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 × deltoides* 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...
suberin at 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 (PIP5), tonoplast (TIP5), Nodulin26-like (NIP5), X (XIP5), 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).

Physiohormones 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 ($L_r$), 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 $L_r$ (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 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 Zwiscek, 2011). Increases in $L_r$ 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 result in the increasing 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-Rodenas 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* × *deltaoides* 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* × *deltaoides*, 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 (17x10 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 with a 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-W/WNI-WW). (2) One compartment was non-inoculated and the other was inoculated with *Laccaria*, and both were well-watered (NI-W/W-Lacc-WW). (3) Both compartments were inoculated and both were well-watered (Lacc-W/W-Lacc-WW). (4) Both compartments were non-inoculated, one was well-watered and the other under drought (NI-W/WNI-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-W/WNI-D). (7) Both compartments were inoculated, one was well-watered and the other under drought (Lacc-W/WNI-D). (8) Both compartments were non-inoculated and under drought (NI-D/Lacc-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 inoculum 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...
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, 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 IQ1-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 μM. 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 QuantiStudio3 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 immunosassay 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 (PIP2280/283). Goat anti-rabbit IgG coupled with horseradish peroxidase (Sigma-Aldrich) was used as the secondary antibody for PIP1 (at 1:10 000). Goat anti-rabbit IgG coupled to horseshadish peroxidase (Sigma-Aldrich) was used as a secondary antibody for PIP2 and PIP2280, PIP2283, and PIP2280/283 (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-zein riboside (t-ZR, and c-ZR), N6- (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/MD Sciex, Ontario, Canada), equipped with an electrospray interface.

Plant physiological measurements

Root and shoot fresh weights, stomatal conductance (g), and leaf relative water content (RWC) were determined at the time of harvest for six plants per treatment combination (i.e. n=6). g, was measured 2 h into the photosynthesis photon flux density (PPFD) of 450–500 µmol m−2 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 KNO3, 0.5 mM KH2PO4, 2.5 mM Ca(NO3)2, 1 mM MgSO4, 23 µM H2BO3, 5 µM MnCl2, 0.3 µM ZnSO4, 0.2 µM CuSO4, 0.01 µM (NH4)2MoO4,2, and 90 µM EDTA-Fe.

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

| Treatments         | Compartment 1 | Compartment 2 |
|--------------------|---------------|---------------|
|                     | L. bicolor    | L. bicolor    |
| 1 Ni-Ww / Ni-Ww    | –             | –             |
| 2 Ni-Ww / Lacc-Ww  | –             | Yes           |
| 3 Lacc-Ww / Lacc-Ww| Yes           | Yes           |
| 4 Ni-Ww / Ni-D     | –             | –             |
| 5 Ni-D / Lacc-Ww   | Yes           | Yes           |
| 6 Ni-Ww / Lacc-D   | Yes           | Yes           |
| 7 Lacc-Ww / Lacc-D | Yes           | Yes           |
| 8 Ni-D / Ni-D      | Yes           | Yes           |
| 9 Ni-D / Lacc-D    | Yes           | Yes           |
| 10 Lacc-D / Lacc-D | Yes           | Yes           |

Nl, 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.

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

| Treatment | Compartment 1 | Compartment 2 |
|-----------|---------------|---------------|
|           | L. bicolor    | L. bicolor    |
| 1 Ni-Ww / Ni-Ww | –             | –             |
| 2 Ni-Ww / Lacc-Ww | –             | Yes           |
| 3 Lacc-Ww / Lacc-Ww | Yes           | Yes           |
| 4 Ni-Ww / Ni-D | –             | –             |
| 5 Ni-D / Lacc-Ww | Yes           | Yes           |
| 6 Ni-Ww / Lacc-D | Yes           | Yes           |
| 7 Lacc-Ww / Lacc-D | Yes           | Yes           |
| 8 Ni-D / Ni-D | Yes           | Yes           |
| 9 Ni-D / Lacc-D | Yes           | Yes           |
| 10 Lacc-D / Lacc-D | Yes           | Yes           |

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

| Treatments         | Compartment 1 | Compartment 2 |
|--------------------|---------------|---------------|
|                     | L. bicolor    | L. bicolor    |
| 1 Ni-Ww / Ni-Ww    | –             | –             |
| 2 Ni-Ww / Lacc-Ww  | –             | Yes           |
| 3 Lacc-Ww / Lacc-Ww| Yes           | Yes           |
| 4 Ni-Ww / Ni-D     | –             | –             |
| 5 Ni-D / Lacc-Ww   | Yes           | Yes           |
| 6 Ni-Ww / Lacc-D   | Yes           | Yes           |
| 7 Lacc-Ww / Lacc-D | Yes           | Yes           |
| 8 Ni-D / Ni-D      | Yes           | Yes           |
| 9 Ni-D / Lacc-D    | Yes           | Yes           |
| 10 Lacc-D / Lacc-D | Yes           | Yes           |
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, \( g_s \), \( \Psi_w \), 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 \(-0.19\pm0.01\) MPa (±SE), while in the drought compartments the mean potential was \(-0.40\pm0.01\) 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 \( \Psi_w \) (\( P=0.010 \)), and in whole-root \( L_{pr} \) (\( P=0.019 \)). The treatment \( \text{Lacc-D/Lacc-D} \) had the highest \( g_s \) and \( L_{pr} \), and the lowest \( \Psi_w \) (Figs 1, 2), while the \( \text{NI-Ww/NI-D} \) treatment had the lowest \( L_{pr} \) values and was among the treatments with the lowest \( g_s \).

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 independently of water stress (Fig. 1B). There was a negative correlation between \( \Psi_w \) 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 \( \Psi_w \) (\( r=-0.141, P=0.28 \)) 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 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 \( L_w \) (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 \( L_w \) (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 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 |
|-----------|-----------------------------------------|--------------|---------------|------------------------|
| PtPIP1;1  | 0.85±0.08 A                             | 0.81±0.09 A  | 0.46±0.10 B   |
| PtPIP1;2  | 1.23±0.10 A                             | 1.46±0.12 A  | 0.83±0.11 B   |
| PtPIP1;4  | 0.07±0.02 A                             | 0.06±0.01 A  | 0.04±0.01 A   |
| PtPIP2;3  | 0.82±0.07 A                             | 0.89±0.08 A  | 0.83±0.08 A   |
| PtPIP2;4  | 0.02±0.01 A                             | 0.05±0.03 A  | 0.02±0.01 A   |
| PtPIP2;5  | 0.25±0.04 B                             | 0.47±0.04 A  | 0.13±0.04 C   |
| PtPIP2;7  | 0.21±0.03 A                             | 0.26±0.03 A  | 0.09±0.03 B   |
| PtPIP2;10 | 0.05±0.01 A                             | 0.06±0.02 A  | 0.05±0.02 A   |
| PtP2      | 0.004±0.0003 AB                         | 0.005±0.0003 AB| 0.004±0.0003 B |
| PtP2\_Ser280 | 0.007±0.0002 A | 0.007±0.0003 A | 0.007±0.0002 A |

Expression of root aquaporins is in relative units using Elongation Factor-1 alpha as the reference gene. Abundance of PtP2 and its phosphorylation state are expressed as mg µg\(^{-1}\) protein. Data are means (±SE), \( n=3 \) Different letters within the same line indicate significant differences as determined using MANOVA (\( \alpha=0.05 \)).

**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 | \( L. \) bicolor treatment applied to split-roots | No \( L. \) bicolor | One side \( L. \) bicolor | Two sides \( L. \) bicolor |
|-----------|-----------------------------------------------|---------------------|---------------------------|---------------------------|
| PtPIP1;1  | 0.68±0.06 A                                  | 0.86±0.11 A         | 0.61±0.07 A               |
| PtPIP1;2  | 1.21±0.10 A                                  | 1.29±0.11 A         | 0.99±0.10 A               |
| PtPIP1;4  | 0.07±0.01 A                                  | 0.07±0.01 A         | 0.02±0.01 B               |
| PtPIP2;3  | 0.03±0.01 A                                  | 0.02±0.01 AB        | 0.01±0.01 B               |
| PtPIP2;4  | 0.25±0.04 A                                  | 0.22±0.03 A         | 0.18±0.04 A               |
| PtPIP2;5  | 0.26±0.04 A                                  | 0.30±0.05 A         | 0.26±0.04 A               |
| PtPIP2;7  | 0.21±0.04 A                                  | 0.25±0.03 A         | 0.19±0.04 A               |
| PtPIP2;10 | 0.08±0.02 A                                  | 0.04±0.01 AB        | 0.03±0.02 B               |
| PtP2      | 0.005±0.0003 A                               | 0.004±0.0003 B     | 0.004±0.0003 B            |
| PtP2\_Ser280 | 0.007±0.0002 A | 0.007±0.0003 A | 0.007±0.0003 A            |

Expression of root aquaporins is in relative units using Elongation Factor-1 alpha as the reference gene. Abundance of PtP2 and its phosphorylation state are expressed as mg µg\(^{-1}\) protein. Data are means (±SE), \( n=3 \) Different letters within the same line indicate significant differences as determined using MANOVA (\( \alpha=0.05 \)).
Calvo-Polanco et al. (P = 0.0002; Supplementary Table S4, Fig. 4) and by the drought treatment (P = 0.006), while the phosphorylation of PIP2 was affected individually by the drought treatment (P = 0.022) and the fungal treatment (P = 0.022; Supplementary Fig. S3). PIP1, PIP2, and PIP2 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 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 × 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 examined 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 detected 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. 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 (±SE), n = 3. Different letters indicate significant differences among the treatments as determined using ANOVA followed by Tukey’s test (α = 0.05).
correlated with \( \text{PtPIP1;4} \) \((r=-0.26, P=0.047)\) (Supplementary Table S7). In addition, \( \text{tZR} \) was positively correlated with \( \text{PtPIP1;4} \) \((r=0.41, P<0.001)\) and negatively correlated with \( \text{PtPIP2;7} \) \((r=-0.20, P=0.032)\) (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 \( L. \text{bicolor} \) 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 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 \( \text{tZR} \) content that were independent of \( L. \text{bicolor} \) inoculation if only half the root system was inoculated (Fig. 5, Table 4), together with an up-regulation of the plant aquaporins \( \text{PtPIP1;1}, \text{PtPIP1;2}, \text{PtPIP2;5}, \) and \( \text{PtPIP2;7} \) (Table 2). \( \text{PIP2;5} \) and \( \text{PIP2;7} \) have been shown to have high water transport capacity in \( \text{Xenopus laevis} \) oocyte swelling assays (Marjanović et al., 2005; Secchi and Zwieniecki, 2010), and \( \text{PIP2;5} \) 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-Rodriguez 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 $L_{w}$ have been reported as either being positive (Veselov 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-Rodriguez 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 $L_{w}$ 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, $L_{w}$ is increased. How these mechanisms interact and are coordinated is important to maintain water uptake under drought seems
to be related to ABA accumulation and coordinated with the overexpression of the fungal aquaporins QF5853595 and QF585595, 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 PtPIP1 and PtPIP2 abundance and PtPIP2 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 PtPIP2 phosphorylated state at serine 280 (PIP2_280Ng) in poplar in the different split-root treatments.

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