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RESEARCH PAPER

Local root ABA/cytokinin status and aquaporins regulate poplar responses to mild drought stress independently of the ectomycorrhizal fungus *Laccaria bicolor*

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Abstract

The relatively better performance of mycorrhizal plants subjected to drought stress has commonly been linked to improved root water uptake through the fungal regulation of plant aquaporins and hormones. In this study, we examined the role of ectomycorrhizal fungi in plant water relations and plant hormonal balance under mild drought using split-root seedlings of *Populus trichocarpa* × *deltooides* either with or without inoculation with *Laccaria bicolor*. The root compartments where the drought treatment was applied had higher ABA and lower cytokinin tZR contents, and greater expression of the plant aquaporins *PtPIP1;1*, *PtPIP1;2*, *PtPIP2;5*, and *PtPIP2;7*. On the other hand, the presence of *L. bicolor* within the roots down-regulated *PtPIP1;4*, *PtPIP2;3*, and *PtPIP2;10*, and reduced the abundance of PIP2 proteins. In addition, expression of the fungal aquaporins *JQ585595* and *JQ585596* were positively correlated with root ABA content, while tZR content was positively correlated with *PtPIP1;4* and negatively correlated with *PtPIP2;7*. The results demonstrate a coordinated plant–fungal system that regulates the different mechanisms involved in water uptake in ectomycorrhizal poplar plants.

Keywords: Abscisic acid, aquaporins, cytokinins, ectomycorrhiza, mild drought, poplar, root water transport.

Introduction

Drought stress is well known to greatly affect the development and yield of plants (Hogg *et al.*, 2008; Allen *et al.*, 2010), and it is expected to be one of the major issues for agriculture and forestry crops in the coming years due to unpredictable climate change (Lesk *et al.*, 2016). Although the exact responses of plants to drying soil are species-dependent (Liu *et al.*, 2010; Pinheiro and Chaves, 2011), a sudden drought event will typically cause stomatal closure to decrease transpiration (Cowan, 1977) and adjustments of root water uptake to avoid desiccation (Perrone *et al.*, 2012).

Water uptake through the roots is tightly controlled by its radial transport, where the presence of apoplastic barriers and cell plasma membranes determine how water is transferred from the soil to the xylem. Following the ‘composite model’ for radial transport (Steudle and Peterson, 1998), water will be transferred from the soil to the root following two main paths: (1) through the cell-wall continuum (apoplastic path), which is driven by the hydrostatic gradient created by the transpiration stream and is controlled by the Casparian strip and

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suberin in the exodermis and endodermis; and (2) through the cell-to-cell path, which includes the transfer of water through plasmodesmata and plasma membranes using the specialized water-channel aquaporin proteins, and which always following the driving force of an osmotic gradient. Plant aquaporins belong to the major-intrinsic proteins family (MIPs) that are present in both plants and fungi (Aroca *et al.*, 2009) and are divided into five subfamilies: plasma membrane (PIPs), tonoplast (TIPs), Nodulin26-like (NIPs), X (XIPs), and small basic (SIPs). Aquaporins have a major role in the control of water transfer under drought stress (Zargar *et al.*, 2017), and their function is closely related to post-translational regulation, in the form of phosphorylation, as well as their coordination with plant hormones (Sánchez-Romera *et al.* 2018).

Phytohormones respond to drought as signal molecules that regulate plant water relations (Wolters and Jürgens, 2009). Among them, abscisic acid (ABA), salicylic acid (SA) (Miura and Tada, 2014), and jasmonic acid (JA) (de Ollas *et al.* 2015) are induced in response to drought, while others such as cytokinins (CK) are typically reduced (reviewed in Cortleven *et al.*, 2019). A complex regulatory network connects the different pathways and enables each hormone to assist or antagonize the others in responding to stress (Peleg and Blumwald, 2011; Munné-Bosch and Müller, 2013). ABA is the most studied and the most abundant under water-deficit conditions, where it is usually implicated in stomatal closure and leaf senescence (Wilkinson and Davies, 2010). ABA is also known to affect root hydraulic conductivity (L_{pr}), both by increasing it (Thompson *et al.*, 2007; Aroca *et al.*, 2008; Parent *et al.*, 2009; Kudoyarova *et al.*, 2011) and by inhibiting it (Beaudette *et al.*, 2007). Other hormones, although less studied in water relations, have also been reported to enhance L_{pr} (JA; Sánchez-Romera *et al.*, 2014) or to decrease it (SA, Quiroga *et al.*, 2018). Information regarding how hormones and their multiple interactions affect water transport requires is scarce.

Plants form mutualistic associations with specialized fungi that affect how they obtain water and nutrients from the soil, including under extreme conditions. Among these different mycorrhizal associations, arbuscular mycorrhizal fungi are the most widespread in the plant kingdom, while ectomycorrhizal fungi are more specific to tree species from temperate forests. Ectomycorrhizal fungi generally form an extensive hyphal network in the soil in symbiosis with trees (Nehls *et al.*, 2010) that is related with improvements in the overall water and nutritional status of the host plant (Rousseau and Reid, 1990; Lehto and Zwiazek, 2011). Increases in L_{pr} in the presence of some ectomycorrhizal fungi have commonly been reported (Marjanović *et al.*, 2005; Yi *et al.*, 2008; Lee *et al.*, 2010, Xu *et al.* 2015), but other studies have found no changes or even reductions (Calvo-Polanco *et al.*, 2008, 2009; Xu *et al.*, 2016). The beneficial effects of ectomycorrhizal fungi on root water transport have been attributed to the resulting increase in the absorbing surface area and the exploration of larger soil volumes by the extramatrical mycelia, and to their effects on the expression of plant aquaporins (Lee *et al.*, 2010; Navarro-Ródenas *et al.*, 2013; Xu *et al.*, 2015). As in arbuscular mycorrhizal fungi (Quiroga *et al.*, 2018; Sánchez-Romera *et al.*, 2018), it is presumed that ectomycorrhizal fungi also play a

major role in regulating plant hormones, although our current knowledge is limited to a few studies that have shown reductions in ABA content under osmotic stress in ectomycorrhizal plants (Rincón *et al.*, 2005) or have demonstrated a priming effect to drought of the ectomycorrhizal fungi on ABA and SA contents in poplar trees (Luo *et al.*, 2009).

In this study, we examine how the interaction of *Populus trichocarpa* × *deltoides* with the ectomycorrhizal fungus *Laccaria bicolor* affects water uptake under drought through aquaporin and hormonal regulation. We used a split-root system in order to determine the responses of the plants to the presence of fungi and water stress. The objectives of the study were to evaluate whether plants can compensate the loss of water in different parts of the root system via the hormonal balance and expression of aquaporins, to evaluate whether ectomycorrhizal fungi can regulate plant hormones and aquaporins, and to test whether fungal aquaporins are involved in root water uptake.

Materials and methods

Plant material and growth conditions

Saplings of hybrid poplar (*Populus trichocarpa* × *deltoides*, clone H11-11, supplied by Dr Uwe Hacke, University of Alberta), were produced from cuttings (4 cm long) with one to two non-flushed buds. The cuttings were immersed in 0.8% indole-3-butyric acid (IBA) solution for 1 min, planted in a mixture of peat moss and vermiculate (2:1), and maintained for 4 weeks under relative high humidity. The roots of the cuttings were then divided into two equal parts and transferred into 1-l pots (17×10 cm) filled with perlite and configured in a split-root system. After transplanting, the different compartments of the split-root system were inoculated with the fungus *Laccaria bicolor* (Orton (R. Maire) and the plants were divided into three groups: (1) plants left as non-inoculated, to which plain Melin-Norkans liquid media was added instead of inoculum; (2) plants with both sides of the split-root system inoculated with *L. bicolor*; (3) plants with only one side of the root compartment inoculated with *L. bicolor*. At 12 weeks after transplanting, plants were subjected to the drought treatment, where half of the different inoculation groups of the split root-system (with or without inoculation) were left without any water for 10 d. The soil water potential was calculated at the time of harvesting in each of the compartments of the split-root system by using a HR-33T dew-point microvoltmeter together with a C-52 sample chamber (Wescor Inc.).

At the time of harvest, there was a total of 10 different treatment groups (Table 1), as follows. (1) Both compartments were non-inoculated and well-watered (NI-Ww/NI-Ww). (2) One compartment was non-inoculated and the other was inoculated with *Laccaria*, and both were well-watered (NI-Ww/Lacc-Ww). (3) Both compartments were inoculated and both were well-watered (Lacc-Ww/Lacc-Ww). (4) Both compartments were non-inoculated, one was well-watered and the other under drought (NI-Ww/NI-D). (5) One compartment was non-inoculated and under drought, and the other was inoculated and well-watered (NI-D/Lacc-Ww). (6) One compartment was non-inoculated and well-watered and the other was inoculated and under drought (NI-Ww/Lacc-D). (7) Both compartments were inoculated, one was well-watered and the other under drought (Lacc-Ww/Lacc-D). (8) Both compartments were non-inoculated and under drought (NI-D/NI-D). (9) One compartment was non-inoculated and the other inoculated and both were under drought (NI-D/Lacc-D). (10) Both compartments were inoculated and under drought (Lacc-D/Lacc-D).

Laccaria bicolor was obtained from the University of Alberta Microfungus Collection and Herbarium (UAMH8232). The inoculation of plant roots was done 1 d after transplanting and again after 4 weeks. The fungus was grown for 4 weeks in modified Melin-Norkans (MMN) liquid medium into 0.5-l Erlenmeyer flasks that were continuously shaken. The inoculum was blended to OD=260 and applied to the roots of the plants at a rate

Table 1. Summary of drought and fungal treatment combinations applied to poplars in the split-root system.

	Treatments	Compartment 1		Compartment 2	
		<i>L. bicolor</i>	Drought	<i>L. bicolor</i>	Drought
1	NI-Ww / NI-Ww	–	–	–	–
2	NI-Ww / Lacc-Ww	–	–	Yes	–
3	LaccWw / LaccWw	Yes	–	Yes	–
4	NI-Ww / NI-D	–	–	–	Yes
5	NI-D / Lacc-Ww	–	Yes	Yes	–
6	NI-Ww / Lacc-D	–	–	Yes	Yes
7	Lacc-Ww / Lacc-D	Yes	–	Yes	Yes
8	NI-D / NI-D	–	Yes	–	Yes
9	NI-D / Lacc-D	–	Yes	Yes	Yes
10	Lacc-D / Lacc-D	Yes	Yes	Yes	Yes

NI, Non-inoculated roots; Lacc, roots inoculated with *L. bicolor*; Ww, well-watered roots; D, roots under drought stress. 'Yes' indicates that the presence of *L. bicolor* or drought in the compartment.

of 20 ml per root compartment. The plants were grown in a controlled-environment growth chamber under a 18/6 h day/night photoperiod with a photosynthetic photon flux density (PPFD) of 450–500 $\mu\text{mol m}^{-2} \text{s}^{-1}$ at 22/18 °C, and 50% relative humidity for 3 months. During the growing period, 40% modified Hoagland's solution was applied twice per week (Epstein, 1972): 2.5 mM KNO_3 , 0.5 mM KH_2PO_4 , 2.5 mM $\text{Ca}(\text{NO}_3)_2$, 1 mM MgSO_4 , 23 μM H_3BO_3 , 5 μM MnCl_2 , 0.3 μM ZnSO_4 , 0.2 μM CuSO_4 , 0.01 μM $(\text{NH}_4)_6\text{Mo}_7\text{O}_{24}$, and 90 μM EDTA-Fe.

Ectomycorrhizal colonization rates

Ectomycorrhizal root colonization rates were estimated by stereomicroscopic examination of the root tips in eight different root sections (3–4 cm) in each of the root compartments within each treatment, with a total of six seedlings per treatment combination being examined. The percentage of root tips with distinct ectomycorrhizal structures was calculated by determining the number of mycorrhizal versus non-mycorrhizal root tips in each of the root compartments per seedling (Brundrett *et al.*, 1996).

Plant physiological measurements

Root and shoot fresh weights, stomatal conductance (g_s), and leaf relative water content (RWC) were determined at the time of harvest for six plants per treatment combination (i.e. $n=6$). g_s was measured 2 h into the photoperiod in the youngest fully developed mature leaf of each plant using a portable AP4 porometer (Delta-T Devices Ltd). RWC was determined in mature, fully developed leaves, which were excised from the main shoot, weighed (W_0), and placed into 15-ml centrifuge tubes (BD Falcon, Fisher Scientific) with a piece of moist cotton for 24 h at 4 °C. The leaves were then weighed again (W_h) and dried at 65 °C for 2 d so that the dry weight could be obtained (W_d). The RWC was then calculated as $[(W_0 - W_d) / (W_h - W_d)] \times 100$. Leaf water potential (Ψ_w) was determined using a pressure chamber (SF-PRES-35, SolFranc Technologies SL, Tarragona, Spain) on six mature leaves per treatment after g_s had been determined. Leaves were excised from the main shoot under water and then pressurized in the chamber until xylem sap was visible at the cut surface (Boyer, 1967).

Root hydraulic conductivity (L_{pr}) was determined in the whole roots of six plants per treatment combination between 4–5 h after the beginning of the photoperiod within the growth chamber. Whole roots were detached from the shoot and connected to a high-pressure flow meter (HPFM, Dynamax, Inc., Houston, TX). Water was pressurized into the roots from 0–0.5 MPa in the transient mode to calculate root hydraulic conductance (K_r). The root volume was determined using the volume displacement method (Kamaluddin and Zwiazek, 2002), and L_{pr} was calculated by dividing K_r by the root volume (Calvo-Polanco *et al.*, 2016).

Although measurements of whole-root hydraulics have been used successfully to determine the water transport capacity of plants under stress (Calvo-Polanco *et al.*, 2016), it should be noted that the technique could be improved by determining the conductivity of each part of the root system (Dodd *et al.*, 2008) as they have been shown to differ from the whole-system conductivity in split-root experiments (Hu *et al.*, 2011).

Quantification of transcript abundance of poplar PIPs and *L. bicolor* aquaporins

Expression of root and fungal aquaporins was determined in each of the compartments of the split-root set-up. After 10 d of drought treatment, the roots of each compartment were harvested, carefully washed, cut in pieces, mixed, and divided into 0.5-g samples that were then stored at –80 °C prior to analysis.

For the detection of poplar aquaporins, total RNA was isolated using the phenol/chloroform protocol as described by Calvo-Polanco *et al.* (2014). Expression was determined for 11 *Populus* PIP genes, using the primers described by Almeida-Rodriguez *et al.* (2011). The expression of the different aquaporins was determined using an iCycler RT-qPCR system (Bio-Rad). Each 23- μl reaction mixture contained 1 μl of cDNA (80 ng), 10.5 μl of Master Mix (Bio-Rad), 8.6 μl of deionized water, and 0.45 μl of each primer pair at a final concentration of 0.2 mM. The PCR program consisted of 3 min incubation at 95 °C, followed by 32 cycles of: 30 s at 95 °C, 30 s of annealing temperature of 58 °C, and 72 °C for 30 s. *Elongation Factor-1 alpha* was used as the reference gene, as it was the most stable one in all the treatments (Almeida-Rodriguez *et al.*, 2011). Three different root RNA samples for each treatment were used for analysis (i.e. $n=3$), with each of them repeated three times. Negative controls without cDNA were used in all the PCR reactions.

For the detection of *L. bicolor* aquaporins, total RNA was isolated following the CTAB-PVP protocol described by Chang *et al.* (1993). The expression levels of the *L. bicolor* aquaporins JQ585592 to JQ585597 (Xu *et al.*, 2015) were determined using a QuantStudio3 RT-qPCR system (Applied Biosystems) with the same reaction mixture as described above except for the use of PowerUp SYBR Green Master Mix (Applied Biosystems). The PCR program consisted of 2 min incubation at 50 °C plus another 2 min at 95 °C, followed by 40 cycles of: 15 s at 95 °C, 30 s of annealing temperature of 58 °C, and 72 °C for 1 min. The *translation elongation factor EF2* was used as the reference gene as it was the most stable across all the samples, as proposed by Xu *et al.*, (2015). For each aquaporin, three different root RNA samples for each treatment were used for the analysis, with each of them repeated three times. Negative controls without cDNA were used in all the PCR reactions.

Root enzyme-linked immunosorbent assays (ELISAs)

Microsomes for the immunoassay determination were isolated and proteins extracted as described by Calvo-Polanco *et al.* (2014). As primary antibodies we used two antibodies (at a dilution of 1:1000) that recognize several PIP1s and PIP2s and three antibodies that recognize the phosphorylation of PIP2 proteins at their C-terminal region at serine 280 (PIP2₂₈₀), serine 283 (PIP2₂₈₃), and at both serine 280 and 283 (PIP2_{280/283}). Goat anti-rat IgG coupled with horseradish peroxidase (Sigma-Aldrich) was used as the secondary antibody for PIP1 (at 1:10 000). Goat anti-rabbit Ig coupled to horseradish peroxidase (Sigma-Aldrich) was used as a secondary antibody for PIP2 and PIP2₂₈₀, PIP2₂₈₃, and PIP2_{280/283} (at 1:10000). Protein quantification was done in three different independent root samples per treatment, repeated three times each.

Root contents of ABA, SA, JA, and CKs

The following hormones were analysed in roots with four replicates per compartment and treatment combination: ABA, SA, JA, and CKs *trans*- and *cis*-zeatin riboside (t-ZR and c-ZR), N6-(Δ^2 -isopentenyl) adenine (iP), and isopentenyl adenosine (iPR). The analyses were done using an HPLC-ESI-MS/MS with a 2795 Alliance HT HPLC device (Waters) coupled to a 3200 Q TRAP LC/MS/MS system (Applied Biosystems/MDS Sciex, Ontario, Canada), equipped with an electrospray interface.

The extraction and purification of ABA and CKs was carried out following the procedure described by Bacaicoa et al. (2009), for JA following the procedure described by Sánchez-Romera et al. (2014), and for SA following the procedure described by Sánchez-Romera et al. (2016).

Statistical analysis

All data were analysed using the MIXED procedure in SAS (v. 9.2, SAS Institute Inc.). MANOVA was used for all the parameters except for the analyses of fungal aquaporins where we used ANOVA directly plus Tukey's test. MANOVA was used in order to detect the main contributions of the two treatments (drought and fungi) to the different parameters studied. Where MANOVA indicated a significant effect, we conducted the following tests. (1) ANOVA and post-hoc Tukey's test for the leaf RWC, g_s , Ψ_w , and whole-root L_{pr} . (2) ANOVA in the split-root system to test if there was a local or long-distance response according to how the treatments (well-watered/drought or non-inoculated/*L. bicolor*) were applied to each side of the compartments (one side or two sides). (3) Pearson correlations analyses with the use of the command 'proc corr' in SAS. (4) Student's *t*-test to compare partial root drying versus total root drying, and partial root inoculation versus complete root inoculation with *L. bicolor*.

Results

Soil conditions and mycorrhizal colonization rates

Drought stress was applied to the selected compartments of the split-root system by leaving them without water for 10 d. The final mean water soil potential of the well-watered compartments was -0.19 ± 0.01 MPa (\pm SE), while in the drought compartments the mean potential was -0.40 ± 0.01 MPa (Supplementary Table S2 at JXB online). The presence of *L. bicolor* within the compartments did not result in any significant difference in the water potential compared with the non-inoculated compartments, either under well-watered or drought conditions.

Examination of colonization rates indicated the presence of mycorrhizal tips in 30–35% of the roots inoculated with *L. bicolor*, while there were none present in the non-inoculated compartments.

Seedling growth and physiological parameters

Although inoculation with *L. bicolor* to at least one compartment of the split-root system tended to increase biomass (Supplementary Table S1), the differences were not large enough to be detected as significant by the MANOVA analyses. Hence, there were no differences among the treatments in FW of roots ($P=0.326$), shoots ($P=0.503$), and leaf RWC ($P=0.380$). However, we were able to detect significant differences in leaf g_s ($P=0.003$), leaf Ψ_w ($P=0.010$), and in whole-root L_{pr} ($P=0.019$). The treatment *Lacc-D/Lacc-D* had the highest g_s and L_{pr} , and the lowest Ψ_w (Figs 1, 2), while the *NI-Ww/NI-D* treatment had the lowest L_{pr} values and was among the treatments with the lowest g_s .

Plants with both compartments inoculated with *L. bicolor* (*Lacc-Ww/Lacc-Ww*, *Lacc-Ww/Lacc-D*, *Lacc-D/Lacc-D*) had a lower mean leaf Ψ_w (-0.42 ± 0.05 MPa) than plants with only one compartment inoculated (*NI-Ww/Lacc-Ww*, *NI-D/Lacc-Ww*, *NI-Ww/Lacc-D*) (-0.25 ± 0.02 MPa; $P=0.008$),

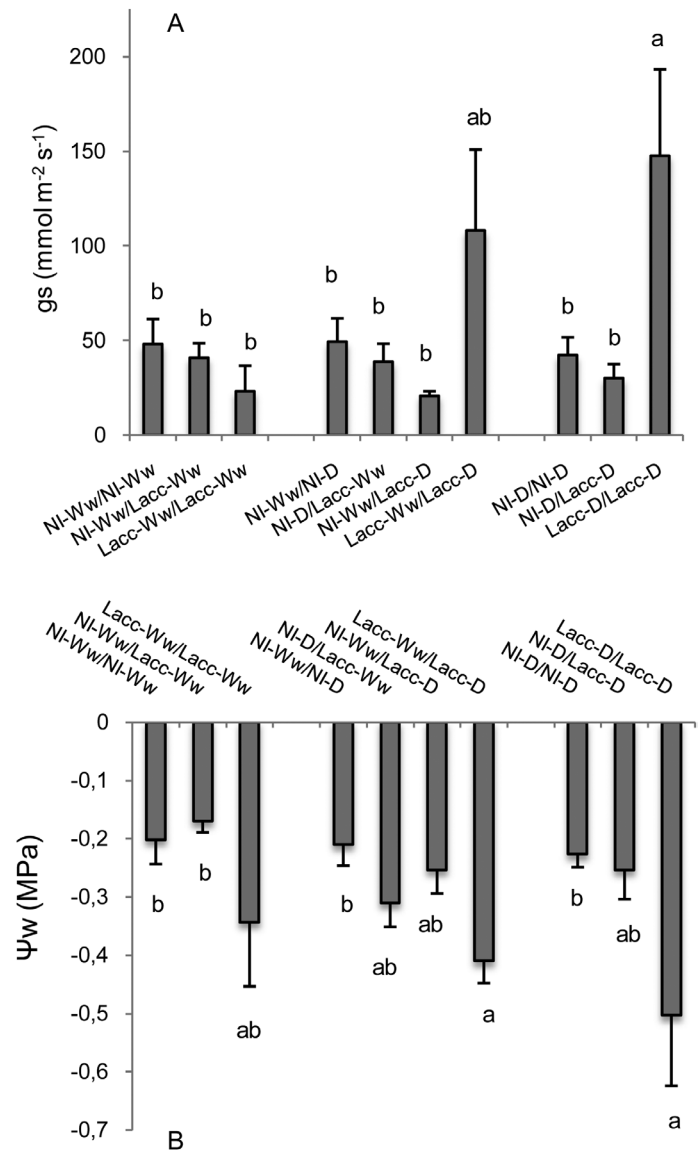


Fig. 1. Effects of drought and inoculation with *L. bicolor* on (A) leaf stomatal conductance (g_s) and (B) leaf water potential (Ψ_w) in poplar seedlings with roots divided between different treatments in a split-root system. The root compartments were either inoculated with *L. bicolor* (*Lacc*) or non-inoculated (*NI*) and either subjected to well-watered (*Ww*) or drought (*D*) conditions in different combinations. Data are means (\pm SE), $n=8$. Different letters indicate significant differences among the treatments as determined using ANOVA followed by Tukey's test ($\alpha=0.05$).

independently of water stress (Fig. 1B). There was a negative correlation between Ψ_w and g_s ($r=-0.562$, $P<0.0001$) that was unaffected by *L. bicolor* inoculation (Fig. 1), while L_{pr} was not correlated either with Ψ_w ($r=-0.141$, $P=0.28$) or g_s ($r=0.145$, $P=0.27$).

Expression of poplar PIPs in roots

The expression of root aquaporins was first tested using MANOVA in order to detect general effects related either to drought treatment, inoculation with *L. bicolor*, or the interaction between the two (Supplementary Table S3). When we found a significant effect using MANOVA, we tested for the individual effects of the presence of drought and *L. bicolor* in

none, one, or both compartments on root aquaporin expression, abundance, and phosphorylation state. The following trends were observed.

Drought treatment resulted in up-regulation of the expression of *PtPIP1;1* ($P=0.0028$), *PtPIP1;2* ($P=0.0001$), *PtPIP2;5* ($P=0.0006$), and *PtPIP2;7* ($P<0.0001$) (Supplementary Table S3). This up-regulation occurred when either one or both of the compartments were subjected to drought (Table 2, Supplementary Fig. S1). The presence of *L. bicolor* in both compartments of the split-root system resulted

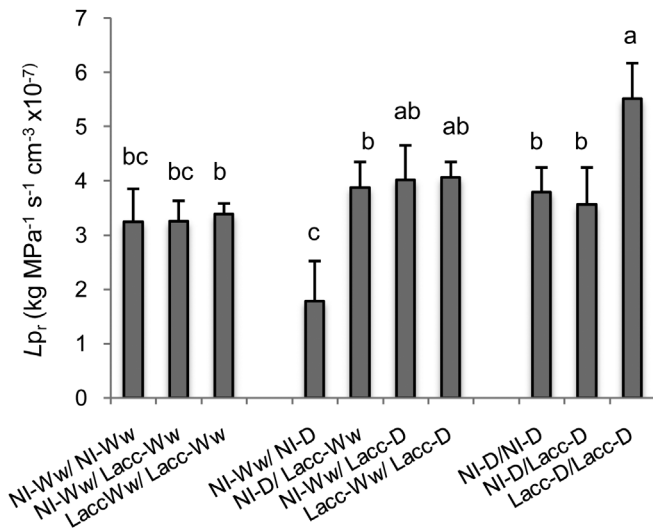


Fig. 2. Effects of drought and inoculation with *L. bicolor* on whole-root hydraulic conductivity (L_{pr}) in poplar seedlings with roots divided between different treatments in a split-root system. The root compartments were either inoculated with *L. bicolor* (Lacc) or non-inoculated (NI) and either subjected to well-watered (Ww) or drought (D) conditions in different combinations. Data are means (\pm SE), $n=6$. Different letters indicate significant differences among the treatments as determined using ANOVA followed by Tukey's test ($\alpha=0.05$).

Table 2. Effects of drought treatments applied to the split-root system on aquaporin expression, abundance of PIP2, and phosphorylation state of PIP2 in poplar roots.

Aquaporin	Drought treatment applied to split-roots		
	One side dry	Two sides dry	Two sides well-watered
<i>PtPIP1;1</i>	0.85 \pm 0.08 A	0.81 \pm 0.09 A	0.46 \pm 0.10 B
<i>PtPIP1;2</i>	1.23 \pm 0.10 A	1.46 \pm 0.12 A	0.83 \pm 0.11 B
<i>PtPIP1;4</i>	0.07 \pm 0.02 A	0.06 \pm 0.01 A	0.04 \pm 0.01 A
<i>PtPIP2;3</i>	0.82 \pm 0.07 A	0.89 \pm 0.08 A	0.83 \pm 0.08 A
<i>PtPIP2;4</i>	0.02 \pm 0.01 A	0.05 \pm 0.03 A	0.02 \pm 0.01 A
<i>PtPIP2;5</i>	0.25 \pm 0.04 B	0.47 \pm 0.04 A	0.13 \pm 0.04 C
<i>PtPIP2;7</i>	0.21 \pm 0.03 A	0.26 \pm 0.03 A	0.09 \pm 0.03 B
<i>PtPIP2;10</i>	0.05 \pm 0.01 A	0.06 \pm 0.02 A	0.05 \pm 0.02 A
PIP2	0.004 \pm 0.0003 AB	0.005 \pm 0.0003 A	0.004 \pm 0.0003 B
PIP2 _{Ser-280}	0.007 \pm 0.0002 A	0.007 \pm 0.0003 A	0.007 \pm 0.0002 A

Expression of root aquaporins is in relative units using *Elongation Factor-1 alpha* as the reference gene. Abundance of PIP2 and its phosphorylation state are expressed as $\mu\text{g } \mu\text{g}^{-1}$ protein. Data are means (\pm SE), $n=3$. Different letters within the same line indicate significant differences as determined using MANOVA ($\alpha=0.05$).

in the down-regulation of *PtPIP1;4* ($P=0.0207$), *PtPIP2;3* ($P=0.037$), and *PtPIP2;10* ($P=0.017$) (Supplementary Table S3). *PtPIP2;10* was also down-regulated when *L. bicolor* was present in only one compartment (Table 3, Fig. S2).

None of the treatments had any effects on the expression of *PtPIP1;5* ($P=0.764$), *PtPIP2;1* ($P=0.630$), and *PtPIP2;2* ($P=0.733$) (Supplementary Table S3).

The only treatment that induced an increase in L_{pr} (*Lacc-D/Lacc-D*) was characterized by up-regulation of aquaporin expression in relation to drought (Table 2, 'two-sides dry' compared with 'two-sides well-watered') and down-regulation of aquaporin expression and reduced PIP2 abundance in relation to *L. bicolor* (Table 3, 'two-sides *L. bicolor*' compared with 'non-inoculated').

Expression of *L. bicolor* aquaporin genes in poplar roots

We examined the expression of six *L. bicolor* aquaporins in the roots of each compartment inoculated with the fungus in the split-root system. Expression was only detected for four of them, namely *JQ585592*, *JQ585594*, *JQ585595*, and *JQ585596* (Fig. 3). Their expression did not follow a regular pattern and instead seemed to vary with the different water conditions applied to each of the compartments. The compartment under drought within the *Lacc-Ww/Lacc-D* treatment had the highest expression levels of *JQ585595* and *JQ585596* (Fig. 3C, D), and the treatment with the highest L_{pr} (*Lacc-D/Lacc-D*), had low expression.

Protein abundance and phosphorylation state of root aquaporins

MANOVA showed that the abundance of PIP2 proteins was affected by the fungus \times drought treatment interaction

Table 3. Effects of the presence of *L. bicolor* in the split-root system on aquaporin expression, abundance of PIP2, and phosphorylation state of PIP2 in poplar roots.

Aquaporin	<i>L. bicolor</i> treatment applied to split-roots		
	No <i>L. bicolor</i>	One side <i>L. bicolor</i>	Two sides <i>L. bicolor</i>
<i>PtPIP1;1</i>	0.68 \pm 0.06 A	0.86 \pm 0.11 A	0.61 \pm 0.07 A
<i>PtPIP1;2</i>	1.21 \pm 0.10 A	1.29 \pm 0.11 A	0.99 \pm 0.10 A
<i>PtPIP1;4</i>	0.07 \pm 0.01 A	0.07 \pm 0.01 A	0.02 \pm 0.01 B
<i>PtPIP2;3</i>	0.03 \pm 0.01 A	0.02 \pm 0.01 AB	0.01 \pm 0.01 B
<i>PtPIP2;4</i>	0.25 \pm 0.04 A	0.22 \pm 0.03 A	0.18 \pm 0.04 A
<i>PtPIP2;5</i>	0.26 \pm 0.04 A	0.30 \pm 0.05 A	0.26 \pm 0.04 A
<i>PtPIP2;7</i>	0.21 \pm 0.04 A	0.25 \pm 0.03 A	0.19 \pm 0.04 A
<i>PtPIP2;10</i>	0.08 \pm 0.02 A	0.04 \pm 0.01 AB	0.03 \pm 0.02 B
PIP2	0.005 \pm 0.0003 A	0.004 \pm 0.0003 B	0.004 \pm 0.0003 B
PIP2 _{Ser-280}	0.007 \pm 0.0002 A	0.007 \pm 0.0003 A	0.007 \pm 0.0003 A

Expression of root aquaporins is in relative units using *Elongation Factor-1 alpha* as the reference gene. Abundance of PIP2 and its phosphorylation state are expressed as $\mu\text{g } \mu\text{g}^{-1}$ protein. Data are means (\pm SE), $n=3$. Different letters within the same line indicate significant differences as determined using MANOVA ($\alpha=0.05$).

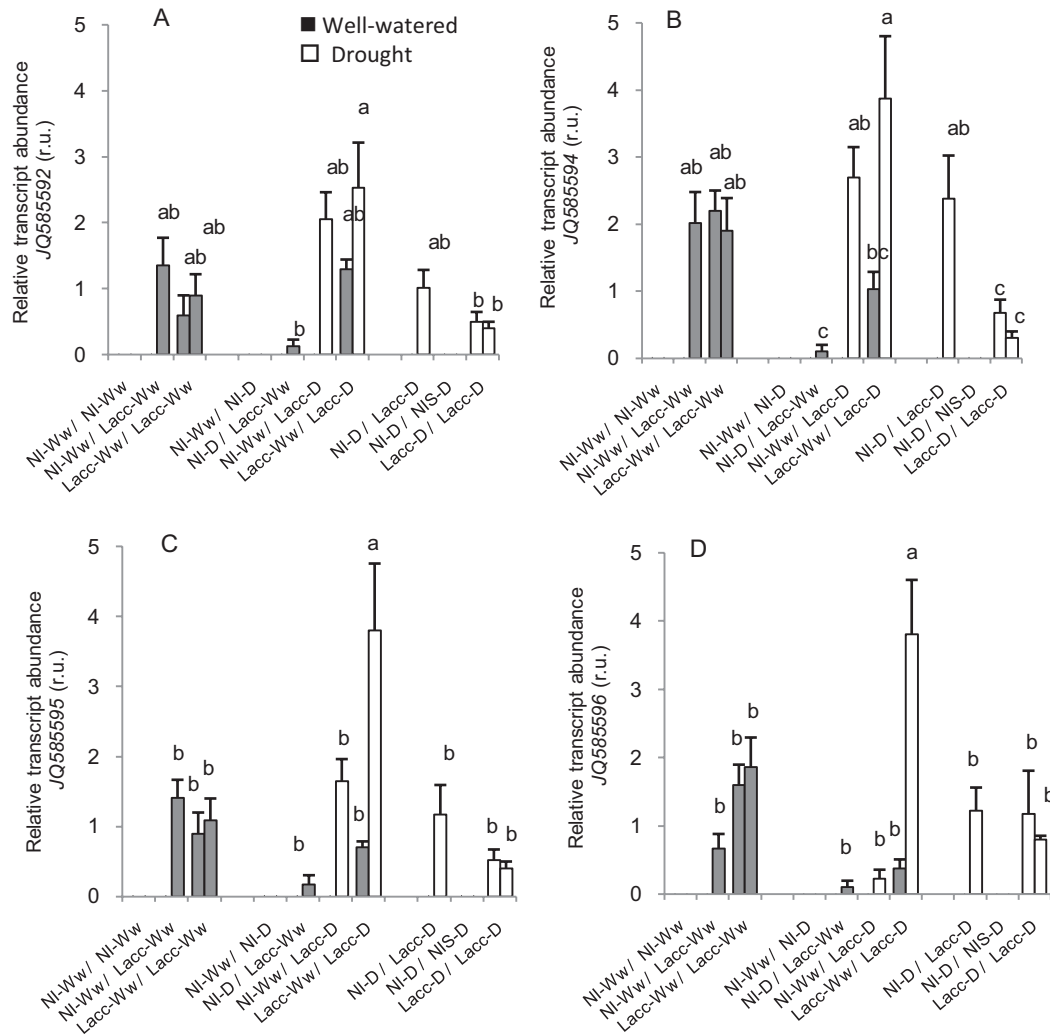


Fig. 3. Relative expression of fungal aquaporins in *L. bicolor* inoculated on roots of poplar seedlings divided between different treatments in a split-root system. The root compartments were either inoculated with *L. bicolor* (Lacc) or non-inoculated (NI) and either subjected to well-watered (Ww) or drought (D) conditions in different combinations. Data from well-watered and drought compartments are presented in grey and white, respectively. (A) *JQ585592*, (B) *JQ585594*, (C) *JQ585595*, and (D) *JQ585596*. Data are means (\pm SE), $n=3$. Different letters indicate significant differences among the treatments as determined using ANOVA followed by Tukey's test ($\alpha=0.05$).

($P=0.0002$; [Supplementary Table S4](#), [Fig. 4](#)) and by the drought treatment ($P=0.006$), while the phosphorylation of PIP2₂₈₀ was affected individually by the drought treatment ($P=0.022$) and the fungal treatment ($P=0.022$; [Supplementary Fig. S3](#)). PIP1, PIP2₂₈₃, and PIP2_{280/283} were not affected by any of the treatments applied ([Supplementary Table S4](#)).

Following MANOVA, we examined whether the treatments applied to the different compartments followed the same general trends that were observed for aquaporin expression. We found only that the abundance of PIP2 increased when both of the compartments were under drought ([Table 2](#), [Fig. 4](#)) and it decreased in the compartments inoculated with *L. bicolor* ([Table 3](#), [Fig. 4](#)).

Root hormonal status

We analysed the contents of ABA, JA, SA, and various CKs in each of the compartments of the split-root system. MANOVA indicated that the drought treatment increased the root

concentrations of ABA ($P<0.0001$), SA ($P=0.014$), and iPR ($P=0.006$), while it reduced tZR ($P<0.0001$; [Supplementary Table S5](#)). There was a significant fungus \times drought interaction in the response of ABA ($P=0.036$), indicating a potential role of *L. bicolor* in the ABA concentrations of the roots.

We examine whether the presence of *L. bicolor* in the different compartments of the split-root system affected root hormone concentrations under different soil water conditions. Under drought stress, we could detect a general reduction of tZR and an increase of ABA concentrations that were independent of the presence of *L. bicolor* in the roots of the plants ([Table 4](#), [Fig. 5](#)). Furthermore, ABA and tZR were negatively correlated ($r=-0.37$, $P=0.001$) ([Supplementary Table S6](#)).

We conducted Pearson correlation analyses with the root hormone contents, the hydraulic conductivity, and aquaporin expression, abundance, and phosphorylation state. We found that ABA was positively correlated with the fungal aquaporins *JQ585595* ($r=0.34$, $P=0.009$) and *JQ585596* ($r=0.29$, $P=0.026$), and that aquaporin *JQ585596* was negatively

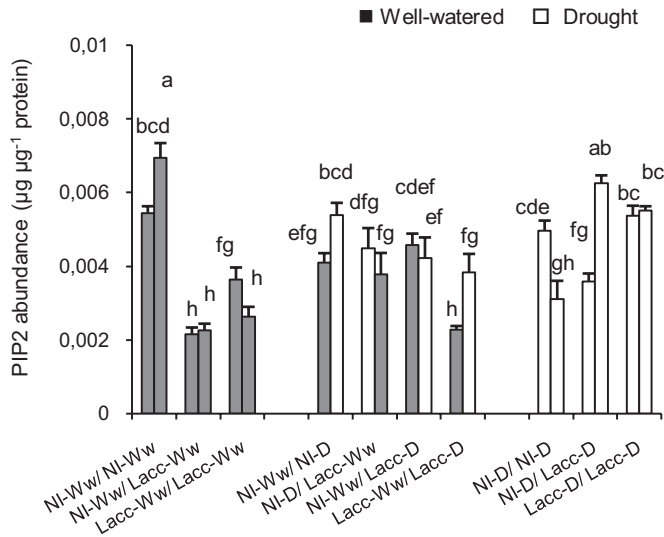


Fig. 4. Effects of drought and inoculation with *L. bicolor* on the root abundance of PIP2 of poplar seedlings with roots divided between different treatments in a split-root system. The root compartments were either inoculated with *L. bicolor* (Lacc) or non-inoculated (NI) and either subjected to well-watered (Ww) or drought (D) conditions in different combinations. Data from well-watered and drought compartments are presented in grey and white, respectively. Data are means (\pm SE), $n=3$. Different letters indicate significant differences among the treatments as determined using ANOVA followed by Tukey's test ($\alpha=0.05$).

Table 4. Effects of drought treatments applied to the split-root system on hormone contents in poplar roots.

Hormone	Drought treatment applied to split-roots		
	One side dry	Two sides dry	Two sides well-watered
tZR	0.27 \pm 0.03 B	0.13 \pm 0.03 C	0.36 \pm 0.03 A
iPR	0.91 \pm 0.06 A	0.84 \pm 0.07 A	0.96 \pm 0.07 A
cZR	0.37 \pm 0.04 A	0.38 \pm 0.04 A	0.37 \pm 0.04 A
ABA	33.81 \pm 4.75 A	40.26 \pm 5.20 A	17.11 \pm 5.48 B
SA	5648.6 \pm 681.3 A	4875.4 \pm 746.3 A	3834.1 \pm 786.7 A

Data are means (\pm SE), $n=4$ (pmoles g^{-1} FW). Different letters within the same line indicate significant differences as determined using MANOVA ($\alpha=0.05$).

correlated with *PtPIP1;4* ($r=-0.26$, $P=0.047$) (Supplementary Table S7). In addition, tZR was positively correlated with *PtPIP1;4* ($r=0.41$, $P<0.001$) and negatively correlated with *PtPIP2;7* ($r=-0.20$, $P=0.032$) (Supplementary Table S6).

Discussion

We used a split-root system to study local and long-distance responses of ectomycorrhizal poplar trees to drought. The mild drought that we imposed allowed us to examine the role of *L. bicolor* in the regulation of root aquaporin expression and hormonal abundance in relation to rebalancing the root hydraulics and whole-plant water status. The results showed that homeostasis of root hydraulic conductivity, L_{pr} , was achieved without drastically affecting plant growth, stomatal conductance, leaf

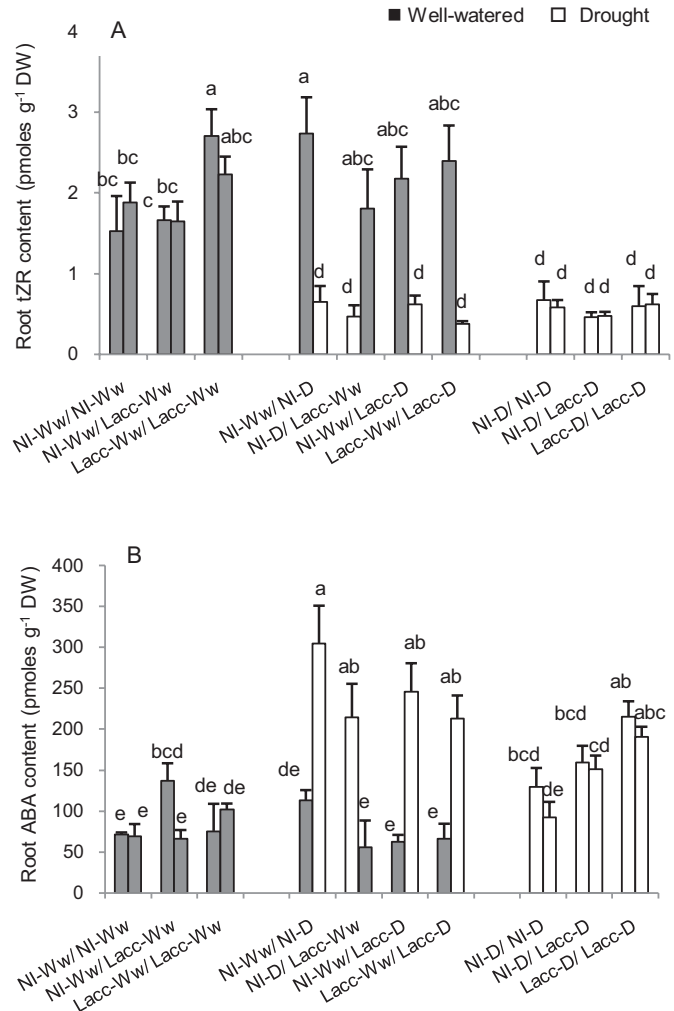


Fig. 5. Effects of drought and inoculation with *L. bicolor* on the root contents of (A) tZR (cytokinin) and (B) ABA of poplar seedlings with roots divided between different treatments in a split-root system. The root compartments were either inoculated with *L. bicolor* (Lacc) or non-inoculated (NI) and either subjected to well-watered (Ww) or drought (D) conditions in different combinations. Data from well-watered and drought compartments are presented in grey and white, respectively. Data are means (\pm SE), $n=4$. Different letters indicate significant differences among the treatments as determined using ANOVA followed by Tukey's test ($\alpha=0.05$).

RWC and water potentials (Fig. 1, Supplementary Table S1). Drought commonly affects the stomatal opening and water balance in the aerial parts of plants, most likely driven by long-distance ABA signaling (Dodd, 2005), although the responses are species-dependent and will be affected by the intensity of the drought (Silim *et al.*, 2009).

The mild drought that we applied to the different root compartments induced a local accumulation of ABA and a reduction of tZR content that were independent of *L. bicolor* inoculation if only half the root system was inoculated (Fig. 5, Table 4), together with an up-regulation of the plant aquaporins *PtPIP1;1*, *PtPIP1;2*, *PtPIP2;5*, and *PtPIP2;7* (Table 2). *PIP2;5*, and *PIP2;7* have been shown to have high water transport capacity in *Xenopus laevis* oocyte swelling assays (Marjanović *et al.*, 2005; Secchi and Zwieniecki, 2010), and *PIP2;5* has been shown

to have a key role in water transport under water stress when overexpressed in poplar plants (Ranganathan *et al.*, 2017). PIP1 aquaporins are channels with low water transport capacity (Marjanović *et al.*, 2005; Almeida-Rodríguez *et al.*, 2011), although it has been demonstrated that they are quite responsive to drought (Mahdieh *et al.*, 2008). In addition, the functionality of PIP1 aquaporins in cell membranes is commonly related to their interaction with PIP2 isoforms (Fetter *et al.*, 2004). The overexpression of aquaporins that we observed under mild drought (Table 2, Supplementary Fig. S1) would indicate a combined response of these aquaporins for the control of water uptake under drought that seems not to be directly regulated by hormone concentrations, as only *PtPIP2;7* was negatively correlated with tZR abundance, and none of the aquaporins was correlated with ABA (Supplementary Table S6).

ABA is one of the main factors affecting the responses of plants to drought as it regulates protective responses by regulating water uptake (Aroca *et al.*, 2003) and by interacting with several other hormones (Peleg and Blumwald, 2011; Zwack and Rashotte, 2015). The effects of ABA on L_{pr} have been reported as either being positive (Veslov *et al.*, 2016) or negative (Beaudette *et al.*, 2007), although it is well established that ABA is a key regulator of aquaporin genes (Jang *et al.*, 2004; Wan *et al.*, 2004; Zhu *et al.*, 2005; Ruiz-Lozano *et al.*, 2009). In our experiments, ABA abundance was not correlated with any of the plant aquaporins (Supplementary Table S6), but it was correlated with the fungal aquaporins *JQ585595* and *JQ585596* (Supplementary Table S7). These are known to have high water-transport capacities (Xu *et al.*, 2015) and *JQ585595* is essential for the formation of mycorrhizas in poplar seedlings (Navarro-Ródenas *et al.*, 2015). The levels of ABA were similar in all the drought-treated root compartments (Fig. 5B); however, plants under partial drought (*NI-Ww/NI-D*, *NI-D/Lacc-Ww*, *NI-Ww/Lacc-D*, *LaccWw-Lacc-D*) had a higher mean ABA content than those under total drought (*NI-D/NI-D*, *NI-D/Lacc-D*, *Lacc-D/Lacc-D*) (244.1 ± 22.6 pmol g⁻¹ DW versus 150.3 ± 11.8 pmol g⁻¹ DW, $P=0.006$). In addition, among the plants subjected to total drought, those with both compartments inoculated with *L. bicolor* (*Lacc-D/Lacc-D*) had a higher ABA content (203.8 ± 9.8 pmol g⁻¹ DW) than those with only one compartment inoculated (*NI-D/Lacc-D*) (155.1 ± 11.7 pmol g⁻¹ DW, $P=0.011$), or with both compartments non-inoculated (*NI-D/NI-D*) (106.2 ± 15.1 pmol g⁻¹ DW, $P<0.001$). This may indicate that plants have different ABA-related mechanisms to cope with drought when inoculated with mycorrhizal fungi and when partial versus total drying is present within the soil. The other hormone that was responsive to drought was the cytokinin tZR, which had lower abundance in the compartments where drought was applied (Fig. 5A). Cytokinins are known for their critical roles in many biological mechanisms in plants (Wybouw and De Rybel, 2019), although their response to water stress is limited to decreases in their abundance (Bano *et al.*, 1993; Goicoechea *et al.*, 1996). In our study, the reduction in abundance of tZR under drought was positively correlated with the plant aquaporin *PtPIP1;4* and negatively correlated with *PtPIP2;7* (Supplementary Table S6). As noted above, *PtPIP2;7* is known for its high water-transport capacity, while *PtPIP1;4* has been shown to have high expression in

root tips and in the cells of the inner cortex, and is related to the water movement from the cortex to the stele (Almeida-Rodríguez *et al.*, 2011). How tZR interacts with *PtPIP1;4* and *PtPIP2;7* under drought stress remains to be determined, as not only their expression but also their localization at the root level and their post-translational regulation will be key elements that determine their functions in water transport.

The presence of ectomycorrhizal fungi in the different compartments of the split-root system added another level of complexity to the system. We found negative effects of the presence of *L. bicolor* on the expression of *PtPIP1;4*, *PtPIP2;3*, and *PtPIP2;10* both under well-watered and drought conditions (Table 3, Supplementary Fig. S2). Xu *et al.* (2016) found the same trend in the same poplar and *L. bicolor* system, where the presence of this specific fungi induced a reduction of the expression of all plant aquaporins under well-watered conditions. The down-regulation of aquaporins has been related to the formation of suberin at the endodermal apoplastic barriers in order to control water transport (Wang *et al.* 2019), although more studies will be needed to understand the role of *L. bicolor* in this respect. We found a negative correlation between the fungal aquaporin *JQ585596* and the poplar aquaporin *PtPIP1;4* (Supplementary Table S7). Negative correlations among fungal and plant aquaporins have been previously shown for other fungus–host systems (Aroca *et al.*, 2009), and they could be integrated into the signaling networks between the two organisms to orchestrate water uptake and the different responses to environmental conditions. What seems to be clear is that different fungal and plant aquaporins form part of a complex system that needs to be coordinated at both the plant and the fungal levels and, at least in the case of poplar, does not seem to be totally controlled by the fungal partner (in this case *L. bicolor*).

Our experiments resulted in only one treatment where L_{pr} increased, which was when both compartments were under drought and inoculated with *L. bicolor* (*Lacc-D/Lacc-D*). In this case, the system presented high ABA and reduced tZR contents (Fig. 5), together with an up-regulation of aquaporins associated with the compartments subjected to drought (Table 2) and a down-regulation of aquaporins associated with *L. bicolor* (Table 3). These observations were quite puzzling and raise the question as to whether the presence of mycorrhizal fungi is necessary at the whole-root level in order to have a faster response to drought or, on the other hand, whether the resources that the fungi demand are so high that the plant is more reactive to external stresses.

In conclusion, our results demonstrate how poplar saplings regulate their water balance by different compensatory mechanisms at the root level in response to different combinations of water stress and inoculation with *L. color*. Under mild drought, ABA concentrations increase and tZR concentrations decrease, whilst the expression of the plant aquaporins *PtPIP1;1*, *PtPIP2;2*, and *PtPIP2;7* is up-regulated. In the presence of *L. bicolor*, expression of *PtPIP1;4*, *PtPIP2;3*, and *PtPIP2;10* is down-regulated. When the whole root system is colonized with *L. bicolor* and is under drought stress, L_{pr} is increased. How these mechanisms interact and are coordinated in order to maintain water uptake under drought seems

to be related to ABA accumulation and coordinated with the overexpression of the fungal aquaporins *QJ585595* and *QJ585595*, while the reduced accumulation of tZR interacts with the plant aquaporins *PtPIP1;4* and *PtPIP2;7*.

Supplementary data

Supplementary data are available at JXB online.

Table S1. Leaf and shoot fresh weights, leaf relative water contents, and root fresh weights in the various treatments.

Table S2. Soil water potentials in the different compartments of the split-root system.

Table S3. MANOVA main factor *P*-values for the expression of root aquaporins in poplar.

Table S4. MANOVA main factor *P*-values for root PIP1 and PIP2 abundance and PIP2 phosphorylation state in poplar.

Table S5. MANOVA main factor *P*-values for root hormone contents in poplar.

Table S6. Pearson correlation matrix among root hydraulic conductivity, aquaporin expression, abundance, and phosphorylation state, and ABA and tZR contents in poplar.

Table S7. Pearson correlations among fungal aquaporin and root aquaporin expression. Abundance, and phosphorylation state, and root ABA and tZR contents in ectomycorrhizal poplar.

Fig. S1. Root aquaporin mRNA expression of *PtPIP1;1*, *PtPIP1;2*, *PtPIP2;5*, and *PtPIP2;7* in response to drought in poplar split-roots.

Fig. S2. Root aquaporin mRNA expression of *PtPIP1;4*, *PtPIP2;3*, *PtPIP2;5*, and *PtPIP2;10* in response to the presence of *L. bicolor* in poplar split-roots.

Fig. S3. Root PIP2 phosphorylated state at serine 280 (PIP2₂₈₀) in poplar in the different split-root treatments.

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