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Research

Overexpression of *Laccaria bicolor* aquaporin *JQ585595* alters root water transport properties in ectomycorrhizal white spruce (*Picea glauca*) seedlings

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Summary

- The contribution of hyphae to water transport in ectomycorrhizal (ECM) white spruce (*Picea glauca*) seedlings was examined by altering expression of a major water-transporting aquaporin in *Laccaria bicolor*.
- *Picea glauca* was inoculated with wild-type (WT), mock transgenic or *L. bicolor* aquaporin *JQ585595-overexpressing* (OE) strains and exposed to root temperatures ranging from 5 to 20°C to examine the root water transport properties, physiological responses and plasma membrane intrinsic protein (*PIP*) expression in colonized plants.
- Mycorrhization increased shoot water potential, transpiration, net photosynthetic rates, root hydraulic conductivity and root cortical cell hydraulic conductivity in seedlings. At 20°C, OE plants had higher root hydraulic conductivity compared with WT plants and the increases were accompanied by higher expression of *P. glauca PIP GQ03401_M18.1* in roots. In contrast to WT *L. bicolor*, the effects of OE fungi on root and root cortical cell hydraulic conductivities were abolished at 10 and 5°C in the absence of major changes in the examined transcript levels of *P. glauca* root *PIP*s.
- The results provide evidence for the importance of fungal aquaporins in root water transport of mycorrhizal plants. They also demonstrate links between hyphal water transport, root aquaporin expression and root water transport in ECM plants.

Introduction

Ectomycorrhizal (ECM) fungi absorb water and nutrients through extensive extraradical hyphal networks, transporting these resources to the mantle and Hartig net where resources are exchanged with the host plant root (Agerer, 2001). Processes involved in water uptake by mycorrhizal plants have received less attention than nutrient acquisition. Improved plant water relations have been frequently attributed to ECM (Plamboeck *et al.*, 2007; Lehto & Zwiazek, 2011) and arbuscular mycorrhizas (AM) (Uehlein *et al.*, 2007; Bárzana *et al.*, 2012). The effects of mycorrhizal associations often include increased root hydraulic conductivity (Muhsin & Zwiazek, 2002a,b; Marjanović *et al.*, 2005), which has been attributed to increased apoplastic (Nylund, 1987; Muhsin & Zwiazek, 2002a; Bárzana *et al.*, 2012) and transmembrane water transport (Marjanović *et al.*, 2005; Porcel *et al.*, 2006; Aroca *et al.*, 2007; Uehlein *et al.*, 2007; Lee *et al.*, 2010). The contribution of extraradical fungal hyphae to root water transport can be significant, as evidenced by decreased root hydraulic conductance following removal of these hyphae (Muhsin & Zwiazek, 2002b). Increased relative apoplastic flow in mycorrhizal plants has also been determined by use of apoplastic tracer dye (Bárzana *et al.*, 2012) and inhibitors of aquaporin activity (Muhsin & Zwiazek, 2002a; Bárzana *et al.*, 2012). However, use of apoplastic tracer dyes and aquaporin inhibitors can be problematic because they also potentially affect hyphal water transport.

Mycorrhizal associations have been reported to increase hydraulic conductivity of root cortical cells (Lee *et al.*, 2010) and alter the expression of root aquaporins in AM and ECM plants (Marjanović *et al.*, 2005; Porcel *et al.*, 2006; Aroca *et al.*, 2007; Uehlein *et al.*, 2007; Dietz *et al.*, 2011; Giovannetti *et al.*, 2012; Navarro-Ródenas *et al.*, 2013). Plant aquaporins are categorized into PIP (plasma membrane intrinsic protein), TIP (tonoplast intrinsic protein), NIP (nodulin-26 like intrinsic protein), SIP (small intrinsic proteins) and XIP (X intrinsic proteins) subfamilies, based on subcellular localization and transport capacities (Maurel *et al.*, 2008). PIPs play a crucial role in facilitating water transport and regulating root (Javot & Maurel, 2002; Aroca *et al.*, 2012; Gambetta *et al.*, 2013) and leaf (Maurel *et al.*, 2008;

Prado & Maurel, 2013) hydraulic conductivity. Their expression and post-translational modifications are sensitive to various environmental factors (Javot & Maurel, 2002; Maurel *et al.*, 2008; Gambetta *et al.*, 2013). The relative contributions of transmembrane and apoplastic water transport pathways in mycorrhizal roots may partly explain the reported lack of effect of mycorrhization on host plant root water flow properties (Coleman *et al.*, 1990; Nardini *et al.*, 2000; Calvo-Polanco *et al.*, 2008; Siemens & Zwiazek, 2008; Yi *et al.*, 2008).

Delineating the precise pathways for water transport from the fungal partner to the host roots in mycorrhizal associations remains a challenge. Some studies support the view that hydrophobic fungal cell walls in the mantle may block the apoplastic water pathway and hinder root water uptake (Duddridge *et al.*, 1980; Unestam & Sun, 1995), whereas others argue that fungal hyphae are more likely to form a water transport highway for plant roots, which substantially increases water availability to the roots (Khalvati *et al.*, 2005; Allen, 2007; Egerton-Warburton *et al.*, 2007; Lehto & Zwiazek, 2011). Because water can be transported in the cell walls of hydrophilic fungi, including *Laccaria bicolor* (Weatherley, 1982; Lehto & Zwiazek, 2011), it could be argued that this route offers the least resistance and thus could be the predominant pathway for water transport to the root cortex. However, the advantages of a symplastic pathway for hyphal water transport include the possibility of hydraulic regulation by fungal aquaporins as water enters and subsequently leaves the hyphae.

In this study, we addressed the question of the contribution of aquaporin-mediated transport in mycorrhizal fungal hyphae to water transport of the host plant. Fungal aquaporins have been described from several fungal taxa, and can be classified into four distinct groups: orthodox fungal water channels, fungal aquaglyceroporins, facultative fungal aquaporins and fungal XIPs (Dietz *et al.*, 2011; Xu *et al.*, 2013). Recent studies have demonstrated the capacity for transport of water and other small molecules by several aquaporins from ECM and AM fungi (Dietz *et al.*, 2011; Navarro-Ródenas *et al.*, 2012; Li *et al.*, 2013), which may play multiple roles in plant–fungal interactions (Maurel & Plassard, 2011). In *L. bicolor* strain S238N, five aquaporin genes heterologously expressed in *Xenopus laevis* oocytes showed strong to moderate water transport capacity; some of these were also permeable to urea, glycerol and ammonia (Dietz *et al.*, 2011). *TcAQP1* from the hypogeous mycorrhizal desert truffle (*Terfezia claveryi*) also showed water and CO_2 transport capacity (Navarro-Ródenas *et al.*, 2013), whereas *GintAQPF1* and *GintAQPF2* from the AM species *Glomus intraradices* showed significant water permeability (Li *et al.*, 2013). The expression of these fungal aquaporins could be altered by mycorrhization or abiotic cues (Dietz *et al.*, 2011; Li *et al.*, 2013; Navarro-Ródenas *et al.*, 2013), suggesting their involvement in water transport of the mycorrhizal partners.

One means to assess the relative significance of the different pathways for water movement in mycorrhizal plants is to alter the aquaporin-mediated water transport properties of the mycorrhizal fungus partner. Accordingly, we selected the aquaporin-encoding *JQ585595* (protein ID AFJ15558.1) from *L. bicolor* strain UAMH8232 for its high water transport capacity and high transcript abundance, and generated transgenic *L. bicolor* overexpressing *JQ585595* to test the role of this fungal aquaporin in facilitating water transport in ectomycorrhizal white spruce (*Picea glauca* [Moench] Voss). *P. glauca* seedlings inoculated with wild-type (WT), *JQ585595*-overexpressing (OE) and mock-transformed strains were examined for the effect of these fungal genotypes on water transport properties of the host plant. We tested the hypothesis that root hydraulic conductivity of mycorrhizal plants would be enhanced by overexpression of the *L. bicolor* aquaporin, reflecting the increased contribution of water transport through fungal hyphae to water transport of the mycorrhizal root system.

Materials and Methods

Cloning and *in silico* analysis of *L. bicolor* aquaporin genes

Laccaria bicolor (Maire) P.D. Orton strain UAMH8232 (University of Alberta Microfungus Collection) mycelia were grown on solid modified Melin-Norkans (MMN) medium (Pham *et al.*, 2004) at 20°C with cellophane placed on the surface for 3 wk before mycelia were harvested and immediately frozen in liquid nitrogen. Mycelia were ground in liquid nitrogen using a mortar and pestle. Total RNA was extracted using the RNeasy Plant Mini Kit (Qiagen), and used for first strand cDNA synthesis (Superscript II; Life Technologies, Carlsbad, CA, USA). Full-length *L. bicolor* UAMH8232 cDNAs corresponding to five of the seven *L. bicolor* aquaporins reported by Dietz *et al.* (2011) were amplified using gene-specific primers designed using *L. bicolor* strain S238N sequence information (Supporting Information Table S1), and ligated into pGEM-T Easy (Promega). Sequences were confirmed by Sanger sequencing, and have been deposited in GenBank (accession numbers JQ585592–JQ585597).

Protein transmembrane secondary structure and subcellular localization was predicted using TMHMM2.0 (Krogh *et al.*, 2001) and Target P (Emanuelsson *et al.*, 2000), respectively. Sequence alignment and phylogenetic analysis was conducted using ClustalW 2.1 (Larkin *et al.*, 2007) and MEGA 5.2.1 (Felsenstein, 1985; Saitou & Nei, 1987; Tamura *et al.*, 2011).

Functional analysis of *L. bicolor* aquaporins

Full-length cDNAs were sub-cloned into the multiple cloning site of pXT7 containing the *T7* promoter and the 5′ and 3′ UTR of the *X. laevis* β-globin gene (Dominguez *et al.*, 1995), between the restriction sites *Xho*I and *Spe*I. Insertion orientation was determined by sequencing, then the expression vector was linearized at the *Nde*I site located downstream of the *Xenopus* β-globin gene. The linearized vector was used for *in vitro* synthesis of capped RNA (cRNA) using *T7* RNA polymerase (mMESSAGE mMACHINE *T7* kit; Ambion).

For the *X. laevis* oocyte swelling assay, healthy Stage V–VI oocytes were treated with collagenase and potassium phosphate (Cao *et al.*, 1992). Ten ng of cRNA or nuclease-free water (as the negative control) was microinjected into each oocyte using an automatic nanoliter injector (Nanoject II; Drummond Scientific,

Broomall, PA, USA). After incubation in 200 mOsmol (kg^{-1} H_2O) modified Barth's solution (MBM) in scintillation vials at 18°C for 48 h, each injected oocyte was transferred into MBM in one well of a four-well Petri dish and viewed under the ×4 objective of an Olympus compound microscope. An initial image was taken with an Olympus QCapture digital camera; upon transfer of an oocyte into a well containing $D = 0.2$ hypotonic MBM (40 mOsmol kg^{-1} H_2O), serial images were captured at 10-s intervals for 3 min to track changes in oocyte volume due to water influx. The diameter and surface area of oocytes were analyzed using ImageJ (v1.44o; Schneider *et al.*, 2012). The initial transmembrane volume flux and osmotic water permeability coefficiency (P_f) were calculated based on Zhang & Verkman (1991) to represent the water permeability of the oocytes injected with cRNAs of each putative aquaporin.

Generation of transgenic *L. bicolor* strains

Transgenic *Laccaria* strains overexpressing *JQ585595* were generated using the pHg/pSILBAγ- plasmid system under hygromycin B selection (Kemppainen & Pardo, 2010). The aquaporin cDNA was liberated from the pGEM-T Easy vector with *Apa*I/*Pst*I, blunt ends were generated with T4 DNA polymerase and the cDNA fragment cloned into *Sna*BI/*Stu*I-digested pSILBAγ between the constitutive *Agaricus bisporus gpdII* promoter and *Aspergillus nidulans trpC* terminator. The correct cDNA orientation in the expression cassette of pSILBAγ was confirmed by sequencing. The full-length pSILBAγ/*JQ585595* -expression vector was cloned as a *Sac*I linearized fragment into the *Sac*I site in the T-DNA of the pHg binary vector to generate the final pHg/pSILBAγ/*JQ585595* transformation/overexpression construct and the vector was introduced into the *Agrobacterium tumefaciens* strain AGL1 by electroporation. The *L. bicolor* UAMH8232 WT strain was transformed with pHg/pSILBAγ/*JQ585595* 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 200 μg ml^{-1} of ceftriaxone in the growth medium. To generate the mock transformant strains, *L. bicolor* UAMH8232 WT was *Agro*-transformed with pHg/pSILBAγ. Thirteen and 12 independent transgenic *Laccaria* strains were obtained for pHg/pSILBAγ/*JQ585595* and pHg/pSILBAγ transformation, respectively. Transformed strains that showed normal phenotypes of the species were selected for further validation.

Southern blot analysis was used to determine transgene copy number. Ten micrograms of gDNA extracted using the DNeasy Plant Maxi Kit (Qiagen) was digested using *Sac*I or *Bam*HI. An 870-bp PCR product was amplified using the 1026-bp hygromycin phosphotransferase gene (*hph*) in the binary vector as the template (primers used are reported in Table S1), and used as both positive control and probe. 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), as previously described (Kemppainen *et al.*, 2008). TAIL-PCR was conducted to identify the T-DNA insertion site in the genome of transgenic strains (Notes S1a; Table S2; Liu, 2012).

Seedling growth and inoculation

Seeds of *Picea glauca* (Moench) Voss (National Tree Seed Centre, Canadian Forest Service, Fredericton, NB, Canada) were surface sterilized with 1% (v/v) sodium hypochlorite and stratified at 4°C according to Groome *et al.* (1991). Stratified seeds were germinated at 20°C on sterile, moistened crepe cellulose paper (Kimpak; Kimberley-Clark, Mississauga, ON, Canada). One week after germination, seedlings were transplanted into autoclaved peat moss : vermiculite (2 : 1) in sterilized 170-ml Spencer-Lemaire root trainers (Spencer-Lemaire Industries Ltd, Edmonton, AB, Canada) covered with plastic domes. Seedlings were grown in a controlled environment growth room with 16 h photoperiod, 22°C : 18°C (day : night) temperature, 400 μmol m^{-2} s^{-1} photosynthetic photon flux density, and 50–60% relative humidity.

Laccaria strains were cultured in liquid MMN medium at 20°C with shaking at 120 rpm for 4 wk. Cultures prepared from the WT strain, one mock strain or two transgenic *JQ585595*-OE strains (designated OE 1 and OE 2) were homogenized in a blender to make liquid inoculum of $OD_{600} = 1.5$. Immediately after transplanting, seedlings were inoculated by injecting 10 ml of homogenized liquid inoculum from one of the four strains described above into the sterilized potting mix. Autoclaved fungal-free liquid MMN was used to treat nonmycorrhizal control seedlings. After 1 month, a second inoculation was conducted by applying 10 ml of the respective inoculum to the soil. Eighteen plants were maintained for each of the five inoculation treatments. Spatial separation of plants minimized the possibility for cross-contamination. Root trainer positions were re-randomized every 3 d to minimize the impact of any growth chamber heterogeneity.

Two months after the second inoculation, mycorrhizal colonization was examined (Fig. S1; Brundrett *et al.*, 1996).

Measurements of gas exchange, growth, and shoot water potential

Net photosynthetic and transpiration rates of lateral branches of 3-month-old seedlings were measured between 09:00 h and 12:00 h using a Li-6400 with a 2 × 3 cm^2 red-blue light chamber (Li-Cor, Lincoln, NE, USA). Subsequent to these measurements, needles were collected, scanned and the total surface area of needles calculated using ImageJ (v1.44o; Schneider *et al.*, 2012). Net photosynthetic (P_n) and transpiration (T_r) rates were expressed as a function of needle surface area. Measurements were carried out for six plants from each inoculation treatment ($n = 6$). Dry mass was determined after oven drying at 80°C for 48 h ($n = 6$). Terminal shoots of 10–15 cm in length were excised at noon and immediately placed into a Scholander pressure chamber for

midday shoot water potential measurements (Scholander *et al.*, 1965) ($n = 6$).

Root hydraulic conductivity

A high pressure flow meter (HPFM; Tyree *et al.*, 1995) was used to determine whole root hydraulic conductivity (Lpr) of 3-month-old seedlings immediately after gas exchange measurements. The root with soil was removed from the root trainer and kept in a plastic bag submerged in a circulating water bath set to 20°C (Thermo Scientific, Hampton, NH, USA) for 30 min before the first measurement of root hydraulic conductance (K_r). Increasing pressure was applied to the root to obtain a smooth linear regression between supplied pressure and flow rate. Slope was calculated as K_r value. The temperature of the circulating water bath was then lowered to 10°C for 30 min before the second measurement and to 5°C for 30 min before the final measurement. To determine root volumes, the peat: vermiculite mixture was gently washed off the roots immediately after K_r measurements. Root volumes were determined using the water displacement method; to calculate L_{pr}, K_r was divided by the root volume (Kamaluddin & Zwiazek, 2002).

Root cortical cell hydraulic conductivity

A cell-pressure probe was used to determine the hydraulic conductivity of individual cortical cells (L_{pc}) in the roots of 3-month-old mycorrhizal and nonmycorrhizal *P. glauca* as previously described (*Lee et al.,* 2010). Roots were collected from six plants per inoculation treatment and kept in a circulating water bath at either 20 or 10°C for 30 min before measurement ($n = 6$). A single cortical cell was punctured at a distance of *c.* 20 mm from the root tip with a silicon oil-filled micro-capillary. The measurements of hydraulic properties were carried out for *c.* 20 min and L_{pc} was calculated (Steudle, 1993).

Quantitative RT-PCR

Three months after the first inoculation, mycorrhizal and nonmycorrhizal root tip segments of *c.* 1 cm in length were collected, stored and ground as described above before total RNA extraction using the RNeasy Plant Mini extraction method (Qiagen), with the addition of 20 mg of polyethylene glycol 8000 ml^{-1} RLT buffer to facilitate the extraction of good quality RNA from the samples. First strand cDNA was synthesized from 1 μg total RNA using Superscript II (Life Technologies), and cDNA of 10 ng μl^{-1} (in a 2.5-μl volume) was used as template for SYBR Green qRT-PCR as described in El Kayal *et al.* (2011). Primers were designed using Primer Express 3.0 (Applied Biosystems, Life Technologies; Table S1).

Quantification of transgene transcript abundance in *JQ585595*-OE *L. bicolor* strains

Transcript abundance of *JQ585595* in transgenic *L. bicolor* mycelia grown on solid MMN medium at 20°C for 3 wk was quantified using the standard curve method of quantification, as previously described (Pfaffl, 2004; El Kayal *et al.*, 2011). Three biological replicates representing independent mycelial cultures were sampled for each strain. Transcript abundance of *JQ585595* was normalized against the geometric mean of transcript abundance of reference genes α-*tubulin* (XM_001876554) and *translation elongation factor EF2* (XM_001887160), which did not change significantly across all tested samples of mycelia in WT and transgenic strains ($P = 0.78$).

Quantification of transcript abundance of *L. bicolor* aquaporins in mycorrhizal root tips

Transcript abundance corresponding to the six *L. bicolor* aquaporins was quantified in roots mycorrhizal with WT, mock and two OE strains of *L. bicolor* using the standard curve method of absolute quantification (Pfaffl, 2004; El Kayal *et al.*, 2011). *EF2* was used as the reference gene, as it exhibited stable expression across all tested samples at 20 and 5°C ($P = 0.81$).

Transcript abundance quantification of *P. glauca PIP*s in mycorrhizal and nonmycorrhizal roots

In order to identify putative *P. glauca PIP*s, phylogenetic analysis and protein secondary structure prediction were conducted as described in Notes S1b (Johanson *et al.*, 2001; Almeida-Rodriguez *et al.*, 2010). Nine putative *PIP*s were selected for transcript profiling by qRT-PCR of *P. glauca* roots sampled at 20°C and 1 h after the treatment of placing root trainers in the circulating water bath at 5°C. *PgCDC2* (*cell division cycle 2*, GQ0197_L17.1, BT106071) was used as the reference gene (Bedon *et al.*, 2009), as its C_t value did not change significantly across all tested samples of mycorrhizal and nonmycorrhizal root tips at 20 and 5°C ($P = 0.84$). The relative transcript abundance of these *PIP*s was calculated using the standard curve method of comparative quantification ($\Delta\Delta C_t$ with efficiency correction) (Livak & Schmittgen, 2001; Pfaffl, 2004). To assess the impact of mycorrhization on expression levels of these *P. glauca PIP*s, the cDNA samples of nonmycorrhizal roots harvested at 20°C were used as calibrator for ratio calculation. To assess the impact of 5°C temperature on expression levels of the *PIP*s, the corresponding samples at 20°C was used as calibrator. To evaluate amplification efficiencies for each primer pair, cDNAs of all samples were pooled to generate a 10× dilution series used as the template for each pair of primers. The slope of standard curves for the target and reference genes ranged between −3.01 and −3.38, corresponding to the range of the efficiencies between 114.9% and 97.8% used in the efficiency correction of $\Delta\Delta C_t$ values.

Statistical analyses

Descriptive statistics and ANOVA were conducted using Origin 8.0 (OriginLab, Northampton, MA, USA). Tukey tests were used to compare means for statistically significant differences ($P = 0.05$).

Results

Characteristics of aquaporin *JQ585595*

Six full-length putative aquaporin cDNAs – designated according to their NCBI accession numbers as *JQ585592, JQ585593, JQ585594, JQ585595, JQ585596,* and *JQ585597* – were cloned from the mycelium of *L. bicolor* UAMH8232. A previously conducted phylogenetic analysis of fungal major intrinsic proteins (MIPs) demonstrated that JQ585592 grouped with orthodox fungal water channels (Cluster I), JQ585593 grouped with fungal aquaglyceroporins (Cluster II), and JQ585594, JQ585595, JQ585596 and JQ585597 all grouped with facultative fungal aquaporins (Cluster III) (Notes S2; Xu *et al.*, 2013). The placement of each of the *L. bicolor* aquaporins within these major subfamilies is shown in Fig. S2. Four of these showed significant water transport capacity in the oocyte swelling assay (Fig. 1a). The water permeability coefficient (P_f) of oocytes microinjected with cRNAs corresponding to *JQ585595* was significantly higher than that of oocytes injected with other *L. bicolor* aquaporins (Fig. 1a).

Transcript abundance profiling by qRT-PCR was carried out for the three facultative fungal aquaporins that showed the greatest water transport capacity (JQ585595, JQ585596 and JQ585597; Fig. 1a), as well as JQ585592 that belonged to the cluster of orthodox fungal water channel. The transcript abundance of *JQ585595* was significantly higher than the other three aquaporins (Fig. 1b). *JQ585595* showed the greatest similarity to the previously characterized *L. bicolor* aquaporin 443240 of *L. bicolor* strain S238N (*L. bicolor* genome v2.0, Joint Genome Institute; annotated as 391485 in genome v1.0; Dietz *et al.*, 2011; Notes S3). The deduced amino acid sequence of JQ585595 was 94% identical to 443240, exhibiting 16 amino acid substitutions over the 312 amino acids of the predicted protein. *In silico* analysis using TMHMM2.0 showed that JQ585595 exhibited canonical aquaporin secondary structure: six transmembrane domains (TMD) and five loops (A–E) (Fig. 2a), with each of the two NPA (asparagine, proline, alanine) signature motifs locating at Loop B and E, respectively (Fig. 2b), aromatic/Arg sites at TMD2, TMD5 and Loop E as the selective filter, and two termini in cytosol. Loops B and E form a seventh half-transmembrane helix and two NPA motifs form a pore for selective transport. Target P predicted a plasma membrane subcellular localization. Accordingly, *JQ585595* was selected for generating transgenic *L. bicolor*, in order to investigate the role of mycorrhizal aquaporins in ECM plant water relations.

Analysis of *L. bicolor* transgenic strains

Seven *L. bicolor* strains transformed with *JQ585595* overexpression construct under control of the constitutive *A. bisporus gpdII* promoter (Fig. 3a) were tested for transgene expression by qRT-PCR. OE 1 and OE 2 showed the highest levels of *JQ585595* transcript abundance, with values *c.* 1.5-fold higher than WT (Fig. 3b). Transcript abundance of *JQ585595* in mock strains as well as OE5 and OE7 strains was slightly lower than that of WT (Fig. 3b). Southern blot analysis using a labeled probe targeting

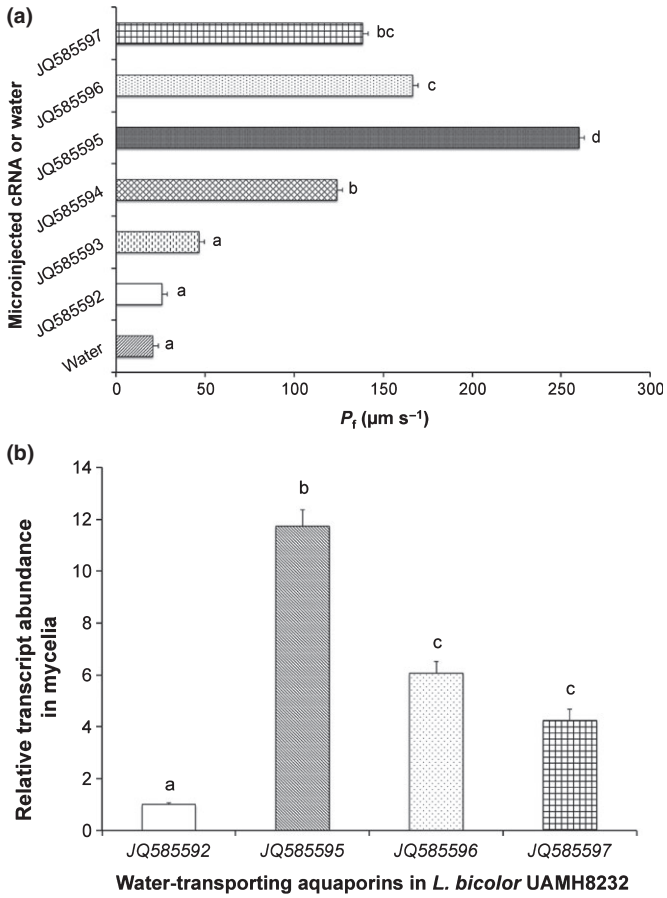

Fig. 1 Functional assay of aquaporins in *Laccaria bicolor* strain UAMH8232. (a) Osmotic permeability coefficient (P_f) values of *Xenopus laevis* oocytes in which the corresponding *L. bicolor* aquaporins were heterologously expressed; either cRNAs of each aquaporin gene or water (as negative control) was microinjected into the oocytes. (b) Relative transcript abundance of selected aquaporins in *L. bicolor* wild-type mycelia measured by a standard curve method of absolute quantification in a qRT-PCR assay. The transcript abundance of the aquaporin genes was normalized against the geometric mean of that of the reference genes, *α-tubulin* and *EF2*. Means, $n = 10$ in (a) and 3 in (b) ± SE are shown. Values with different letters are significantly different at $P \leq 0.05$ (ANOVA, Tukey's test).

the *hph* gene confirmed that the OE1, OE2 and Mock 2 strains each harbored a random single insertion of the transgenic cassette (Fig. 3c,d). Using TAIL-PCR, we demonstrated that for all strains, the transgene cassette was inserted into different scaffold locations. None of the insertion sites disrupted ORFs of known genes within the *L. bicolor* genome (Notes S1a). Based on these analyses, OE1, OE2 and Mock 2 were chosen as the OE strains and mock control, respectively.

Root colonization and impact on seedling growth, gas exchange and shoot water potentials

All seedlings treated with *L. bicolor* were successfully inoculated. Evident and similar mantle and Hartig net structures were found in *c.* 90% of the 30 sampled root tips from inoculated plants (Fig. S1e–h). There were no significant differences between

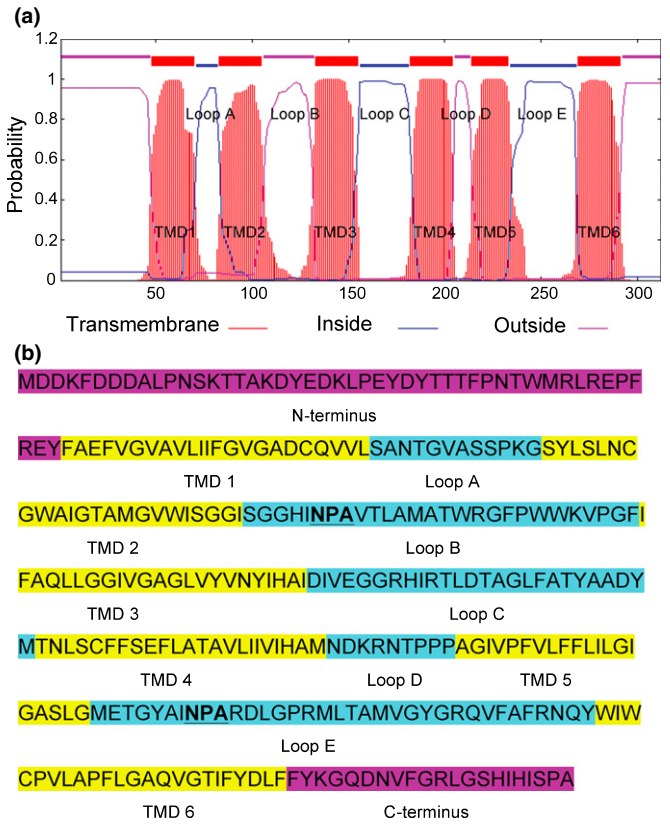

Fig. 2 Deduced amino acid sequence of *Laccaria bicolor* JQ585595 predicted as a canonical aquaporin with six-transmembrane-domain structure and NPA signature motifs in the *in silico* assay. (a) Transmembrane secondary structure of the protein predicted using TMHMM. (b) Deduced amino acid sequence fragments in termini, transmembrane domains TMD 1-6 and Loop A–E, with two NPA motifs (in bold and underlined) in Loop B & E, respectively.

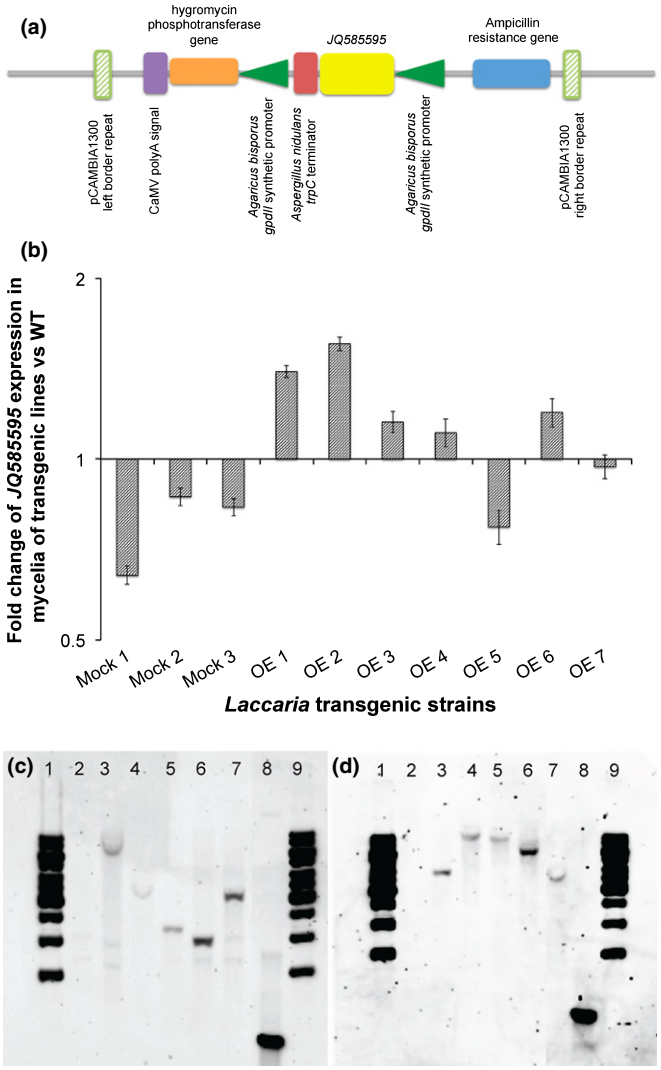

Fig. 3 Construction and selection of *Laccaria bicolor* transgenic strains overexpressing *JQ585595*. (a) The transgenic cassette rendering *JQ585595* overexpression and hygromycin resistance randomly inserted into the genome of *L. bicolor* UAMH8232 via *Agrobacterium*-mediated transformation. (b) The qPCR SYBR-Green assay showing *JQ585595* expression in mycelia of the overexpression (OE), mock and wild-type (WT) ($n = 3 \pm$ SE). (c, d) Southern blot showing the transgenic strains for single copy insertion events in the genomic DNA digested by *Sac*I (c) and *Bam*HI (d); one clear band of hybridization indicating single copy insertion; DNA ladder and digested genomic DNA were loaded in the following order: Lanes 1 and 9, for 1 kb DNA ladder; Lane 2, WT; Lane 3, Mock 1; Lane 4, Mock 2; Lane 5, Mock 3; Lane 6, OE 1; Lane 7, OE 2; Lane 8, 0.2 ng of 870 bp PCR amplicon of hygromycin phosphotransferase gene as positive control. 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 4 h. Chemiluminescence generated via an alkaline phosphatase reaction was detected by CCD sensor using a 30-min exposure time (BioRad ChemiDoc).

L. bicolor strains in terms of colonization rates: 93.3 ± 4.1% (± SE), 86.7 ± 3.3%, 90 ± 4.1% and 93.3 ± 4.1% for WT (Fig. S1e), mock (Fig. S1f), OE1 (Fig. S1g) and OE2 (Fig. S1h), respectively. There was neither extraradical mycelia in soil nor distinct ectomycorrhizal structures in thin sections of the root tips observed in noninoculated plants (Fig. S1i).

Seedlings inoculated with OE1 strain had lower dry mass compared with the noninoculated plants, and both OE1 and OE2 plants also had lower dry mass compared with the mock strain (Fig. 4a). Mycorrhizal plants had higher shoot water potential (Fig. 4b), net photosynthesis (Fig. 4c) and transpiration rates (Fig. 4d) than nonmycorrhizal plants. However, there was no significant difference in these parameters between the different inoculation treatments (Fig. 4).

Fungal aquaporin gene expression in mycorrhizal root tips

Of the fungal aquaporins, *JQ585595* exhibited the highest transcript abundance in mycorrhizal root tips, followed by *JQ585593* and *JQ585594* (Fig. 5). The transcript abundance levels of *JQ585592*, *JQ585596* and *JQ585597* were 5- to 10-fold lower than those of *JQ585595*.

The expression of *JQ585592* in mycorrhizal root tips was low and not significantly different between the different strains at each examined temperature, and increased in all strains with the decrease in temperature from 20 to 5°C (Fig. 5a). The expression of *JQ585593* was also low at 20°C and not significantly different between mycorrhizal treatments; however, in all strains, it

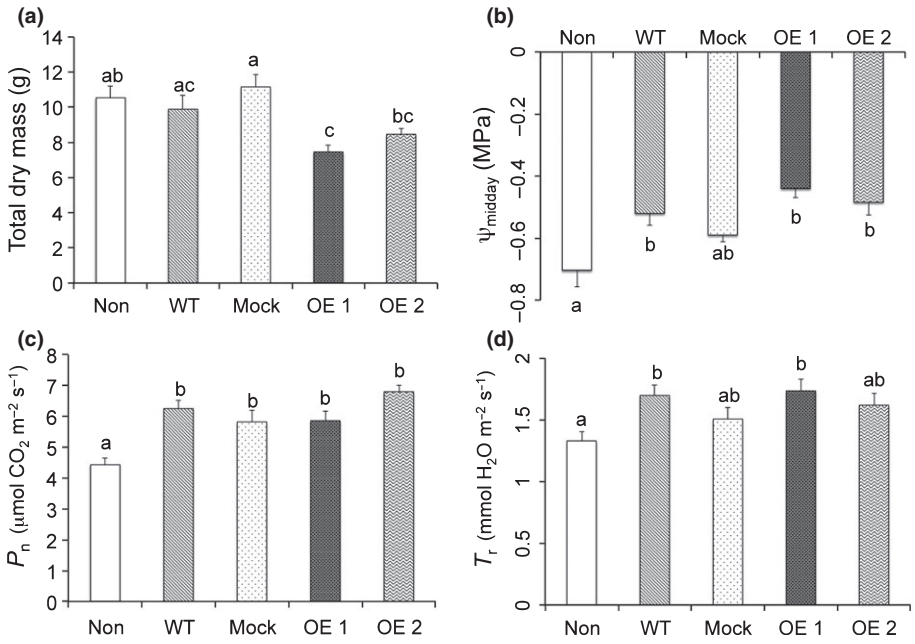

Fig. 4 The effects of mycorrhization with *Laccaria bicolor* on (a) total dry mass, (b) midday shoot water potential ψ_{midday}, (c) net photosynthetic rate P_n and (d) transpiration rate T_r of *Picea glauca* seedlings. The treatments were nonmycorrhizal (Non), and mycorrhized with wild-type *L. bicolor* (WT), mock (Mock) and two *JQ585595*-overexpression strains (OE 1 and OE 2). Means ($n = 6$) ± SE are shown. Different letters indicate significant difference at $P \leq 0.05$ (ANOVA, Tukey's test).

increased by more than 10-fold with the decrease in temperature to 5°C (Fig. 5b). Transcript abundance of *JQ585594* at 20°C was similar in all strains with the exception of a small, but statistically significantly higher level in OE1 compared with the mock strain (Fig. 5c). In all strains, *JQ585594* levels were higher at 5°C compared with 20°C (Fig. 5c). Transcript abundance of *JQ585595* was significantly higher in OE strains than in WT and mock strains at 20°C (Fig. 5d). In all strains, a decrease in temperature from 20 to 5°C induced a significant increase in *JQ585595* expression (Fig. 5d). All strains maintained low expression levels of *JQ595596* and *JQ585597* at 20 and 5°C (Fig. 5e,f). Temperature decrease from 20 to 5°C had little effect on the expression levels of these aquaporins with the exception of small, but statistically significant decreases observed in OE2 (Fig. 5e,f).

Root hydraulic conductivity (L_{pr}) and root cortical cell hydraulic conductivity (L_{pc})

At 20°C, L_{pr} in mycorrhizal seedlings of the WT and mock strains was *c.* 2-fold higher than L_{pr} in the nonmycorrhizal control seedlings (Fig. 6a). In both OE lines, L_{pr} was more than 50% higher than in the WT and mock lines, and *c.* 3-fold higher compared with nonmycorrhizal control (Fig. 6a).

When root temperature was decreased from 20°C first to 10°C and then 5°C, only small decreases in L_{pr} were measured in nonmycorrhizal plants and in the mock-mycorrhizal plants (Fig. 6a). There was no effect of the decreased temperatures on L_{pr} in WT plants (Fig. 6a). However, in both OE lines, the decline in temperature from 20 to 10°C and 5°C resulted in a greater than two-fold decrease in L_{pr} (Fig. 6a).

At 20°C, L_{pc} was *c.* 2-fold higher in WT-mycorrhized plants compared with nonmycorrhizal control and more than three-fold higher in both OE lines (Fig. 6b). There was no significant effect on L_{pc} in nonmycorrhizal and WT plants when the temperature was decreased to 10°C (Fig. 6b). However, in both OE lines the decrease in temperature from 20 to 10°C lowered L_{pc} by more than three-fold which brought the L_{pc} levels to approximately those that were measured in nonmycorrhizal plants (Fig. 6b).

Gene expression of *P. glauca PIP*s in root tips

Thirteen full-length or near full-length *P. glauca PIP* cDNAs were identified in a large-scale expressed gene resource (Fig. S3; Notes S2; Rigault *et al.*, 2011). Most of the nine *PIP*s selected for transcript abundance profiling were represented in sequenced cDNA libraries of *P. glauca* root tissue, taken as evidence of being expressed in roots (Rigault *et al.*, 2011). *In silico* analysis showed that the deduced amino acid sequences of the nine putative *PIP*s that were assayed by qRT-PCR exhibited the canonical aquaporin transmembrane structure (Notes S4). The most highly expressed *PIP*s among these nine genes in the nonmycorrhizal roots at 20°C were *GQ03401_M18.1*, *GQ03703_H07.1* and *GQ02905_E13.1*, followed by *GQ03610_A06.1*, *GQ03010_E09.1*, *GQ03001_P18.1* and *GQ02901_B20.1*. Transcript abundance of *GQ03002_G07.1* and *GQ03111_E12.1* was low (Fig. S4).

Expression profiles of the nine *P. glauca PIP*s showed varying responses to mycorrhization with WT, mock, OE1 and OE2 strains. Mycorrhization with WT and mock strains resulted in an three- to four-fold increase in *GQ03401_M18.1* transcript abundance, whereas in both OE lines, *GQ03401_M18.1* transcript abundance increased by 40- to 56-fold (Fig. 7a). Both OE lines also showed a strong increase in *GQ03703_H07.1* expression, whereas the opposite was observed for expression of *GQ03610_A06.1* (Fig. 7a). Expression of *GQ03001_P18.1* was decreased by mycorrhization (Fig. 7a). Transcript abundance of most of the *P. glauca PIP*s in roots was significantly

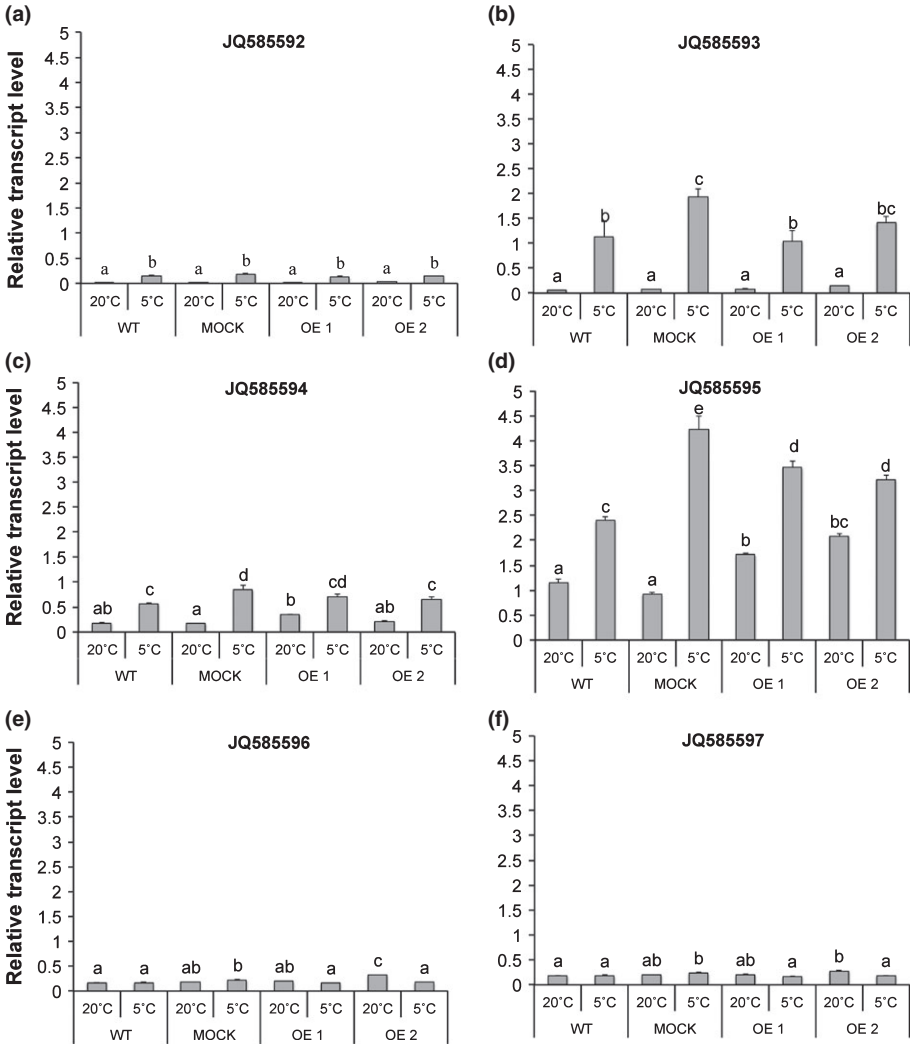

Fig. 5 Relative transcript abundance of *Laccaria bicolor* aquaporins JQ585592 (a), JQ585593 (b), JQ585594 (c), JQ585595 (d), JQ585596 (e) and JQ585597 (f) in roots of *Picea glauca* mycorrhized with the wild-type (WT), mock (Mock), and two overexpression (OE 1 and OE 2) strains of *L. bicolor* and exposed to root temperatures of 20 and 5°C. The transcript abundance of target aquaporins was normalized to that of the reference gene *EF2*. Different letters indicate significant differences at $P \leq 0.05$ determined with ANOVA, Tukey's test ($n = 3 \pm$ SE). The standard curve template was prepared as a series of dilutions of the mixture of the PCR amplicons of each analyzed gene, with the template concentration ranging from 1.6×10^2 to 1.6×10^8 molecules ml^{-1} for each gene. The number of molecules was calculated from the mass (ng) and the molecular weight (g mol^{-1}) of the PCR amplicons of each gene, given that the fragment size (base pairs) of PCR amplicons was known, and the average molecular weight of each base pair is 660 g mol^{-1} and there is 6.02×10^{23} of molecules mol^{-1}.

downregulated at 5°C compared with 20°C in all inoculation treatments (Fig. 7b). With the exception of a higher *GQ03610_A06.1* transcript abundance 5 to 20°C ratio in both OE lines compared with the other inoculation treatments, there was no clear pattern showing consistent differences in the temperature responses between the inoculation treatments (Fig. 7b).

Discussion

Taking advantage of the genome sequence of the *L. bicolor* model strain S238N (Martin *et al.*, 2008), we obtained six aquaporin genes from *L. bicolor* UAMH8232. Of these, JQ585595 demonstrated the highest water transport capacity in the heterologous *X. laevis* oocyte expression system, and the highest transcript abundance in mycelium grown on MMN medium. In another study carried out using the same *X. laevis* oocyte assay in the same time frame, JQ585595 demonstrated 40% higher water-transporting capacity compared to the maize ZmPIP2;8 aquaporin and *c.* 160% higher water-transporting capacity than the ZmTIP2;2 aquaporin (Lawrence *et al.*, 2013). JQ585595 shared 94% amino acid sequence identity with the aquaporin 443240 from v2.0 of the *L. bicolor* strain S238N genome assembly, equivalent to 391485 from v1.0 (Notes S3). Dietz *et al.* (2011) demonstrated that the aquaporin 391485 showed relatively high water transport capacity as well as capacity to transport glycerol and ammonia, and that the expression of this aquaporin was upregulated upon mycorrhization with *Populus tremuloides* grown on MS medium.

Based upon the findings above, we selected *JQ585595* for construction of OE transgenic strains. Similar to earlier studies (Kemppainen *et al.*, 2005, 2008; Kemppainen & Pardo, 2010), we found that *Agrobacterium*-meditated transformation was effective in yielding successful *L. bicolor* transgenic strains. In the transgenic cassette, the constitutive *A. bisporus gpd*II promoter was used to drive expression of *JQ585595* (Fig. 3a). Previous studies have demonstrated the effectiveness of gene expression induced by this promoter in transgenic basidiomycete fungi (Burns *et al.*, 2006; Kilaru *et al.*, 2006; Ding *et al.*, 2011). Compared with WT, the OE strains did not demonstrate a multiple-fold increase in transcript abundance for *JQ585595* (Fig. 3b), probably because the transcript abundance for the endogenous aquaporin gene was already high in WT grown on MMN

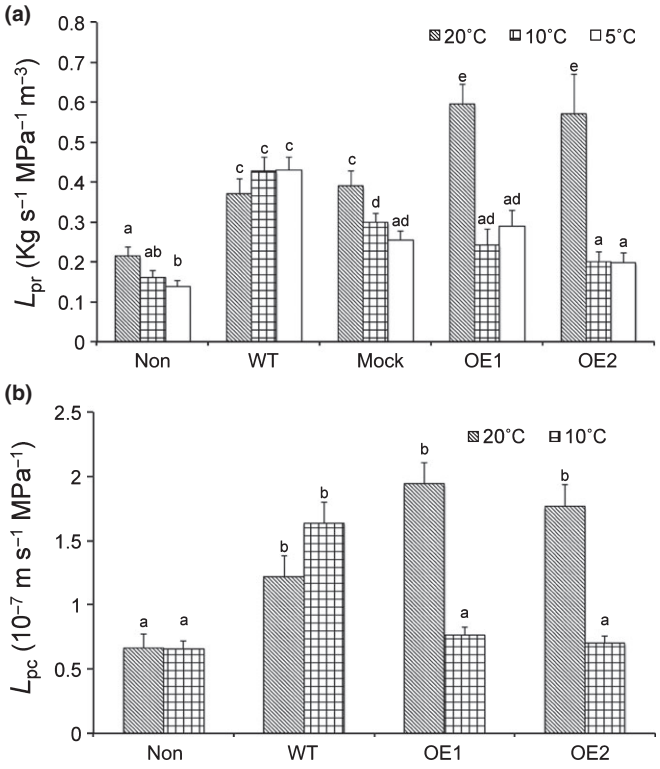

Fig. 6 Root hydraulic conductivity (L_{pr}) (a) and cell hydraulic conductivity of root cortical cells (L_{pc}) (b) in nonmycorrhizal (Non) *Picea glauca* seedlings and in seedlings inoculated with the wild-type (WT), mock (Mock), and two overexpression (OE 1 and OE 2) strains of *Laccaria bicolor*. Means ($n = 6$) ± SE are shown. Different letters indicate significant differences at $P \leq 0.05$ (ANOVA, Tukey's test).

medium at 20°C (Fig. 1b). Two selected OE strains demonstrated *c.* 1.5-fold greater transcript abundance of *JQ585595* compared with WT (Fig. 3b). Considering the high expression levels of *JQ585595* in WT, the 50–100% observed increase represents a considerable increase in transcript quantity (Fig. 5d). Importantly, the elevated level of transcript abundance was sufficient to produce significant functional effects (Fig. 6).

In general, the higher expression of *JQ585595* in OE strains did not cause significant changes in transcript abundance of other *L. bicolor* aquaporins at 20°C compared with the WT and mock strains, with the exception of *JQ585594* in OE1 (Fig. 5c), and *JQ585596* and *JQ585597* in OE2 (Fig. 5e,f). Transcript profiles of the six *L. bicolor* aquaporins responded differently to mycorrhizal treatments and temperature decline (Fig. 5). At 20°C, transcript profiles were not significantly different between WT and mock strains, indicating that the mock strain behaved as an appropriate control. By contrast, at 5°C, five of the six *L. bicolor* aquaporins showed significantly higher transcript abundance in the mock line than WT. We demonstrated using TAIL-PCR that no known ORF was disrupted by the insertion in the mock (Notes S1a; Table S2; Fig. S5); thus, this effect does not appear to be due to unintended interruption of gene function. It remains unclear whether the insertion sites had an impact on gene expression at low temperature via possible mechanisms such as chromatin modification. Interestingly, the enhanced expression of these *L. bicolor* aquaporins – especially *JQ585595* – in the mock strain at 5°C corresponded to a greater decrease in L_{pr} of mock-inoculated roots at 5°C compared to WT-inoculated roots (Fig. 6a). This partly explained why the L_{pr} profile of mock-inoculated roots was different than that of WT-inoculated roots, but similar to that of the OE-inoculated roots as a function of temperature. The mechanism by which *JQ585595* might contribute to this process remains to be explored.

Mycorrhization with OE1 reduced seedling dry mass compared with noninoculated plants. Both OE1- and OE2-inoculated seedlings also showed reduced dry mass compared with mock strain-inoculated seedlings (Fig. 4a). The effects of mycorrhization on plant growth vary, depending on mycorrhization stage and various abiotic and biotic environmental factors (Smith & Read, 2008). Growth reductions may occur due to increased carbohydrate demand by the mycorrhizal fungus (Tinker *et al.*, 1994).

Increased net photosynthetic rates of mycorrhizal plants were paralleled by increased transpiration rates (Fig. 4c,d), suggesting that stomatal factors were likely largely responsible for differences in photosynthetic rates. Similar increases in transpiration and photosynthetic rates of mycorrhizal plants were previously reported for ECM and AM associations (Allen *et al.*, 1981; Dosskey *et al.*, 1990; Caravaca *et al.*, 2003; Birhane *et al.*, 2012). In our study, shoot water potentials were higher in mycorrhizal plants despite higher transpiration rates, likely due to higher L_{pr}. Increased shoot water potential and higher rates of gas exchange due to AM mycorrhization were also observed in squash (*Cucurbita*), soybean (*Glycine max*) and maize (*Zea mays*) (Subramanian *et al.*, 1997; Porcel & Ruiz-Lozano, 2004; Augé *et al.*, 2008). Leaf water potential was stable despite increased transpiration rate in *Citrus jambhiri* (Levy & Krikun, 1980). This indicated that water supply to photosynthetic tissues of mycorrhizal plants enabled sufficient stomatal opening for gas exchange to meet the carbon needs of both symbionts.

In the composite model of root water transport, L_{pr} is a function of apoplastic and cell-to-cell (transmembrane and symplastic) pathways (Steudle & Peterson, 1998). Water flow follows the least resistance pathway and this resistance is controlled largely by the transmembrane pathway through the transcriptional and post-translational regulation of *PIP*s (Törnroth-Horsefield *et al.*, 2006; Maurel *et al.*, 2008). These changes can be determined by the direct measurements of L_{pc} in root cortical cells (Steudle, 1993; Javot & Maurel, 2002). In our study, mycorrhization increased L_{pr} and L_{pc} by a similar magnitude in *P. glauca* seedlings, suggesting that the decreased resistance of the transmembrane pathway was likely responsible for the increased root water transport capacity. Similar enhancements of L_{pr} and L_{pc} were previously reported for mycorrhizal plants and may involve both apoplastic and cell-to-cell pathways (Muhsin & Zwiazek, 2002a,b; Marjanović & Nehls, 2008; Lee *et al.*, 2010; Bárzana *et al.*, 2012).

Similar to the differences in *L. bicolor* aquaporin expression observed in WT- and mock-inoculated seedlings, differences in expression of some *P. glauca* aquaporins were observed between WT- and mock-inoculated seedlings. Although expression profiles of *L. bicolor* aquaporins were not significantly different between OE1 and OE2 mycorrhizal strains, greater differences

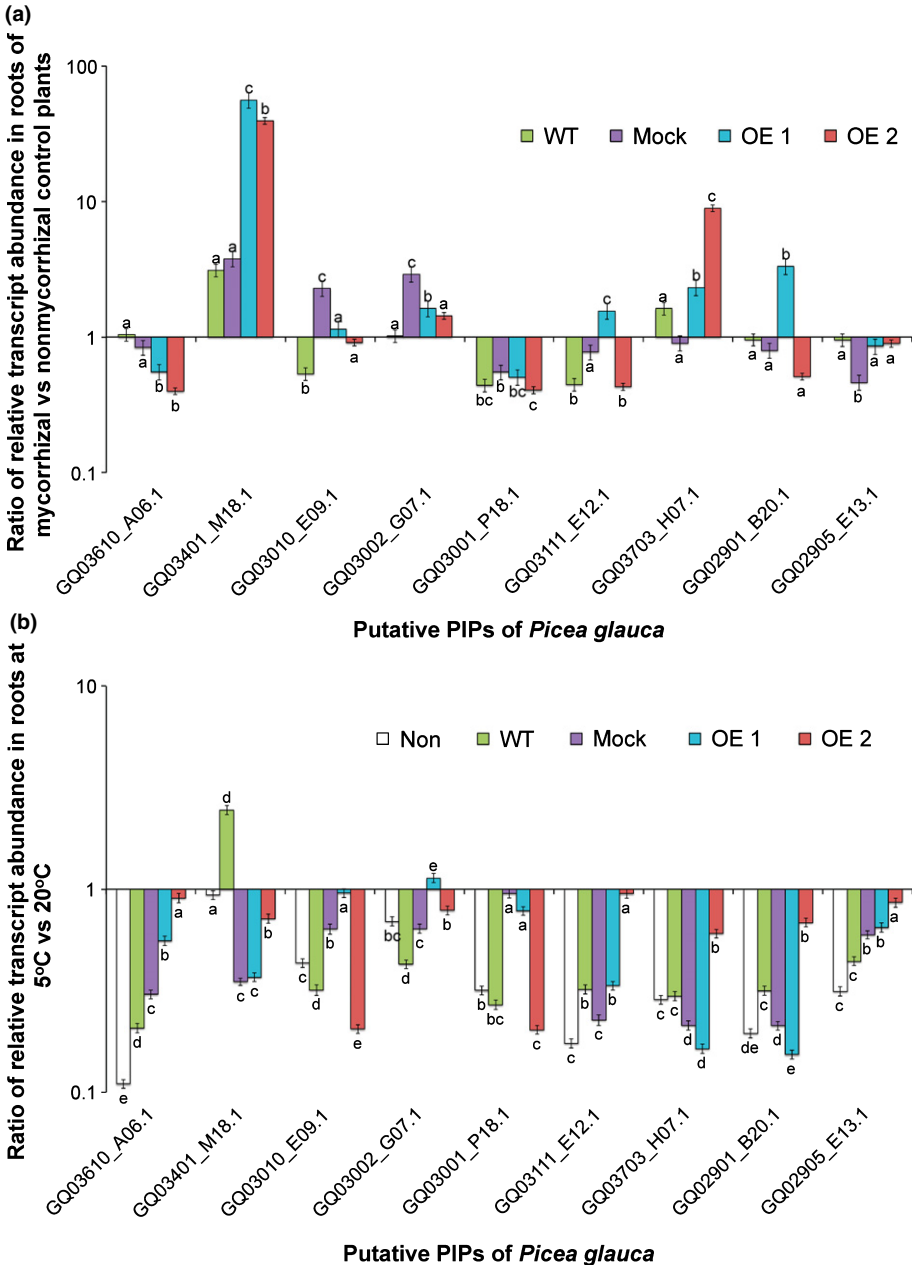

Fig. 7 Changes in transcript abundance of nine putative *PIP* genes in *Picea glauca* nonmycorrhizal (Non) root tips and in root tips mycorrhized with the *Laccaria bicolor* wild-type (WT), mock (Mock), and two overexpression strains (OE1 and OE2) due to (a) mycorrhizal inoculation and (b) temperature decrease from 20 to 5°C. Relative transcript abundance was measured for three biological replicates in SYBR-Green qPCR assay using a standard curve method of comparative quantification with *PgCDC2* as the reference gene. Fold change is displayed on the log scale. Different letters indicate significant differences at $P \leq 0.05$ determined with ANOVA, Tukey's test ($n = 3 \pm$ SE).

were observed between expression profiles of some *P. glauca* aquaporins in OE1- and OE2-inoculated root tips. One possible explanation for these observations is that there may have been differences in fungal–plant dynamics between plants inoculated with OE1 vs OE2. Future studies should include longer-term low temperature treatments and examine root tissue distribution of PIPs in response to temperature to explain the reasons why the expression of some root *PIPs* was not always consistent between the plants inoculated with different strains.

GQ03401_M18.1 was annotated as *PIP1;1* in a recent study of the *P. glauca* aquaporin gene family (Laur & Hacke, 2014). The increase in its transcript abundance upon mycorrhization, particularly with OE strains, was accompanied by increased L_{pc} and L_{pr} of mycorrhizal *P. glauca*. *PttPIP1;1* and *PttPIP2;5* transcript abundance were proposed to be the principal factors responsible for the increase in L_{pr} of ectomycorrhizal *Populus tremula* × *tremuloides* (Marjanović *et al.*, 2005). However, the signaling pathways leading to this response are not known. Regulation of aquaporin-mediated water transport involves changes in the abundance of aquaporins in cell membranes and aquaporin gating, which is affected by various factors including protein phosphorylation and dephosphorylation (Johansson *et al.*, 1998; Kline *et al.*, 2010), protonation (Tournaire-Roux *et al.*, 2003; Fischer & Kaldenhoff, 2008), divalent cations (Gerbeau *et al.*, 2002; Verdoucq *et al.*, 2008), trafficking (Prak *et al.*, 2008; Maurel *et al.*, 2009; Zelazny *et al.*, 2009), heteromerization (Fetter *et al.*, 2004), as well as turgor pressure, solute gradients and temperature (Chaumont *et al.*, 2005). Increased water availability in root extracellular space was postulated to be a significant factor triggering *PIP* transcriptional and post-translational regulation in

root cells (Steudle & Peterson, 1998; Javot & Maurel, 2002). Because the water transporting capacity of mycorrhizal roots increases with the increasing volume of fungal hyphae (Duddridge *et al.*, 1980; Plamboeck *et al.*, 2007), it is plausible that an increase in root hydration by the fungal hyphae may provide a positive feedback mechanism regulating root aquaporin expression and/or function. Additional evidence in support of this hypothesis is provided by the increases at 20°C in L_{pr} and L_{pc} and in *P. glauca PIP GQ03401_M18.1* root expression in OE-inoculated plants compared with the plants mycorrhized with WT and mock strains (Fig. 7a). The expected outcome of *JQ585595* fungal aquaporin expression was an increase in the hyphal water flow, likely leading to an increase in hydration at the hyphal–root interphase.

Interestingly, the stimulating effects of the OE mycorrhizas on L_{pr} and L_{pc} were totally abolished at low temperatures. Although low soil temperature inhibits root water uptake in most plants, including many boreal tree species (Wan *et al.*, 1999, 2001; Lee *et al.*, 2005, 2012; Aroca *et al.*, 2012), low temperature-tolerant plants, including *P. glauca*, show little responsiveness of root hydraulic properties to low temperature (Landhäusser *et al.*, 2002). In our study, when temperature was decreased to 10°C, L_{pr} and L_{pc} were little affected in the nonmycorrhizal and WT-mycorrhizal seedlings. Similar tolerance of L_{pc} to low temperature was reported for chilling-tolerant figleaf gourd, contrary to chilling-sensitive cucumber (Lee *et al.*, 2005). The responses of L_{pc} to low temperature have been explained by the aquaporin gene expression and inhibition of aquaporin phosphorylation and/or dephosphorylation (Lee *et al.*, 2005, 2012). In our study, the L_{pc} in nonmycorrhizal and WT-inoculated plants was not affected despite the reductions in the expression levels of the examined *PIP*s, pointing to possible gating processes in *P. glauca* being responsible for low temperature tolerance of transmembrane water transport as in figleaf gourd (Lee *et al.*, 2005) and rice (Matsumoto *et al.*, 2009). The overexpression of fungal aquaporin *JQ585595* increased the sensitivity of root water transport to low temperature. Because the effect of low root temperature on root hydraulic properties was accompanied by inconsistent differences in root *PIP* expression compared with the WT and mock lines (Fig. 7b), it is possible that the overexpression of *JQ585595* could have affected the root aquaporin gating processes, as previously reported for chilling-sensitive plants (Aroca *et al.*, 2005; Lee *et al.*, 2005, 2012; Murai-Hatano *et al.*, 2008). It is also worth noting that contrary to root *P. glauca PIP*s, most of the *L. bicolor* aquaporins exhibited increased transcript abundance when subjected to low temperatures. It can be speculated that the functionality of aquaporin-mediated transport is important to hyphal water transport, and its protection under unfavorable environmental conditions is among the priorities for the fungus.

Conclusions

Our study has demonstrated the enhancement of L_{pc} and L_{pr} in *P. glauca* roots mycorrhized with *L. bicolor* overexpressing aquaporin *JQ585595*. We propose that the contribution of *L. bicolor* hyphae to root water transport in *P. glauca* involves increased apoplastic water transport in the root intercellular spaces, which may lead to increased hydration at the fungal–root interface and, consequently, impact aquaporin expression and cell-to-cell water transport in mycorrhizal roots. During chilling, *PIP* post-translational regulation may influence L_{pc} in *P. glauca* roots mycorrhized with *L. bicolor* strains overexpressing *JQ585595*, as increased fungal aquaporin transcription may alter hydration in the root intercellular spaces and, consequently, affect root *PIP* regulation and root hydraulic dynamics.

Acknowledgements

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Supporting Information

Additional supporting information may be found in the online version of this article.

Fig. S1 Ectomycorrhizal association between *Picea glauca* and *Laccaria bicolor* was induced.

Fig. S2 Aquaporins of *Laccaria bicolor* UAMH8232 in the phylogenetic tree of 229 fungal major intrinsic proteins (MIPs) from 88 fungal species representing four phyla.

Fig. S3 Phylogenetic analysis of putative *Picea glauca* MIPs using 36 MIPs of *Arabidopsis thaliana* and 57 MIPs of *Populus* as reference proteins.

Fig. S4 Relative transcript abundance of nine *Picea glauca PIPs* in nonmycorrhizal root tips at 20°C.

Fig. S5 Gel electrophoresis of TAIL-PCR products to amplify the part of the T-DNA right border and its flanking sequence from the genome of *Laccaria bicolor* transgenic strains.

Table S1 Polymerase chain reaction primers used in this study

Table S2 TAIL-PCR conditions

Notes S1 Additional materials and methods.

Notes S2 Deduced amino acid sequences used in phylogenetic analysis on plant MIPs and fungal MIPs.

Notes S3 Alignment of aquaporin amino acid sequences of *Laccaria bicolor* strain UAMH8232 with their counterpart aquaporins of strain S238N.

Notes S4 *In silico* protein secondary structure prediction on deduced amino acids of putative *PIP*s of *Picea glauca* analyzed in this study.

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