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ORIGINAL ARTICLE

Hydraulic conductivity and aquaporin transcription in roots of trembling aspen (*Populus tremuloides*) seedlings colonized by *Laccaria bicolor*

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Abstract Ectomycorrhizal fungi have been reported to increase root hydraulic conductivity (L_{pr}) by altering apoplastic and plasma membrane intrinsic protein (PIP)-mediated cell-to-cell water transport pathways in associated roots, or to have little effect on root water transport, depending on the interacting species and imposed stresses. In this study, we investigated the water transport properties and *PIP* transcription in roots of aspen (*Populus tremuloides*) seedlings colonized by the wild-type strain of *Laccaria bicolor* and by strains overexpressing a major fungal water-transporting aquaporin *JQ585595*. Inoculation of aspen seedlings with *L. bicolor* resulted in about 30 % colonization rate of root tips, which developed dense mantle and the Hartig net that was restricted in the modified root epidermis. Transcript abundance of the aspen aquaporins *PIP1;2*, *PIP2;1*, and *PIP2;2* decreased in colonized root tips. Root colonization by *JQ585595*-overexpressing strains had no significant impact on seedling shoot water potentials, gas exchange, or dry mass; however, it led to further decrease in transcript abundance of *PIP1;2* and *PIP2;3* and the significantly lower L_{pr} than in non-inoculated roots. These results, taken together with our previous study that showed enhanced root water hydraulics of *L. bicolor*-colonized white spruce (*Picea glauca*), suggest that the impact of *L. bicolor* on root hydraulics varies by the ectomycorrhiza-associated tree species.

Keywords Aquaporins · Ectomycorrhiza · Plasma membrane intrinsic proteins · Root water transport

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Introduction

Symbiotic ectomycorrhizal associations are commonly formed between roots of woody plants and certain species of basidiomycete and ascomycete fungi (Kottke and Oberwinkler 1986). Hundreds of ectomycorrhizal fungal species have been found in the upper layers of the soil in boreal and temperate forests interacting with trees of the Pinaceae, Fagaceae, Dipterocarpaceae, and Caesalpinoidaceae families (Marjanović and Nehls 2008; Smith and Read 2008), where they function as a crucial component of forest ecosystems. Ectomycorrhizal fungi colonize plant roots by typically forming a mantle (hyphal sheath) that envelops the root tips, in conjunction with a Hartig net comprising hyphae in the intercellular space surrounding the epidermal and outer cortical cells (Smith and Read 2008). The nutrients and water can be transported through different exploration types of hyphae within the extensive hyphal network of extraradical fungal mycelium, which provides the roots of the plant hosts with improved mineral nutrition and water relations (Agerer 2001; Lehto and Zwiazek 2011). In return, the plant host supplies the fungal partners with carbohydrates (Martin and Nehls 2009). However, the degree of mutual benefit depends on the interacting species and their growth, and the developmental stages of the interaction, as well as environmental stresses

imposed on the plant and fungal partners (Boyle and Hellenbrand 1991; Smith and Read 2008).

Root tips are a determinant of root water permeability (Steudle and Peterson 1998). Ectomycorrhizal fungal colonization of root tips, therefore, may significantly impact root water uptake by altering both apoplastic and cell-to-cell pathways (Smith and Read 2008; Lehto and Zwiazek 2011). The nature by which the fungal partner impacts the apoplastic pathway largely depends on the affinity of fungal cell walls for water. Cell walls of fungi in the mantle that have low water affinity (hydrophobic) may block the root apoplastic water pathway and hinder root water uptake (Duddridge et al. 1980; Unestam and Sun 1995). In contrast, fungal cell walls that have higher affinity for water (hydrophilic) offer relatively little resistance to water flow. Therefore, in this latter case, the cell wall space is likely to be the predominant pathway for water transport to the root cortex of mycorrhizal plants (Weatherley 1982; Agerer 2001; Lehto and Zwiazek 2011). Given the improved rhizosphere hydration and water delivery to roots (Querejeta et al. 2003, 2007; Egerton-Warburton et al. 2008), fungal hyphae have been proposed to serve as a water transport highway from soil to roots (Khalvati et al. 2005; Allen 2007; Egerton-Warburton et al. 2007; Lehto and Zwiazek 2011). Although the extent of water transport through the cell-to-cell pathway within the mycorrhizal fungus has not been well studied in different mycorrhizal associations, current evidence suggests that the entry and exit of water in the hyphal cells are largely regulated by aquaporins, major intrinsic proteins (MIPs) that facilitate transmembrane transport of water, and that fungal aquaporins play important roles in mycorrhizal root water transport (Uehlein et al. 2007; Aroca et al. 2009; Ruiz-Lozano et al. 2009; Dietz et al. 2011; Hacquard et al. 2013; Li et al. 2013; Navarro-Ródenas et al. 2013; Nehls and Dietz 2014; Xu et al. 2015). For example, in roots of *Picea glauca* seedlings colonized with *Laccaria bicolor*, the fungal aquaporin *JQ585595* had a significant impact on root hydraulics (Xu et al. 2015). This study demonstrated that use of *Laccaria bicolor* transgenic strains mis-expressing functionally characterized fungal aquaporins (Dietz et al. 2011; Xu et al. 2015) provides exceptional opportunities to study water transport properties in ectomycorrhizal associations.

In the composite model of root water transport, root hydraulic conductivity (L_{pr}) is a function of apoplastic and cell-to-cell (transmembrane and symplastic) pathways (Steudle and Peterson 1998). Mycorrhizal effects on root water transport may involve both root apoplastic pathway (Nylund 1987; Muhsin and Zwiazek 2002a; Bárzana et al. 2012) and cell-to-cell water transport. The latter is mediated by plant aquaporins, especially by plasma membrane intrinsic proteins (PIPs) and tonoplast membrane intrinsic protein (TIPs) (Marjanović et al. 2005; Ruiz-Lozano et al. 2009; Navarro-Ródenas et al. 2013; Xu et al. 2015), which are the determinants of the transmembrane water transport in roots (Javot and Maurel 2002; Aroca et al. 2012). Ectomycorrhizas often result in increased L_{pr} in tree species (Landhäusser et al. 2002; Muhsin and Zwiazek 2002a, b; Marjanović et al. 2005; Lee et al. 2010; Xu et al. 2015), as changes in ectomycorrhizal root anatomy and internal surfaces can substantially alter the properties of the root apoplastic pathway (Muhsin and Zwiazek 2002a). From the perspective of cell-to-cell root water transport, enhanced L_{pr} of mycorrhizal plants has generally coincided with the increase in root cortical cell hydraulic conductivity L_{pc} (Lee et al. 2010; Xu et al. 2015) and in root *PIP* expression (Marjanović et al. 2005; Uehlein et al. 2007; Ruiz-Lozano et al. 2009; Xu et al. 2015), presumably triggered by the increased hydration at the fungal–root interface. However, no effects of ectomycorrhizas on root hydraulic properties have also been frequently reported (Coleman et al. 1990; Nardini et al. 2000; Calvo-Polanco et al. 2008; Siemens and Zwiazek 2008; Yi et al. 2008). In addition, ectomycorrhizal effects on the expression of root aquaporins vary by specific aquaporins, interacting ectomycorrhizal species and presence of environmental stresses (Marjanović et al. 2005; Navarro-Ródenas et al. 2013; Xu et al. 2015). The discrepancies between these findings, which may be caused by differences in environmental factors and plant–fungal partnership (Calvo-Polanco et al. 2008; Siemens and Zwiazek 2008; Calvo-Polanco et al. 2009), are not clearly understood.

Sharing the well-characterized and considerably conserved MIP family in *Populus* spp. (Secchi et al. 2009; Secchi and Zwieniecki 2010; Lopez et al. 2012; Cohen et al. 2013), trembling aspen (*Populus tremuloides*, aspen) has been frequently used to study water transport in trees (Wan and Zwiazek 1999; Wan et al. 1999; Wan et al. 2001; Kamaluddin and Zwiazek 2002; Landhäusser et al. 2002; Siemens and Zwiazek 2004; Liu et al. 2014). Water relations of aspen and their hybrids may be altered by ectomycorrhizal fungi such as *Laccaria bicolor*, *Amanita muscaria*, *Hebeloma crustuliniforme*, and *Leccinum aurantiacum* (Cripps and Miller 1993; Landhäusser et al. 2002; Marjanović et al. 2005; Dietz et al. 2011). For example, an increased L_{pr} has been observed in aspen mycorrhized with *Leccinum aurantiacum* (Landhäusser et al. 2002) and in hybrid aspen *Populus tremula* × *Populus tremuloides* mycorrhized with *Amanita muscaria* (Marjanović et al. 2005). However, the results of our preliminary experiments showed no effect of root colonization with *Laccaria bicolor* on root water flow dynamics in trembling aspen. Since the inconsistent effects of mycorrhization on water relations have been frequently reported and the reasons for such inconsistency are little understood, we followed up with a detailed study to shed more light on these processes in mycorrhizal trembling aspen. The main objectives of the present study were to examine (i) the effects of mycorrhization on root *PIP* transcript abundance in ectomycorrhizal trembling aspen plants which do not show a positive L_{pr} response to fungal colonization, (ii) the contribution of hyphal water transport to L_{pr} in

these plants by inoculating them with the aquaporin-overexpressing fungal strains, and (iii) the effects of low temperature, one of the common limiting factors for root water transport, on L_{pr} and root *PIP* expression. Since increased hydration at the root–hyphal interface has been proposed to be the likely primary factor leading to increased aquaporin expression in ectomycorrhizal roots (Xu et al. 2015), we tested the hypothesis that aquaporin overexpression in the mycorrhizal fungus would induce root *PIP* expression in the ectomycorrhizal plants in which mycorrhization with the wild-type strain does not affect L_{pr}.

Materials and methods

Preparation of plant and fungal materials

Populus tremuloides Michx. (trembling aspen, aspen) seeds, which were collected in Juniper, NB, Canada, were provided by the National Tree Seed Centre, Canadian Forest Service, Fredericton, NB, Canada. The seeds were surface-sterilized with 1 % (*v*/*v*) sodium hypochlorite and germinated on the surface of autoclaved peat moss: vermiculite (2:1) in sterilized 1-L pots covered with plastic domes. Seedlings were grown in a 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. The seedlings were fertilized with 25 % modified Hoagland's solution (Epstein 1972) weekly in the first 2 weeks and biweekly afterward.

The mycelial fragments of *Laccaria bicolor* (Maire) P. D. Orton dikaryotic wild-type fungus UAMH8232 (University of Alberta Microfungus Collection) and its genetically modified strains were grown in liquid modified Melin–Norkans (MMN) medium (Marx 1969; Pham et al. 2004) at 20 °C, 0.8×*g* for 4 weeks. For root inoculation, homogenized inocula of the wild type, two transgenic strains each overexpressing *JQ585595* under control of the constitutive *Agaricus bisporus gpd*II promoter (designated OE1 and OE2), and a mock strain expressing the empty vector pHg/pSILBAγ were prepared as previously described (Xu et al. 2015). Homogenized liquid inoculum (10 mL) containing one of the four strains was added to autoclaved potting mix (peat moss/vermiculite, 2:1 by volume) in each pot before planting the seeds. The second inoculation was conducted 1 month later by injecting 10 mL of the respective inoculum into the potting mix around the rhizosphere. Autoclaved fungal-free liquid MMN was used to treat non-inoculated control seedlings. Eighteen plants were maintained for each of the five inoculation treatments for 2 months. The plants were repositioned every 3 days to minimize the impact of non-uniform conditions within the growth room.

Mycorrhizal colonization was determined by microscopic examination of 30 randomly sampled root tips from five seedlings in each mycorrhizal treatment ($n = 5$, six root tips per seedling) (Brundrett et al. 1996; Peterson et al. 2004). The root segments for microscopy were fixed in formalin-acetic acid-alcohol (FAA). Fixed root tips were embedded in paraffin and sectioned at 5 μm using a microtome (model RM2125 RTS, Leica; Solms, Germany). Thin sections were stained with toluidine blue and photographed with the MacroFire Digital Camera (Optronics; Goleta, CA, USA) under ZEISS AXIO compound light microscope (Carl Zeiss; Jena, Germany). The root colonization rate for each examined seedling was calculated as the percentage of root tips with distinct ectomycorrhizal structures out of the total number of root tips that were examined.

Seedling gas exchange, shoot water potential, and dry mass

Three-month-old aspen seedlings with stem heights of 76–91 cm and leaf area of 670–730 cm^2 were measured for net photosynthetic (P_n) and transpiration (E) rates. P_n and E of the first fully expanded leaves were measured between 09:00 and 12:00 using a Licor-6400 infrared gas analyzer equipped with the 2×3 cm^2 red-blue light chamber (LI-COR, Lincoln, NB, USA). Transient water use efficiency (*WUE*) was calculated by dividing P_n by E. Measurements were carried out in six plants from each inoculation treatment ($n = 6$). Distal shoots of 10–15 cm in length were excised at noon and immediately placed into a Scholander pressure chamber (PMS instruments; Corvallis, OR, USA) for mid-day shoot water potential (Ψ_{shoot}) measurements (Scholander et al. 1965; Wan et al. 1999) ($n = 6$). Dry mass was determined after oven drying at 80 °C for 48 h ($n = 6$).

Root hydraulic conductivity

A high-pressure flow meter (HPFM) was used to determine whole-root hydraulic conductivity (L_{pr}) immediately after Ψ_{shoot} measurements (Landhäusser et al. 2002). The root with potting mix was removed from the pot and kept in a plastic bag submerged in a circulating water bath (Thermo Scientific; Hampton, NH, USA). Root hydraulic conductance (K_r) was measured after temperature treatment for 1 h at 20 or 5 °C, respectively (Xu et al. 2015). Root volumes were determined using the water displacement method after the potting mix was gently washed off from the roots immediately following K_r measurements. L_{pr} was calculated by dividing K_r by the root volume (Kamaluddin and Zwiazek 2002).

Transcript abundance of root PIPs

The relative transcript abundance was analyzed for seven PIP genes first characterized from roots of the hybrid aspen, *Populus tremula* × *Populus tremuloides* (Marjanović et al. 2005), and recently examined in *Populus tremuloides* (Liu et al. 2014): *PtrePIP1;1* (NCBI accession number *AJ849323*), *PtrePIP1;2* (*AJ849322*), *PtrePIP2;1* (*AJ849324*), *PtrePIP2;2* (*AJ849325*), *PtrePIP2;3* (*AJ849326*), *PtrePIP2;4* (*AJ849327*), and

PtrePIP2;5 (*AJ849328*). Root tip segments of about 1 cm in length were collected from 3-month-old aspen seedlings after a 1-h treatment in the water bath at 20 or 5 °C. From the inoculated plants, only the mycorrhizal root tips were collected. Root tip samples were flash frozen in liquid nitrogen and stored at −80 °C, until they were ground in liquid nitrogen with mortar and pestle prior to total RNA extraction using the RNeasy Plant Mini kit (Qiagen, Valencia, CA, USA), with the addition of 20 mg of polyethylene glycol 8000 mL^{-1} RLT buffer to facilitate extraction of good-quality RNA. First-strand complementary DNA (cDNA) synthesis and qRT-PCR were conducted as described (Xu et al. 2015). The transcript abundance of target *PtrePIP*s was normalized against that of the reference gene *PtreJIP1*, corresponding to the *Populus tremula* × *Populus tremuloides* gene *PttJIP1* (*AJ407583*) (Grunze et al. 2004; Liu et al. 2014). The C_t value for *PtreJIP1* did not change significantly across all tested samples of inoculated and non-inoculated root tips at 20 and 5 °C ($P \approx 0.96$). Relative transcript abundance of *PtrePIP*s was calculated using the $\Delta\Delta C_t$ method of comparative quantification (Livak and Schmittgen 2001; Pfaffl 2004). 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 −3.25 and −2.86, corresponding to the range of the efficiencies between 103.2 and 123.7 %. The standard curve efficiencies were used in the efficiency correction of $\Delta\Delta C_t$ values. To assess the impact of mycorrhization on transcript abundance of these *PtrePIP*s, the cDNA samples of non-inoculated roots harvested at 20 °C were used as calibrator for ratio calculation. To assess the impact of 5 °C temperature on transcript abundance of the *PtrePIP*s, the corresponding samples at 20 °C were used as calibrator.

Statistical analysis

Descriptive statistics and ANOVA were conducted using Origin 8.0 (OriginLab, Northampton, MA, USA). Tukey's test was used to compare means for statistically significant differences ($P \leq 0.05$). One-way ANOVA was conducted to analyze the impacts of mycorrhiza on seedling gas exchange, Ψ_{shoot}, and dry mass, and impacts of mycorrhiza or temperature on root *PIP* transcript abundance. Two-way ANOVA was conducted to analyze the impacts of mycorrhiza, temperature, and their interaction on L_{pr}.

Results

Root colonization and root structure

Mantle and Hartig net structures were found in 33.3 ± 5.3 % (mean ± SE) of the seedlings inoculated with wild-type strain (Fig. 1a), and 30 ± 6.2 %, 33.3 ± 7.5 %, and 26.7 ± 4.1 % for mock, OE1, and OE2 strains, respectively. Neither difference in colonization rate was statistically significant between the strains, nor were differences in their ectomycorrhizal structures noticeable. While large mass of hyphae grew around the epidermal layer and formed a thick and dense mantle, Hartig net development was restricted to the intercellular spaces of the epidermis and the outermost cortex layer. The epidermis was largely modified and partially replaced by the mantle and Hartig net, which were often indistinguishable (Fig. 1a–d). No hyphal structures or structure alterations were observed in the root tips of non-inoculated seedlings (Fig. 1e–f).

Effects of ectomycorrhizas on shoot water potentials, gas exchange, seedling dry mass, and root hydraulic conductivity

Mycorrhizal inoculation had no significant effect on Ψ_{shoot} (Fig. 2a). There was also relatively little effect of mycorrhization on P_n and E (Fig. 2b, c). However, the small increase in P_n of OE1-inoculated aspen compared with non-inoculated and mock-inoculated seedlings was statistically significant (Fig. 2b). Mycorrhizal inoculation had no significant effect on *WUE* (Fig. 2d), total seedling dry mass (Fig. 2e), root/shoot dry mass ratios (Fig. 2f), or seedling height or leaf area (data not shown).

Although there was no effect of seedling inoculation with wild-type and mock strains on L_{pr} (Fig. 3), inoculation with both OE strains significantly reduced L_{pr} in seedlings ($P \approx 0.009$ for OE1 and 0.013 for OE2). There was neither significant temperature effect in any of the inoculation treatments ($P \approx 0.27$), nor significant effect of the interaction between mycorrhizal and temperature treatments ($P \approx 0.97$).

Effects of ectomycorrhizas on *PtrePIP* transcript abundance in root tips

Of the seven *PtrePIP*s that were profiled in root tips of *Populus tremuloides*, the transcript abundance of *PtrePIP2;4* was the highest in non-inoculated root tips, followed by transcript abundance corresponding to *PtrePIP1;2*, *PtrePIP1;1*, and *PtrePIP2;5*. In contrast, the transcript abundance corresponding to *PtrePIP2;1*, *PtrePIP2;2*, and *PtrePIP2;3* was relatively low (Fig. 4a).

Transcript profiling in root tips revealed a general downregulation of *PtrePIP*s due to mycorrhization with all the strains. This downregulation was significant for *PtrePIP1;2*, *PtrePIP2;1*, and *PtrePIP2;2* in root tips, with the changes in *PtrePIP1;2* and *PtrePIP2;2* being the most pronounced (Fig. 4b). Mycorrhization with mock and the two OE strains also was associated with significant downregulation of *PtrePIP2;3* and *PtrePIP2;4* (Fig. 4b). The decrease in transcript abundance of *PtrePIP1;2* and *PtrePIP2;3* was more pronounced in the two OE

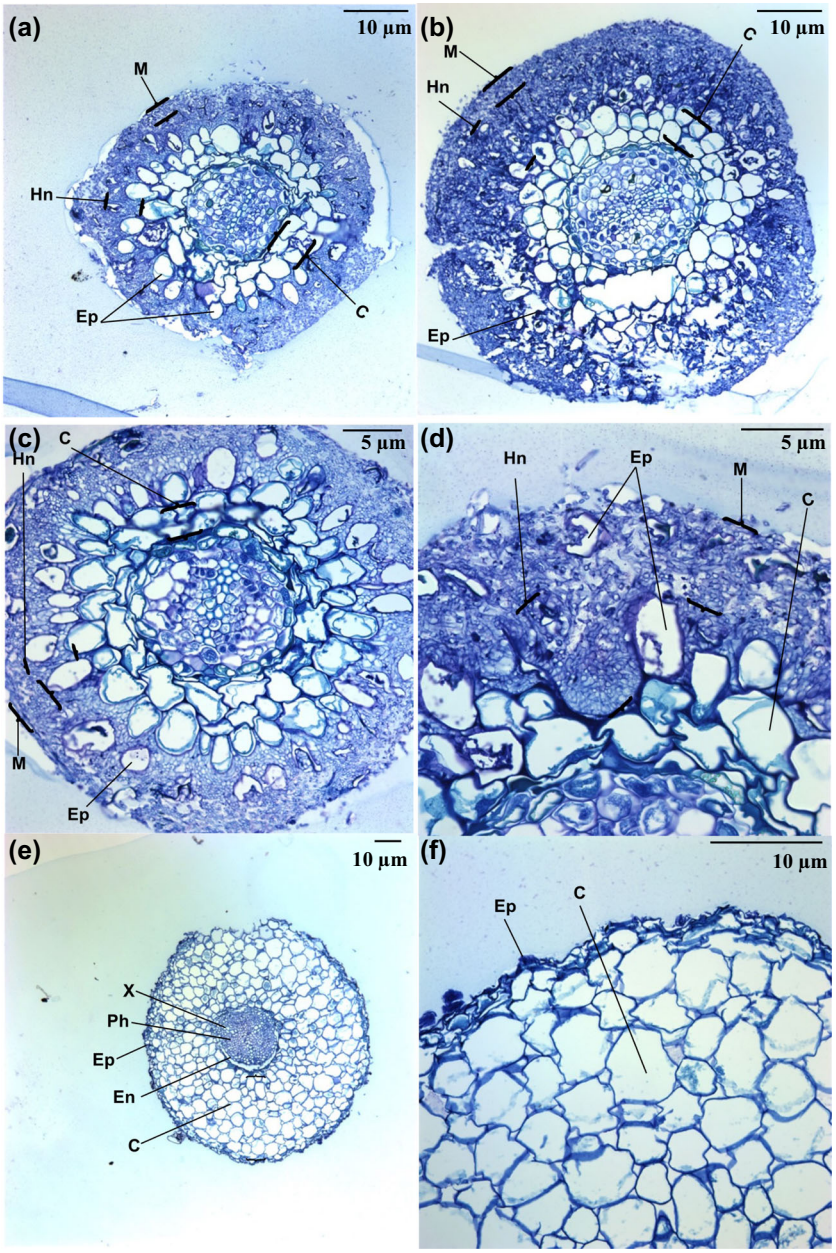


Fig. 1 Cross sections of mycorrhizal and non-mycorrhizal root tips of *Populus tremuloides* seedlings. Roots were inoculated with *Laccaria bicolor* strains: **a** wild-type strain, **b** mock strain, two *JQ585595* overexpression strains **c** OE1 and **d** OE2, or **e**, **f** non-inoculated. Sections were stained with toluidine blue. Images are representative of 30 sectioned root tips. Ep, epidermis; C, cortex; En, endodermis; Ph, phloem; X, xylem; M, mantle; Hn, Hartig net

strains (Fig. 4b). In contrast, transcript abundance for *PtrePIP1;1* or *PtrePIP2;5* was altered to only a limited extent by mycorrhization, with the only exception being a small decrease in *PtrePIP1;1* in OE2-inoculated root tips (Fig. 4b).

Decreasing root temperature from 20 to 5 °C significantly increased transcript abundance of *PtrePIP1;1*, *PtrePIP1;2*, *PtrePIP2;1*, and *PtrePIP2;4* in root tips inoculated with wild-type, mock, and at least one of the two OE strains (Fig. 4c). The temperature decrease also caused a decrease in transcript abundance corresponding to *PtrePIP2;2*, *PtrePIP2;3*, and *PtrePIP2;5* in non-inoculated root tips, but there was no change in transcript abundance of other *PtrePIP*s (Fig. 4c). The transcript abundance of *PtrePIP2;2* and *PtrePIP2;3* was also affected by the temperature decrease in all inoculation treatments, but relatively less compared with non-inoculated control (Fig. 4c). The effect of temperature drop on transcript abundance of *PtrePIP*s was not significantly different between the mycorrhization with the four strains, with the only exception of a slightly greater decrease in *PtrePIP2;3* in OE1 compared with wild type and mock (Fig. 4c).

Of all examined *PtrePIP*s, the relative transcript abundance of *PtrePIP1;2* and *PtrePIP2;2* at 5 °C was the most affected by inoculation treatments (Fig. 4d). In plants inoculated with the OE strains, the transcript abundance corresponding to *PtrePIP1;1*, *PtrePIP1;2*, *PtrePIP2;3*, and *PtrePIP2;4* at 5 °C was significantly lower compared with the other inoculation treatments (Fig. 4d).

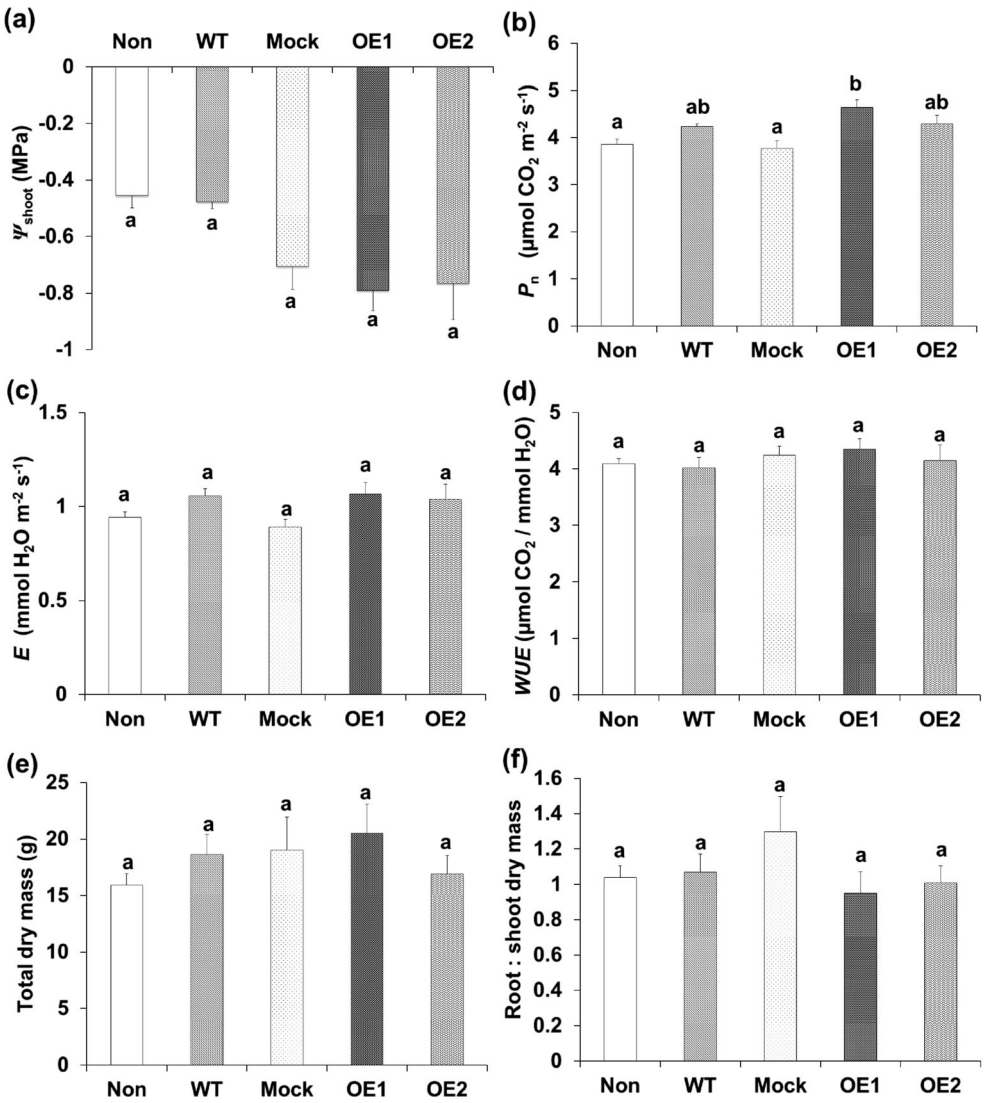
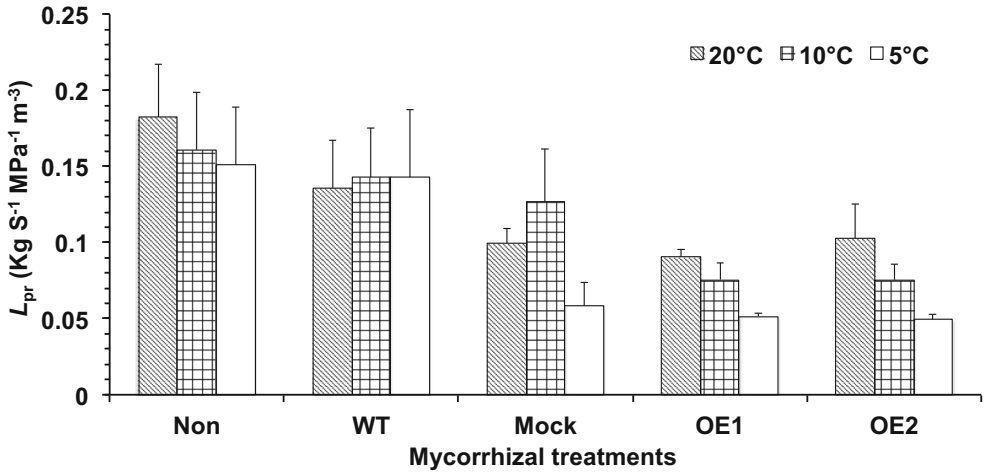


Fig. 2 The effects of mycorrhization with *Laccaria bicolor* on **a** mid-day shoot water potential Ψ_{shoot}, **b** net photosynthetic rate P_n, **c** transpiration rate E, **d** transient water use efficiency *WUE*, **e** total dry mass, and **f** root/shoot dry mass ratio of *Populus tremuloides* seedlings. The treatments were inoculation with *Laccaria bicolor* wild-type strain (WT), mock strain (Mock), two *JQ585595* overexpression strains (OE1 and OE2), and non-inoculated (Non). Means (n = 6) ± SE are shown. Different letters indicate significant difference at $P \leq 0.05$ (ANOVA, Tukey's test)

Discussion

Mycorrhization is commonly reported to result in positive effects on plant growth and is generally attributed to improved mineral nutrition and water uptake (Smith and Read 2008). Inoculation with ectomycorrhizal fungi also frequently improves plant water relations and increases root hydraulic conductivity (Landhäusser et al. 2002; Muhsin and Zwiazek 2002a, b; Marjanović et al. 2005; Lee et al. 2010; Xu et al. 2015). The increase in L_{pr} may be attributed to enhanced apoplastic pathway in ectomycorrhizal roots, which can be examined using apoplastic tracer dyes (Siemens and Zwiazek 2003; Bárzana et al. 2012) or inhibitors of aquaporin activity (Muhsin and Zwiazek 2002a; Siemens and Zwiazek 2004; Bárzana et al. 2012), and altered cell-to-cell water transport which can be measured by examining cell hydraulic conductivity L_{pc} (Lee et al. 2010; Lee and Zwiazek 2015; Xu et al. 2015). However, plant growth responses to mycorrhization depend on the balance between the benefits provided by the fungus and the energy costs to the plant (Boyle and Hellenbrand 1991; Jonsson et al. 2001; Kivlin et al. 2013). Therefore, the effects of ECM on plant growth may differ depending on the interacting species, their developmental stages, and various environmental factors (Nguyen et al. 2006; Smith and Read 2008; Kivlin et al. 2013). The colonization rates by *Laccaria bicolor* vary between different *Populus* species (Quoreshi and Khasa 2008; Tschaplinski et al. 2014; Plett et al. 2015). In an earlier study with aspen seedlings, inoculation of roots with *Laccaria bicolor* resulted in only 8–10 % colonization rates and no noticeable effects on seedling growth (Quoreshi and Khasa 2008). Consistent with those findings, in the present study, inoculation of aspen seedlings with *Laccaria bicolor* resulted in a colonization rate of about 30 % and did not significantly affect Ψ_{shoot}, gas exchange parameters, or plant dry mass.

Similarly to the present results, no beneficial effects of ectomycorrhizas on root water flow properties have been



P values of means comparison by Tukey test					
		Non	*WT*	*Mock*	*OE1*
Factor A Mycorrhizal treatments	*WT*	0.88772			
	Mock	0.07705	0.42276		
	OE1	0.00904*	0.09218	0.91675	
	OE2	0.01303*	0.12243	0.95376	0.99993
		5°C	*10°C*		
Factor B Temperatures	*10°C*	0.42691			
	20°C	0.28299	0.95719		
Overall ANOVA	*Factor A*	0.00251*			
	Factor B	0.2717			
	Interaction	0.97082			

Fig. 3 Responses of root hydraulic conductivity (L_{pr}) to 5, 10, and 20 °C root temperatures in *Populus tremuloides* seedlings under mycorrhizal treatments. Roots were sampled from the seedlings inoculated with *Laccaria bicolor* wild-type strain (WT), mock strain (Mock), two *JQ585595* overexpression strains (OE1 and OE2), and non-inoculated (Non). Means (n = 6) ± SE are shown. *P* values with asterisk indicate significant difference at $P \leq 0.05$ by two-way ANOVA, Tukey's test

reported for various forest tree species, including *Pseudotsuga menziesii* (Coleman et al. 1990), *Quercus ilex* (Nardini et al. 2000), *Ulmus americana* (Calvo-Polanco et al. 2008), *Populus balsamifera* (Siemens and Zwiazek 2008), *Populus tremuloides*, and *Betula papyrifera* (Yi et al. 2008). It could be hypothesized that the net effect of a mycorrhizal fungus on root hydraulic properties may vary in different fungal–host systems depending on the balance between the fungal effect on apoplastic and cell-to-cell water transport. However, this notion requires additional experimental support. In this study, the expression of *PtrePIP1;2*, *PtrePIP2;1*, and *PtrePIP2;2* decreased in wild-type and mock-inoculated root tips (Fig. 4b), whereas these two *Laccaria bicolor* strains did not significantly affect L_{pr} (Fig. 3). In a previous study, ectomycorrhizal association with *Amanita muscaria* enhanced L_{pr} in *Populus tremula* × *Populus tremuloides*, which was accompanied by an increased transcript abundance for *PttPIP1;1*, and the water-transporting *PttPIP2;3* and *PttPIP2;5*, (Marjanović et al. 2005). In *Populus tremula* × *Populus tremuloides*, *PttPIP1;1* was the most highly expressed *PIP* in roots, and *PttPIP2;5* was found to encode an aquaporin with the highest water transport capacity in *Xenopus laevis* oocyte swelling assays; hence, the upregulation of these *PIP*s was proposed to be responsible for the enhanced L_{pr} in ectomycorrhizal hybrid aspen (Marjanović et al. 2005). Therefore, it is plausible that, in our study, comparatively stable expression of *PtrePIP1;1* and *PtrePIP2;5* in aspen root tips in response to mycorrhization with *Laccaria bicolor* is among the factors responsible for an overall lack of effect of fungal inoculation on L_{pr}.

In contrast to earlier reports (Wan and Zwiazek 1999; Wan et al. 1999, 2001), the decline of root temperature from 20 to 10 °C and 5 °C did not significantly affect L_{pr} of aspen seedlings of any inoculation types (Fig. 3). These results suggest that root temperature responses may widely vary, even in the same species of trees. Possible genetic influences or impacts of environmental history on root hydraulic responses of trees have received little attention to date. Despite no significant

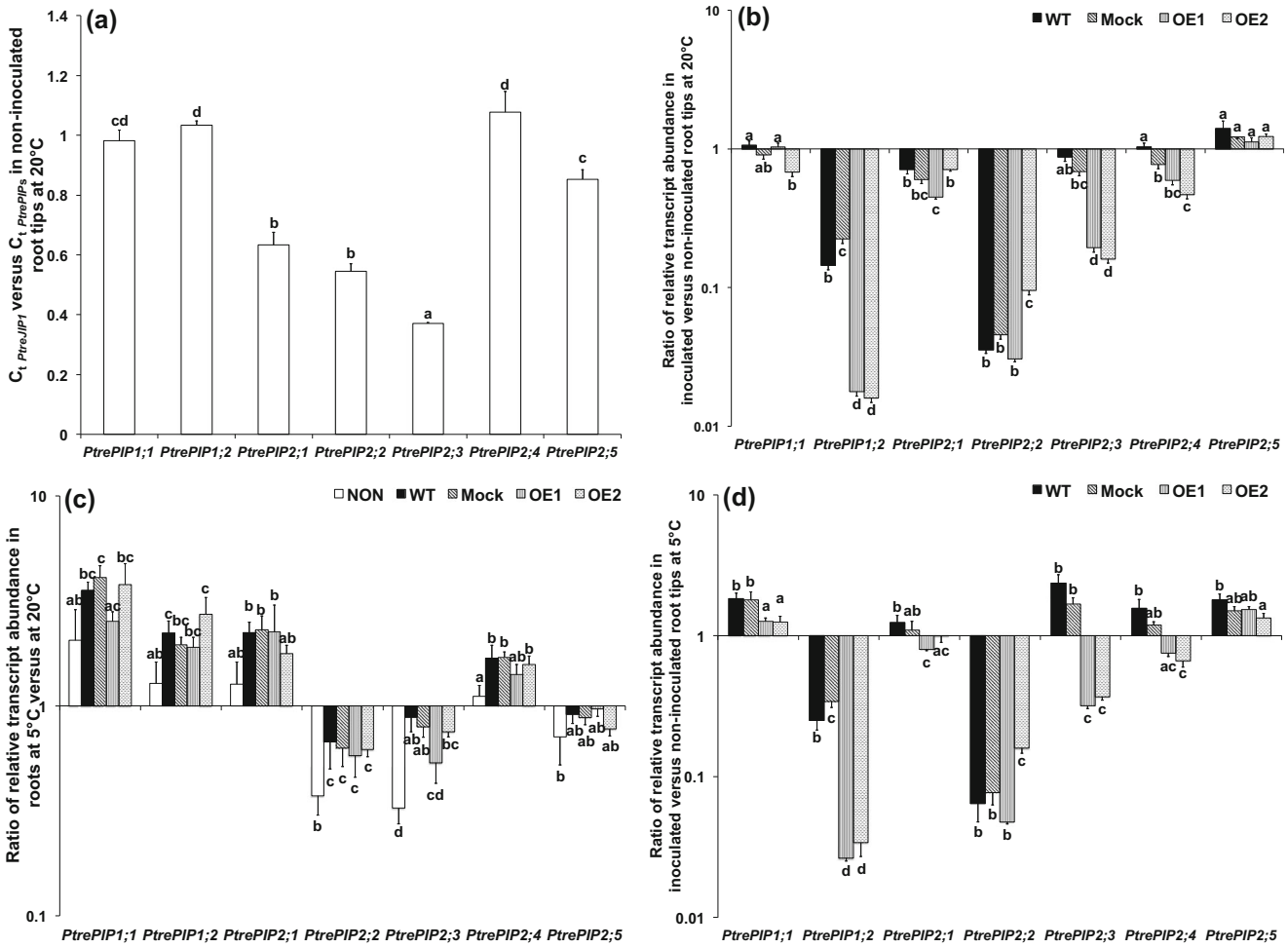


Fig. 4 Relative transcript abundance of seven *PtrePIP* genes in root tips of non-inoculated *Populus tremuloides* seedlings (Non) and in seedlings inoculated with *Laccaria bicolor* wild-type (WT), mock (Mock), and two *JQ585595* overexpression (OE1 and OE2) strains and exposed to different root temperatures. **a** Relative transcript abundance of *PtrePIP* genes in non-inoculated plants at 20 °C root temperature, **b** relative transcript abundance of *PtrePIP* genes following mycorrhizal inoculation, and **c**, **d** relative transcript abundance of *PtrePIP* genes at 5 °C root temperature compared with 20 °C. Relative transcript abundance was measured for three biological replicates in a SYBR-Green qRT-PCR assay using the $\Delta\Delta C_t$ method of comparative quantification with *PtreJIP1* (*AJ407583*) as the reference gene. Fold change is displayed on the log scale. The calibrators used in fold change calculation in (**b**), (**c**), and (**d**) were non-inoculated root tips at 20 °C, root tips of corresponding inoculation treatments at 20 °C, and non-inoculated root tips at 5 °C, respectively. The letter "a" indicates the relative transcript abundance in the samples did not significantly change from the calibrator. Different letters indicate significant differences at $P \leq 0.05$ determined with ANOVA, Tukey's test (n = 3 ± SE)

effect of temperature on L_{pr}, several aquaporins including *PtrePIP2;2*, *PtrePIP2;3*, and the major water-conducting poplar aquaporin *PtrePIP2;5* (Marjanović et al. 2005) were significantly downregulated in the root tips of non-inoculated seedlings at 5 °C compared with 20 °C. The decrease in abundance of important water-transporting aquaporins would generally be expected to lead to higher resistance in cell-to-cell water transport pathway in roots (Javot and Maurel 2002) and therefore decrease in L_{pr}. However, the downregulation of *PtrePIP2;5* that encodes the strongest water transporter in poplar roots (Marjanović et al. 2005) might be compensated by other *PIP*s that were not examined in this study and, therefore, did not translate into a significant decline in L_{pr} at 5 °C. The impact of 5 °C root temperature on expression of *PtrePIP2;2*, *PtrePIP2;3*, and *PtrePIP2;5* was less pronounced in ectomycorrhizal root tips than in non-inoculated root tips (Fig. 4c). Furthermore, *PtrePIP1;1*, *PtrePIP1;2*, *PtrePIP2;1*, and *PtrePIP2;4* were upregulated at 5 °C, although the extent of regulation was not always consistent between wild type and mock, or between the two OE lines (Fig. 4c).

In the MIP family of *Laccaria bicolor* dikaryotic strain UAMH8232, JQ585595 demonstrated the highest water transport capacity in the oocyte swelling assay and played a significant role in hyphal water status and root hydraulic properties of associated *Picea glauca* (Xu et al. 2015). Based on these results, we had hypothesized that since aquaporin overexpression in the mycorrhizal fungus would likely increase the hydration of the root–hyphal interface, inoculation of

aspen roots with fungal strains, which were shown to constitutively and consistently overexpress *JQ585595* aquaporin in different types of mycelia (Xu et al. 2015), would stimulate *PIP* transcription in roots and increase L_{pr}. However, this hypothesis was not confirmed by the study presented here. Instead, the inoculation by the OE strains reduced L_{pr} and *PIP* expression in roots, suggesting that increased water transport capacity of the fungal hyphae may not always favor hydraulic conductivity of mycorrhizal roots. Expression of *PtrePIP1;2* and *PtrePIP2;3* was significantly lower in OE-inoculated root tips than in non-inoculated root tips at 20 °C (Fig. 4b) and 5 °C (Fig. 4d), which coincides with lower L_{pr} in OE-inoculated roots than in non-inoculated roots (Fig. 3). The transcript abundance of *PtrePIP1;2* and *PtrePIP2;3* was also significantly lower in OE-inoculated root tips than in wild-type and mock-inoculated root tips (Fig. 4b, d). Moreover, L_{pr} in wild-type and mock-inoculated roots was not significantly different from that in non-inoculated roots. Therefore, it can be inferred that the decrease in L_{pr} in OE-inoculated roots was caused by the effect of *JQ585595* overexpression rather than by mycorrhization in general. Interestingly, *JQ585595* overexpression significantly upregulated the *Picea glauca* gene *PgPIP1;1* (*GQ03401_M18.1*) in ectomycorrhizal root tips of white spruce (Xu et al. 2015), which is an opposite effect observed on *PtrePIP1;2* expression in aspen roots (Fig. 4b). This may contribute to different response of L_{pr} in aspen and white spruce to mycorrhization with *Laccaria bicolor*.

Water permeability of the mantle may be determined by its thickness, the degree of hydrophobicity in hyphal cell walls (Duddridge et al. 1980; Weatherley 1982; Unestam and Sun 1995), and the abundance of fungal aquaporins (Lehto and Zwiazek 2011). It can largely influence the hydration level of the apoplastic space in the root epidermis and cortex and, in turn, affect transcriptional and posttranscriptional regulation of root *PIP*s and water uptake (Lehto and Zwiazek 2011). Therefore, changes in mycorrhizal root tip structure may determine the responses of L_{pr} to mycorrhization in a species-dependent manner. In this study, the epidermis of mycorrhizal aspen root tips was largely disintegrated by the development of a thick mantle of more than eight layers of hyphal cells and the Hartig net, whereas the Hartig net did not develop extensively in the inner cortex and did not form a distinct interface between the hyphal and cortical cells (Fig. 1). A similar epidermal Hartig net development was observed in mycorrhizal root tips of *Populus tremula* associated in vitro with *Laccaria bicolor*, with the thin and loosely arranged mantle in the in vitro system that comprised less than five layers of hyphal cells (Langer et al. 2008). It is conceivable that when compared with the well-developed Hartig net and the thin, three- to five-cell-layer mantle in ECM root tips of *Picea glauca*, the absence of Hartig net in the cortex and the thick, compact, mantle in aspen root tips could potentially hamper root water transport. However, our present knowledge concerning the effects of mycorrhizal development on the root water transport properties is severely limited.

In addition to fungal–plant compatibility, nutrient availability may also lead to different ectomycorrhizal structures and root colonization rate (Quoreshi and Khasa 2008). Resource competition rather than mutualistic resource exchanges may cause an antagonistic interaction between the interacting species at certain stages and result in changes in root anatomy. The plant–fungus interaction can dynamically shift from mutualism to antagonism, since the nature of the interaction depends on the benefits and costs to the host plant and is sensitive to resource availability and environmental stresses (Kivlin et al. 2013). For example, low nutrient availability could change the thickness and density of ectomycorrhizal fungal mantle and the structure of aspen root epidermis, causing impaired fungal growth and poor plant survival (Langer et al. 2008). In contrast, gymnosperm species are usually less nutrient-demanding than angiosperm species and therefore tend to nurture higher nutrient availability in rhizosphere and more mutualistic interaction with ectomycorrhizal fungi. This could explain why the percentage of mycorrhizal root tips in *Picea glauca*, where *Laccaria bicolor* produced strong L_{pr} responses, was over 90 % (Xu et al. 2015), which was about threefold higher compared with aspen examined in this study (i.e., about 30 % of all root tips). The comparatively reduced colonization rate of aspen relative to white spruce could explain why the effect of ectomycorrhizas on L_{pr} of the entire aspen root was much less pronounced than the effect on *PIP* transcript abundance in mycorrhizal root tips, since the latter was determined in colonized root tips. Future studies should attempt a more complete transcript profiling of *PIP*s in both mycorrhizal and non-mycorrhizal root tips of the colonized plants. It would also be important to examine the apoplastic hydration status with apoplastic tracer dyes and inhibitors of aquaporin activity for a more comprehensive understanding of the dynamic relationship between *PIP*s and water transport properties of the mycorrhizal partners.

In summary, inoculation of aspen seedlings with the *Laccaria bicolor* wild-type strain, the mock-transformant strain, and the strains that overexpress *JQ585995* fungal aquaporin has resulted in relatively low root colonization rates and little impact on L_{pr}, Ψ_{shoot}, gas exchange, and dry mass of aspen seedlings. Expression of *PIP1;2*, *PIP2;1*, and *PIP2;2* was reduced in root tips of inoculated plants, possibly due to reduced hydration in root apoplastic space caused by the thick mantle. The decrease of *PIP* transcript abundance in mycorrhizal roots did not significantly affect L_{pr} of the whole root, which can be explained by the low colonization rate of roots by the fungus. In roots inoculated with *Laccaria bicolor* overexpressing *JQ585995*, the decreased expression of *PIP1;2* and *PIP2;3* and the lower L_{pr} compared with non-inoculated roots suggest the involvement of *Laccaria bicolor JQ585595*

in root water transport pathways, in a way that is contrary to our original hypothesis. At 5 °C, *PIP2;2*, *PIP2;3*, and *PIP2;5* were significantly downregulated in the root tips of non-inoculated seedlings, whereas the fungal inoculation appeared to buffer this down-regulatory effect by low temperature. Temperature had little effect on L_{pr}, likely due to the compensatory regulation of the unanalyzed root *PIP*s. Differential transcript abundance of root *PIP*s regulated by ectomycorrhizal formation indicates a possible mechanism of *PIP* interaction or compensation as responses to external stimuli, which should be further explored in the context of tissue-specific transcription and posttranslational regulations of *PIP*s.

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