease of *MiSSP7* transcript levels on day 5 in the mycorrhizal structures formed by the *LbAQP1* knock-down strains cannot be simply explained by lack of the Hartig net tissue in mantle-covered roots as *MiSSP7* is known to be strongly expressed both in the mantle and the Hartig net (Plett *et al*. 2011).

The *LbAQP1* expression preceded the up-regulation of *MiSSP7* in mycorrhizal formation. We have demonstrated that both indirect fungus–root contact, which inhibited *LbAQP1* expression, and *LbAQP1* RNAi knock-down, resulted in identical and abnormal *MiSSP7* expression pattern. In addition, such knock-down strains were incapable of forming typical functional mycorrhizal structures. Our results strongly indicate that function of the LbAQP1 protein is an absolute pre-requirement for the fungus–plant interaction to proceed from its pre-symbiotic phase to functional symbiosis. This LbAQP1 action is not likely to be water transport, but it must involve transport of some other bioactive molecule with capacity of initiating the correct symbiosis-associated gene regulation cascade. Neither can a putative LbAQP1-mediated nutrient efflux be postulated to be behind the detected effects on symbiosis regulation, as *LbAQP1* expression peak coincides with very early developmental phases of symbiotic structures when no apoplastic space of nutrient exchange has developed yet.

Some AQPs appear to play an important role in facilitating plasma membrane passage of small molecules with molecular signalling capacity in cells (Wu & Beitz 2007; Verdoucq *et al*. 2014). The LbAQP1 is included in the phylogenetic cluster I: orthodox fungal water channels and it is actually very little effective in water transport when expressed in *Xenopus laevis* oocytes (Xu *et al*. 2015). However, we have demonstrated that LbAQP1 is capable of facilitating NO, H_2O_2 and CO_2 passage through biological membranes when heterologously expressed in *Saccharomyces cerevisiae*. The two highly conserved NPA motifs are one of the most important structural domains of AQPs and play a crucial role in water-selective permeation (Borgnia *et al*. 1999). Nevertheless, the two NPA motifs of LbAQP1 were altered to NPN and NSA, respectively, which might be responsible for its decreased water permeability and enhanced transport capacity of the three examined small molecules. The mostly likely subcellular localization of LbAQP1 was predicted to be in secretory pathways (TargetP; Emanuelsson *et al*. 2007). AQP Lacbi1:392091 of *L. bicolor* strain S238N also locates in cluster I and shares 99.04% identity with the deduced amino acid sequence of LbAQP1. This AQP showed low capacity for water transport and no capacity for the transport of ammonium/ammonia, urea or glycerol (Dietz *et al*. 2011). A closely related mycorrhizal fungal AQP, TcAQP1 (JF491353) from *Terfezia claveryi*, was also characterized as a functional water and CO_2 channel in the heterologous *S. cerevisiae* expression system (Navarro-Ródenas *et al*. 2012).

Carbon dioxide has been found to produce synergistic effects with root exudates on growth stimulation in *Gigaspora rosea* (Bécard & Piché 1989). It may be involved with other factors in defining the transition from the asymbiotic to presymbiotic developmental stage. It has been reported that CO_2 can act as a key signalling molecule in a wide range of biological processes of fungi, including growth and differentiation of pathogenic fungi (Bahn & Mühlschlegel 2006), as well as in sporocarp development (Stamets 2000). Moreover, the role of CO_2 as a signalling molecule is not restricted to fungi (Kim *et al*. 2010; Oliver *et al*. 2012; Cummins *et al*. 2014). The exact mechanism by which it operates is still not fully understood, but most studies have pointed to cAMP (Hall *et al*. 2010) as a secondary messenger since CO_2/HCO_3^- appears to be capable of directly activating adenylyl cyclase proteins (Hall *et al*. 2010). Participation of NO and H_2O_2 as signalling molecules has been documented for various processes in plants including stress responses (Neill *et al*. 2002; Gaupels *et al*. 2008) and establishing plant symbiotic relationships with *Rhizobium* and arbuscular mycorrhizal fungi (Weidmann *et al*. 2004; Lafranco *et al*. 2005; Moche *et al*. 2010; Puppo *et al*. 2013). These molecules have also been shown to be transported through some aquaporins (Herrera *et al*. 2006; Bienert *et al*. 2007; Wang & Tajkhorshid 2010) including LbAQP1 in the present study. Therefore, we postulate that LbAQP1 plays a role in ectomycorrhizal development in trembling aspen by facilitating the transmembrane transport of the small signalling molecules regulating *MiSSP7* expression.

In conclusion, the present report demonstrates that LbAQP1 from *L. bicolor* is required for the development of functional ectomycorrhizal structures in *P. tremuloides* roots. Expression of *LbAQP1* was strongly and rapidly up-regulated after direct contact with the root tissues and during mantle formation. This up-regulation was required for the correct expression of the fungal effector *MiSSP7* and for the formation of functional ectomycorrhizal structures. We propose that the molecule(s) that are transported from plant roots through LbAQP1 may act as a signal in the fungal hyphae triggering the events leading to the regulation of the *MiSSP7* expression and ectomycorrhizal development. NO, H_2O_2 and CO_2 are among the candidate molecules that may be involved in these signalling processes; however, more research is needed to support this notion. Our results reveal a novel and fundamental regulatory role for a fungal AQP in symbiotic interaction.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Table S1. List of primers used in this study.

Table S2. Statistically significant differences for gene expression shown in Figs 1, 2, 3 and 5. Different letters in the same column (the same day) indicate significant differences at $P \leq 0.05$ determined by ANOVA and Tukey's test.

Figure S1. Transcript abundance of *LbAQP1* in mycelia of *Laccaria bicolor* transgenic mock and RNAi strains. Different letters indicate significant differences at $P \leq 0.05$ determined with ANOVA, Tukey's test ($n = 3 \pm$ SE).

Figure S2. Southern blot analysis of *Laccaria bicolor* genomic DNA digested by restriction enzymes *Sac*I (A) or *Bam*HI (B). DNA ladder and digested genomic DNA were loaded as described below: lanes 1 and 15 for 1 Kb DNA ladder (GeneRuler®; Fermentas); lane 2 for WT; lane 3 for Mock 2; lane 10 for RL3-4, lane 11 for RL3-9; lane 4–9 and 12–13 for other transgenic strains; lane 14 for 0.2 ng of 870 bp PCR amplicon of hygromycin phosphotransferase gene as positive control. One clear, strong signal of hybridization band using a labeled probe targeting the *hph* gene indicates single copy insertion in the genomic DNA of transgenic strains.

Figure S3. Morphological characteristics (A) and dry mass (B) of mycelia of WT, mock and RNAi strains grown on MMN medium at 20 °C and 5 °C for 3 weeks. Different letters indicate significant differences at $P \leq 0.05$ determined with ANOVA, Tukey's test ($n = 6 \pm$ SE).

Figure S4. Cross sections of *Populus tremuloides* roots showing time course of colonization by different strains of *Laccaria bicolor* in an *in vitro* sandwich culture. (A and E) Wild type, (B and F) mock, (C and G) *LbAQP1* knock-down L3-4 and (D and H) *LbAQP1* knock-down L3-9 strains on day 1 (A to D) and 5 (E to H). Bars are 100 μM. Co, cortex; E, epidermis; Hn, Hartig net; M, mantle.