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Aquaporin regulation in roots controls plant hydraulic conductance, stomatal conductance, and leaf water potential in *Pinus radiata* under water stress

Juan Rodríguez-Gamir[1,2] | Jianming Xue[2] | Michael J. Clearwater[3] | Dean F. Meason[4] | Peter W. Clinton[2] | Jean-Christophe Domec[5,6]

[1] Departamento de Suelos y Riegos, Instituto Canario de Investigaciones Agrarias (ICIA), Ctra de El boquerón s/n. 38270. San Cristóbal de La Laguna, Tenerife, Canary Islands, Spain

[2] Forest Systems, Scion, PO Box 29237, Christchurch 8440, New Zealand

[3] Environmental Research Institute, University of Waikato, Private Bag 3105, Hamilton, New Zealand

[4] Forest Systems, Scion, Private Bag 3020, Rotorua 3046, New Zealand

[5] Bordeaux Sciences Agro, UMR INRA ISPA 1391, Gradignan, France

[6] Nicholas School of the Environment, Duke University, Durham, North Carolina, USA

Correspondence
Dr Juan Rodríguez Gamir, Instituto Canario de Investigaciones Agrarias (ICIA), Ctra. de el Boquerón s/n, 38270. San Cristóbal de la Laguna, Tenerife, Canary Islands, Spain.
Email: jrodriguez@icia.es

Dr Jean-Christophe Domec, Bordeaux Sciences Agro, UMR INRA ISPA 1391, Gradignan, France.
Email: jc.domec@agro-bordeaux.fr

Present Address
Juan Rodríguez-Gamir, Instituto Canario de Investigaciones Agrarias (ICIA), Ctra. de El Boquerón s/n, 38270. San Cristóbal de la Laguna, Tenerife, Spain.

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Abstract

Stomatal regulation is crucial for forest species performance and survival on drought-prone sites. We investigated the regulation of root and shoot hydraulics in three *Pinus radiata* clones exposed to drought stress and its coordination with stomatal conductance (g_s) and leaf water potential (Ψ_{leaf}). All clones experienced a substantial decrease in root-specific root hydraulic conductance ($K_{root\text{-}r}$) in response to the water stress, but leaf-specific shoot hydraulic conductance ($K_{shoot\text{-}l}$) did not change in any of the clones. The reduction in $K_{root\text{-}r}$ caused a decrease in leaf-specific whole-plant hydraulic conductance ($K_{plant\text{-}l}$). Among clones, the larger the decrease in $K_{plant\text{-}l}$, the more stomata closed in response to drought. Rewatering resulted in a quick recovery of $K_{root\text{-}r}$ and g_s. Our results demonstrated that the reduction in $K_{plant\text{-}l}$, attributed to a down regulation of aquaporin activity in roots, was linked to the isohydric stomatal behaviour, resulting in a nearly constant Ψ_{leaf} as water stress started. We concluded that higher $K_{plant\text{-}l}$ is associated with water stress resistance by sustaining a less negative Ψ_{leaf} and delaying stomatal closure.

KEYWORDS

aquaporin activity, drought stress, isohydric

1 | INTRODUCTION

Pinus radiata D. Don is one of the world's most extensively planted exotic softwood species for forest production (Stone, Penman, & Turner, 2012). A decrease in future precipitation and an increase in the frequency of hot-dry days per year have been predicted for many *P. radiata* growing regions, potentially constraining its productivity (IPCC, 2013). Structural and physiological adaptations to drought determine the growth and survival of forest tree species in dry climates (Tenhunen, Lange, & Pearcy, 1987). On a short time scale, this regulation is primarily physiological rather than structural. At the physiological level, *P. radiata* drought response includes decreased leaf

water potential and reduced stomatal conductance, transpiration, and photosynthesis rate (De Diego, Pérez-Alfocea, Cantero, Lacuesta, & Moncaleán, 2012). Leaf water potential reflects whole-plant water status (Martínez-Vilalta et al., 2009), and less negative leaf water potential under water stress is associated with drought resistance (Levitt, 1972; Pantuwan et al., 2004; Turner, 1982). Stomatal closure regulates the decrease in leaf water potential (Oren et al., 1999) and provides the most obvious and important mechanism for plants to control water loss under drought conditions. In this regard and like most conifers, *P. radiata* is classified as a strongly isohydric species (Brodribb & McAdam, 2013), meaning that stomata close early during drought within a very narrow range of leaf water potentials.

Most of the models that try to explain stomatal behaviour are generally incapable of providing an insight into the mechanisms through which stomata respond to water stress (Damour, Simonneau, Cochard, & Urban, 2010). It has been proposed that the integration of a drought-induced variable whole-plant hydraulic conductance is necessary to explain plant behaviour (Baert, De Schepper, & Steppe, 2015) and stomatal regulation mechanisms under water stress (Vandeleur et al., 2009). A number of studies have indicated a functional relationship between stomatal conductance and hydraulic conductance of the leaf (Brodribb, Holbrook, Zwieniecki, & Palma, 2005; Sack, Tyree, & Holbrook, 2005; Sadok & Sinclair, 2010), shoot (Yang & Tyree, 1993), stem (Martorell, Díaz-Espejo, Medrano, Ball, & Choat, 2014; Nardini & Salleo, 2000) or root (Domec & Pruyn, 2008; Perrone et al., 2012; Rodríguez-Gamir et al., 2011; Rodríguez-Gamir, Intrigliolo, Primo-Millo, & Forner-Giner, 2010). Those studies focused on separate plant components (leaves, stems, and roots), which has led to a lack of understanding about the integration of whole-plant hydraulics and the repercussions of this on stomatal regulation and plant water status, especially under water stress conditions (Domec et al., 2009; Pratt, North, Jacobsen, Ewers, & Davis, 2010). Therefore, understanding the dynamics of the whole hydraulic system in trees holds great potential for explaining stomatal regulation and the control of plant transpiration under conditions of low soil water availability (McCulloh & Woodruff, 2012).

From the point of view of hydraulic architecture, the effect of higher or lower plant hydraulic efficiency (i.e., high or low leaf-specific whole-tree hydraulic conductance) on plant physiology under water stress conditions is controversial. It has been suggested that low hydraulic conductance is related to drought resistance (Oliveras et al., 2003). Namely, limiting sap flow from roots to leaves promotes conservative water use and favours a better water balance in the plant (Yamada, Katsuhara, Kelly, Michalowski, & Bohnert, 1995). This can be caused by having smaller tracheid diameters (Oliveras et al., 2003) or by decreasing membrane permeability to water flow, to preserve water content of the cells (Johansson et al., 1998; Suga, Komatsu, & Maeshima, 2002; Yamada et al., 1995). However, it is also argued that high hydraulic efficiency improves plant water status by maintaining lower xylem water potential gradients and lower xylem embolism (Corcuera, Gil-Pelegrín, & Notivol, 2012; Martínez-Vilalta et al., 2009; Peguero-Pina et al., 2011). This controversy, apart from being determined by different adaptation mechanisms among species, may be due to the fact that many studies have been performed only at root, leaf, or even at plant specific tissue level without considering their respective and integrated effects at the whole-plant scale.

On a short-time scale, aquaporins (plasma membrane proteins) can regulate hydraulic conductance of different organs. This is achieved through changes in abundance or activity in response to a number of environmental cues, including water stress (Gilliham et al., 2011; Maurel, Verdoucq, Luu, & Santoni, 2008). The role of these aquaporins is widely demonstrated in roots and leaves (Javot & Maurel, 2002; Tyerman, Niemietz, & Bramley, 2002) and more recently in stems (Almeida-Rodriguez & Hacke, 2012; Steppe, Cochard, Lacointe, & Améglio, 2012). In both herbaceous and woody plants, aquaporin inhibition results in a large decrease in water flux and a reduction in hydraulic conductance by more than 60% (Adiredjo, Navaud, Grieu, & Lamaze, 2014; Gambetta et al., 2013; Johnson, Sherrard, Domec, & Jackson, 2014). Although it is known that aquaporins are involved in plant adaptation and resistance against water stress (Luu & Maurel, 2005), there is no general consensus about the pattern and physiological significance of aquaporin expression or activity in response to this stress. Thus, both down-regulation and up-regulation of aquaporin activity and/or expression have been reported under water stress conditions and may potentially be most useful once water becomes available again after a drought (Gambetta, Knipfer, Fricke, & McElrone, 2016). This issue is made more complex by the differences that exist within and between species, and between plant compartments (see reviews, Aroca, Porcel, & Ruiz-Lozano, 2012; Chaumont & Tyerman, 2014).

The growth, form, and wood properties of commercially grown *P. radiata* have been improved through generations of breeding, and deployment of selected clones has become more common (Baltunis & Brawner, 2010). Existing forest tree breeding programmes mostly rely on improving wood traits favourable for timber and pulp as well as disease and pest resistance (reviewed by Mullin et al., 2011, for conifers). However, adaptation to a changing climate is generally considered indirectly by evaluating growth or survival (Marguerit et al., 2014). Given the predicted increase in drought frequency and severity, the importance of plantation forestry on the hydrological cycle, and the increasing demand for water from rural and urban sectors, the issue of water use by forests will likely be one of the most important questions in many countries in the coming decades (Dunningham, Kirschbaum, Payn, & Meason, 2012; Dvorak, 2012). The New Zealand commercial forestry sector is increasingly interested in tree species and genotypes that are more drought tolerant and are more water efficient in growing wood. Improving understanding of the hydraulic and physiological mechanisms controlling drought stress responses in *P. radiata* from commercial breeding programmes will contribute to this goal.

This study assessed the response to drought stress of several commercially available *P. radiata* genotypes and determined how this stress affected the root and shoot hydraulic properties and their relative contributions to the whole-tree hydraulic conductance as the soil dries. Using greenhouse experiments with three different genotypes of *P. radiata*, we tested the following hypotheses: (a) Changes in the hydraulic conductance of root and/or shoot affect the partitioning of whole-tree hydraulic conductance; (b) the aquaporin-mediated regulation of hydraulic conductance is linked with the stomatal dynamics; and (c) plant hydraulic efficiency (i.e., leaf-specific plant hydraulic conductance) regulates the decline in leaf water potential and stomatal conductance under water stress.

2 | MATERIAL AND METHODS

2.1 | Plant material, growth conditions, and experimental design

We used three 1-year-old *P. radiata* genotypes (thereafter Clone 15, Clone 44, and Clone 48) supplied by Forest Genetics Ltd. (Rotorua, New Zealand). These genotypes belong to a set of clonally tested progenies developed by Forest Genetics Ltd. from the control-crossed parent trees identified in the Radiata Pine Breeding Company programme (Dungey et al., 2009), which represent a subset of the genetic entities in New Zealand's deployment population available to forest growers. The clones were selected for the following traits: improved growth rate, stem form, wood properties (i.e., corewood stiffness and wood density) and resistance to *Dothistroma pini* (M. Carson, pers. comm.). Clonal stoolbeds were established from somatic seedlings ("emblings"), and stem cutting technology was used for propagating these clones. Two hundred uniform ramets of each of three 1-year-old genotypes were potted individually in 2-L plastic pots with a potting mix (15% bark at 5–15 mm, 50% pine A Grade fines, 15% cocoa fibre—coir, 20% pumice 7 mm) and grown in a glasshouse in Christchurch, New Zealand (43°33′S, 172°47′E) in Spring (September). Before the experiment, plants were watered twice per week and fertilized at the beginning of the growing season with a commercial slow-release fertilizer (18:18:18 N:P:K).

A water stress experiment was conducted during the late summer (January–February) with an average photoperiod of 14 daylight hours. The plants were maintained in the glasshouse where day time average temperature was 25 ± 2°C and relative humidity was 50 ± 5% throughout the experimental period. Plants were separated in two groups and labelled: 15C, 44C, and 48C for the control plants (no water stress) and 15WS, 44WS, and 48WS for the water-stressed plants. The drought experiment lasted 7 weeks, and plants were randomly distributed throughout the glasshouse and surrounded by some buffering plants that were not used for the study. During the experiment, the pots with plants were covered with a sheet of plastic to prevent soil evaporation. Control plants were irrigated with 3 L of water once per week, which was enough to saturate the substrate. For the water-stressed plants, watering was withheld from the start until the end of the experiment. Every week, midday leaf water potential (Ψ_{leaf}), stomatal conductance (g_s), and whole-plant transpiration (T_p) were measured. Hydraulic conductance of roots (K_{root}) and shoots (K_{shoot}) and volumetric soil water content (θ) were measured in Weeks 1, 2, 5, and 7. The aquaporin contribution to K_{root} (AQP contribution) was determined in Week 2. For each clone and at each sampling date, all the parameters were determined in at least six individual plants per treatment.

Additionally, after 1 week of water stress, one set of 15WS plants were rewatered at 10:00 a.m. until the substrate was saturated (15WS-R). During the same day and the following day, K_{root}, AQP contribution, and g_s were measured periodically from 10:00 a.m. to 4:00 p.m.

At the end of the experiment, the mean growth rates (g week^{-1}) of control and water-stressed plants were calculated by dividing the increase in plant dry mass between the beginning and the end of the experiment by the number of weeks the experiment lasted (7 weeks). Further, in Week 7, a dye staining experiment on stem of Clone 48 was performed to assess the extent of embolized xylem area.

2.2 | Hydraulic conductance of root, shoot, and whole plant

Root hydraulic conductance (K_{root}) and shoot hydraulic conductance (K_{shoot}) were measured using a High Conductance Flow Meter (HCFM; Dynamax Inc., Houston, Texas, USA). These measurements allowed us to look at the hydraulic conductances and the partitioning of resistances between different components (i.e., root and shoot) of the whole-plant water transport pathway. Values of K_{root} and K_{shoot} for a given plant were obtained from the same plant. The plants were cut at 5 cm above the soil surface, and the cut ends of the shoots and roots were connected to the HCFM. This equipment perfuses degassed water through the root or shoot system by applying pressure to a water-filled bladder contained within the unit. The flow rate of water through root or shoot was determined using the HCFM under transient mode (Bogeat-Triboulot, Martin, Chatelet, & Cochard, 2002; Tyree, Patiño, Bennink, & Alexander, 1995), with flow measured under increasing pressure applied by a nitrogen gas cylinder. The applied pressure was increased gradually from 0 to approximately 300 KPa over the course of approximately 1 min and the flow rate logged every 2 s using the Dynamax software. Flow rates measured at increasing pressure are less vulnerable to flow rate reductions caused by the plant's wound response than flow rates measured at constant pressure (Judd, Jackson, Fonteno, & Domec, 2016; Li & Liu, 2010; Tyree et al., 1995). Once the transient curve was constructed, hydraulic conductance (K) was calculated using the formula: $K = Q_v/P$; where Q_v is the volumetric flow rate (kg s^{-1}) and P is the applied pressure (MPa). Temperature was automatically recorded by the HCFM, and all conductance measurements were corrected to values at 25°C. Because the HCFM operates under high pressure, the measured K_{root} and K_{shoot} represent maximum values of conductances, that is, in the absence of embolized conduits. To minimize the potential impact of diurnal periodicity on K_{root} and K_{shoot} (Tsuda & Tyree, 2000), all measurements were taken between 10:00 a.m. and 4:00 p.m. at ambient temperature and inside the glasshouse where the plants were kept during the experiment. Once K_{root} was measured, the volumetric soil water content (θ) in the pot was determined with a time domain reflectometry MiniTRASE Kit (Soilmoisture Equipment Corp., Santa Barbara, California, USA).

Using K_{root} and K_{shoot}, whole-plant hydraulic conductance (K_{plant}) was calculated as (Domec, Palmroth, & Oren, 2016):

$$1/K_{plant} = 1/K_{root} + 1/K_{shoot}. \quad (1)$$

After measuring hydraulic conductance, the total projected leaf area of all the needles of each plant was measured with a Li-Cor Li-3100 Area Meter (Li-Cor Biosciences Inc., Lincoln, Nebraska, USA), and all plant fractions were dried in a forced-draft oven at 60°C for 48 hr and weighed. All hydraulic parameters were normalized by leaf area or dry biomass. We calculated root-specific root hydraulic conductance ($K_{root\text{-}r}$) by dividing K_{root} by the root dry weight (DW). Leaf-specific shoot and plant hydraulic conductance ($K_{shoot\text{-}l}$ and $K_{plant\text{-}l}$) were

obtained by dividing K_{shoot} and K_{plant}, respectively, by the total projected leaf area. Shoot and root contributions to the whole-plant conductance were evaluated through the K_{root}/K_{shoot} ratio.

2.3 | Aquaporin contribution to K_{root}

The aquaporin contribution to K_{root} (AQP contribution) was quantified by using hydroxyl radicals (*OH) to inhibit root aquaporin activity (Henzler, Ye, & Steudle, 2004; Ye & Steudle, 2006). We produced hydroxyl radicals using the Fenton reaction by mixing equal parts of 0.6-mM H_2O_2 and 3-mM $FeSO_4$. For measuring K_{root} with the aquaporin activity inhibited ($K_{root\text{-}inh}$), approximately 10 ml of *OH solution was introduced, instead of water, into the existing compression couplings between the root system and the HCFM (Johnson et al., 2014; McElrone et al., 2007). We calculated the aquaporin contribution to K_{root} as

$$\text{AQP contribution} = (K_{root} - K_{root\text{-}inh})/K_{root}^{*} 100 \quad (2)$$

In order to demonstrate the effectiveness of hydroxyl radicals in detecting changes in aquaporin activity, the kinetics of aquaporin-mediated inhibition of K_{root} was investigated after total shoot decapitation (Vandeleur et al., 2014). To do so, an independent experiment was performed with well-watered plants of Clone 15. To reduce the effect of possible diurnal variation in K_{root}, plants were periodically decapitated at 10 cm above the soil surface from 10:00 a.m. to 4:00 p.m. Measurements were made between 12:00 p.m. to 4:00 p.m. During this period, the measurement of plants with a different time span between decapitation and measuring time was randomized. Plants were recut at 5 cm above the soil surface at the moment of connecting to the HCFM. K_{root} was measured in 25 plants by perfusing water through the root system and in another 25 plants by perfusing *OH solution.

2.4 | Cavitation curves and dye staining experiments

Dye staining experiments on stems were performed to assess the extent of embolized xylem area (Mayr et al., 2014). Both control plants and water-stressed plants harvested 7 weeks after the beginning of the experiment were sampled. Fresh stem segments (12 cm long) were cut under water and connected to a reservoir with a solution of 2% (*w*/v) Phloxine-B solution (Sigma Chemicals). After staining for 10 min under a pressure head of 7.5 KPa, stems were cut in the centre and pictures of cross sections taken. Stained areas indicate functional xylem, whereas embolism blocked the flow of staining solution and resulted in unstained areas (Mayr et al., 2014). In addition, to test that the Phloxine-B solution did not cause refilling of the stem when being perfused, cavitation was induced in control plants of Clone 48 with a cavitation chamber (Model 1505D-EXP Pressure Chamber Instrument, PMS Instrument Company, USA). After inducing cavitation at different pressures (between 1 and 9 MPa), stem segments were connected to the Phloxine-B solution for about 10 min and pictures of cross sections taken. In parallel, loss of hydraulic conductivity was also measured on a subsample of unstained but pressurized stems to create vulnerability to embolism curves (Sperry & Saliendra, 1994).

2.5 | Whole-plant transpiration (T_p)

To measure whole-plant transpiration (T_p), the pots were kept covered with plastic sheets and weekly transpiration of each control plant was calculated as the difference between the weight of the watered pot (after draining) and the weight of the pot before watering the following week. Stressed plants were weighed every week at the same time as control plants.

2.6 | Stomatal conductance and water potential

Stomatal conductance (g_s) was measured weekly with a portable photosynthesis system (LI-6400XT, Li-Cor, Lincoln, NE, USA) equipped with a light source. For each tree, three fascicles taken from the central part of each plant were placed across the 2 × 3 cm cuvette to avoid shading between needles. Temperature in the cuvette was maintained at 25°C, whereas the leaf-to-air vapour pressure deficit was maintained around 1 kPa to limit its negative effect on g_s (Oren et al., 1999). The needles were left to equilibrate at a CO_2 concentration of 400 μmol mol^{-1} and saturating irradiance (1500 μmol m^{-2} s^{-1}). Measurements were recorded after the values of g_s were stable. For the 7-week-long water stress experiment, the measurements were taken between 10:00 a.m. to 2:00 p.m. All measurements are presented on a surface area basis. The foliage surface area of the fascicles used for each measurement was calculated for fascicles consisting of three needles by $S = (d\ l(3+\pi))/2$, where S is the fascicle surface included in the Li-Cor cuvette, d is the fascicle diameter, and l is the length of the needles segment clipped in the cuvette (i.e., 3 cm; Bown, Watt, Mason, Clinton, & Whitehead, 2009).

Midday (Ψ_{leaf}) leaf water potentials were measured in needles similar to those used for g_s measurement with a Model 600 Pressure Chamber Instrument (PMS Instrument Company, Albany, OR, USA).

2.7 | Statistical analyses

All measured parameters were tested by multiple analysis of variance, with *clone*, (water stress) *treatment*, and the *week of measurement* as factors, with mean separation by the Tukey's test at 95% confidence level, with R software version 3.1.0 (Pumpkin Helmet).

The *week of measurement* and its interactions with the other factors had no significant effect ($P > 0.05$) on the variance of the normalized hydraulic parameters ($K_{root\text{-}r}$, $K_{shoot\text{-}l}$, and $K_{plant\text{-}l}$) and on the variance of the K_{root}/K_{shoot} ratio (Table S1). This allowed the results for these parameters to be expressed as averages over the total experimental period for the factors *clone* and *treatment*.

3 | RESULTS

3.1 | Plant biomass

Clone 15 had higher biomass ($P < 0.05$) at the end of the experiment (Table 1) and higher growth rate (0.64 g $week^{-1}$) than the other two clones (0.27 and 0.49 g $week^{-1}$ for Clones 44 and 48, respectively).

TABLE 1 Plant, leaf, root, and stem dry weights (DWs) and leaf/root ratio (L/R) for Clones 15, 44, and 48 of *Pinus radiata* at the beginning ($15_{initial}$, $44_{initial}$, and $48_{initial}$) and the end of the experiment, in control plants ($15C_{final}$, $44C_{final}$, and $48C_{final}$) and in water-stressed plants ($15WS_{final}$, $44WS_{final}$, and $48WS_{final}$

Clone-treatment	Plant DW (g)	Leaf DW (g)	Root DW (g)	Stem DW (g)	L/R
$15_{initial}$	21.68 ± 0.59	9.11 ± 0.26	7.59 ± 0.24	4.98 ± 0.18	1.20 ± 0.020
$15C_{final}$	25.52 ± 0.87	10.59 ± 0.33	8.47 ± 0.28	6.47 ± 0.33	1.25 ± 0.021
$15WS_{final}$	22.44 ± 0.74	9.33 ± 0.35	7.92 ± 0.29	5.19 ± 0.21	1.18 ± 0.022
$44_{initial}$	20.70 ± 0.60	8.19 ± 0.33	7.96 ± 0.24	4.55 ± 0.14	1.03 ± 0.032
$44C_{final}$	22.32 ± 0.64	8.71 ± 0.35	7.98 ± 0.19	5.63 ± 0.18	1.08 ± 0.032
$44WS_{final}$	20.98 ± 0.69	8.10 ± 0.25	7.63 ± 0.33	5.26 ± 0.17	1.07 ± 0.029
$48_{initial}$	19.49 ± 0.32	7.27 ± 0.16	8.36 ± 0.17	3.86 ± 0.14	0.87 ± 0.022
$48C_{final}$	22.45 ± 0.61	8.44 ± 0.33	9.20 ± 0.26	4.81 ± 0.36	0.93 ± 0.048
$48WS_{final}$	20.19 ± 0.59	7.59 ± 0.24	8.43 ± 0.37	4.17 ± 0.13	0.91 ± 0.038
Multifactorial ANOVA performed for the biomass data at the end of the experiment					
Factors					
Clone	***	***	**	***	***
Treatment	***	***	*	***	n.s.
Clone × Treatment	n.s.	n.s.	n.s.	n.s.	n.s.

Note. Values are means ± SE (n = 12). n.s., not significant.

*P < 0.05.

**P < 0.01.

***P < 0.001.

Water stress significantly reduced growth of all three genotypes (P < 0.001). For all the clones, this decrease in plant growth was attributed to a reduction in leaf DW (P < 0.001), root DW (P < 0.05), and stem DW (P < 0.001) biomass. Despite this reduction in plant size, the leaf/root ratio was not affected (P > 0.38) by the water stress treatment.

3.2 | Plant hydraulic system regulation

Whole-plant hydraulic conductance (K_{plant}) for control plants of two (Clones 15 and 48) out of three clones increased (P < 0.05) over the experimental period (Figure 1). This increase was positively correlated with their increasing size, with r^2 values ranging from 0.41 to 0.56. After the first week of stress, K_{plant} for all three clones sharply decreased (P < 0.001), and this was maintained at lower values than in control plants throughout the experiment.

At any given date, leaf-specific whole-plant hydraulic conductance ($K_{plant\text{-}l}$) was significantly lower (P < 0.001) for all three clones growing under water stress conditions than under control conditions (Figure 2a). The overall reduction in $K_{plant\text{-}l}$ was 16.2% for Clone 15, 19.4% for Clone 44, and 24.4% for Clone 48 (Figure 2b). Under water stress conditions, 15WS had the highest $K_{plant\text{-}l}$ and 48WS the lowest (P < 0.001). No significant differences (P > 0.05) in $K_{shoot\text{-}l}$ were found between the control and stressed plants for each clone (Figure 2c,d). In contrast to shoots, water stress reduced root-specific hydraulic conductance ($K_{root\text{-}r}$; P < 0.001) by 35–50% as soon as the water stress was imposed (Figure 2e,f).

For the three clones studied, the reduction in K_{root} following the water stress treatment caused a significant (P < 0.001) decrease in the K_{root}/K_{shoot} ratio (Figure 3), making the relative contribution of the shoot to the total plant conductance larger. There was no time effect on K_{root}/K_{shoot} in water-stressed plants as water stress progressed (P > 0.05; Table S1). For both control and stressed plants, this ratio was always greater than one, indicating that the resistances to water movement were larger in shoots than in roots.

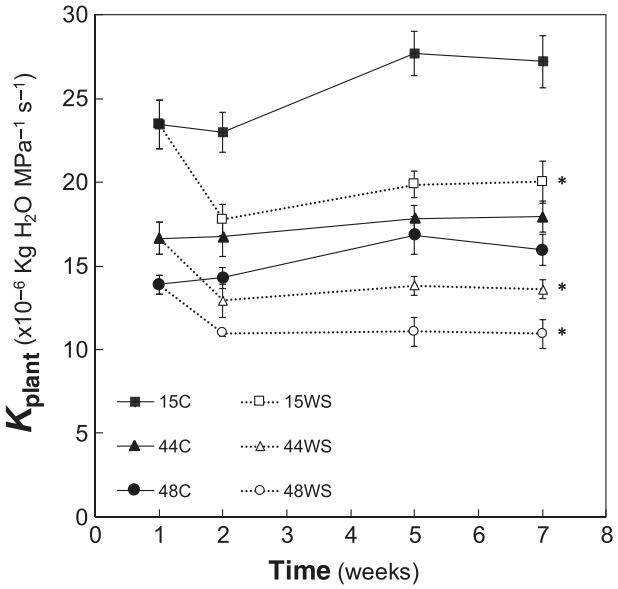

FIGURE 1 Time course of whole-plant hydraulic conductance (K_{plant}) of Clones 15, 44, and 48 of *Pinus radiata* for control plants (15C [black squares], 44C [black triangles], and 48C [black circles]) and water-stressed plants (15WS [white squares], 44WS [white triangles], and 48WS [white circles]). Values are means ± SE (n = 6). The asterisks indicate statistically significant differences between control and stressed plants for each clone (P < 0.05)

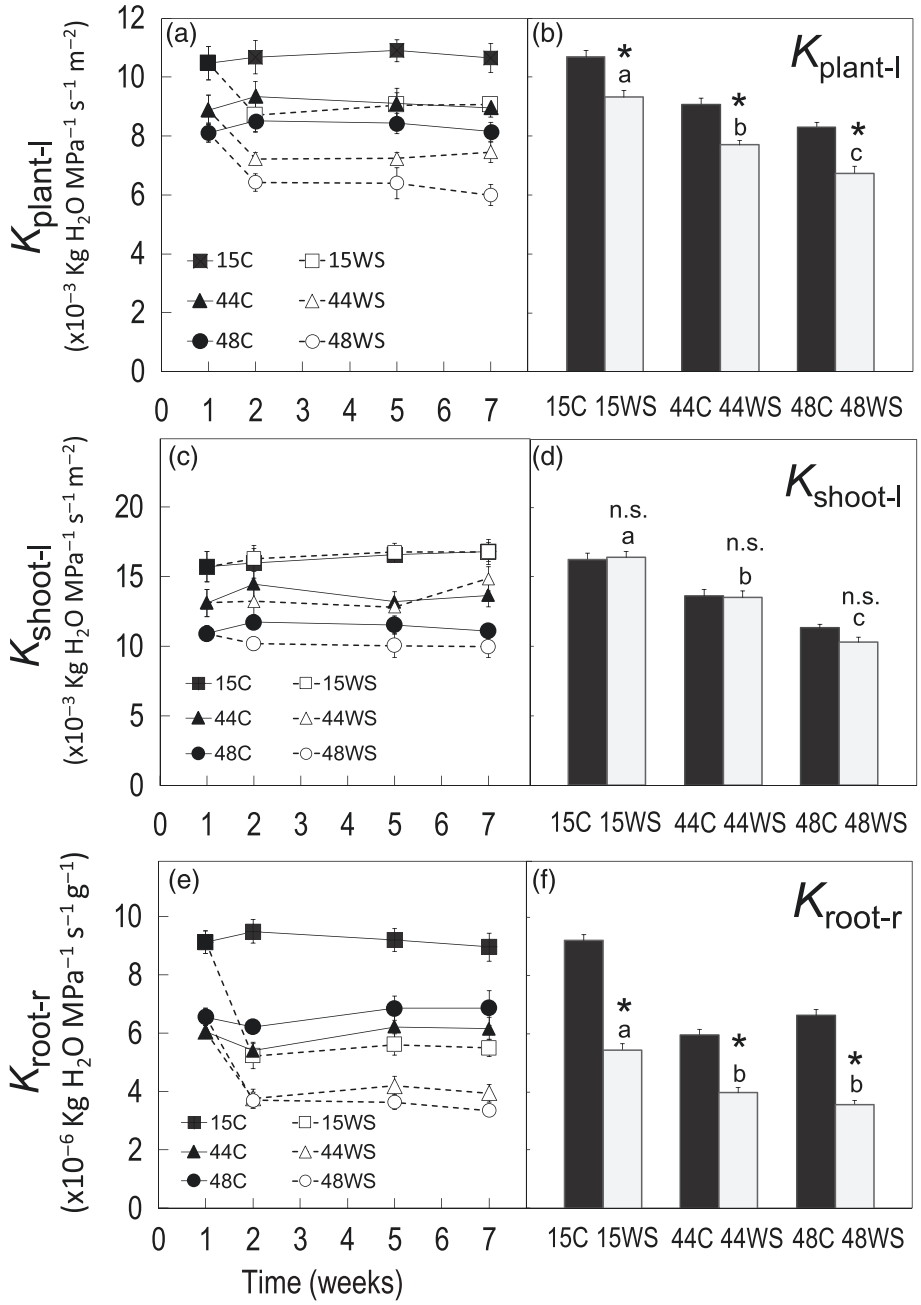

FIGURE 2 Left panels: Time courses of (a) leaf-specific whole-plant hydraulic conductance ($K_{plant-l}$), (c) leaf-specific shoot hydraulic conductance ($K_{shoot-l}$), and (e) root-specific root hydraulic conductance (K_{root-r}) of Clones 15, 44, and 48 of *Pinus radiata* for control plants (15C [black squares], 44C [black triangles], and 48C [black circles]) and water-stressed plants (15WS [white squares], 44WS [white triangles], and 48WS [white circles]). Values are means ± SE (n = 6). Right panels: Mean values across the 7-week-long measurements of (b) $K_{plant-l}$, (d) $K_{shoot-l}$, and (f) K_{root-r} of Clones 15, 44, and 48 for control plants (15C, 44C, and 48C; black bars) and water-stressed plants (15WS, 44WS, and 48WS; grey bars). Values are means ± SE (n = 18). The asterisks indicate statistically significant differences between control and stressed plants of each clone (P < 0.05). Different letters indicate differences between the clones under water stress conditions (P < 0.05).

Stem vulnerability to embolism curves were similar between clones and combined with stained stem cross sections indicated that significant loss of conductivity occurred when xylem pressures were lower than −5 MPa (Figure S1A). However, in the water stress experiment, no differences were found in the stem stained cross sectional area between control and water-stressed plants after 7 weeks of treatment (Figure S1B), indicating that embolism and loss of stem hydraulic conductivity did not occur during the drought period.

3.3 | Aquaporin contribution to K_{root}

Results of the independent experiment for testing the effectiveness of hydroxyl radicals to inhibit the aquaporin activity showed that total shoot decapitation caused a linear time-dependent reduction in K_{root-r}. K_{root-r} decreased to approximately 20% of the starting value by 3 hours after shoot removal (Figure 4). When hydroxyl radicals were perfused through the root system, $K_{root-inh}$ was similar

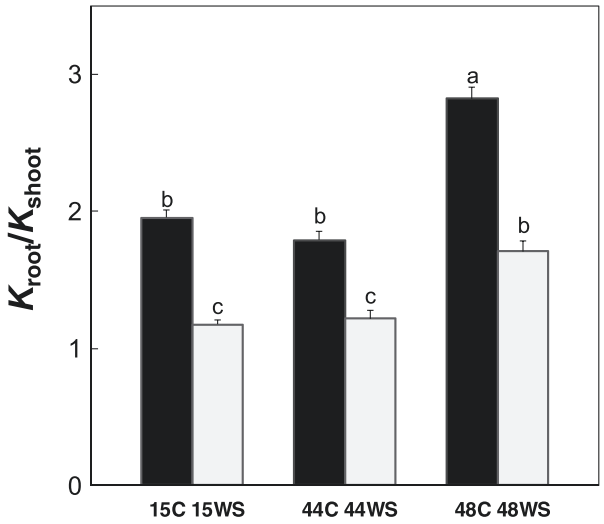

FIGURE 3 Mean values across the 7-week-long measurements of K_{root}/K_{shoot} ratio of Clones 15, 44, and 48 of *Pinus radiata*, for control plants (C15, C44, and C48; black bars) and water-stressed plants (15WS, 44WS, and 48WS; grey bars). Values are means ± SE ($n = 18$). Different letters indicate statistically significant differences at $P < 0.05$

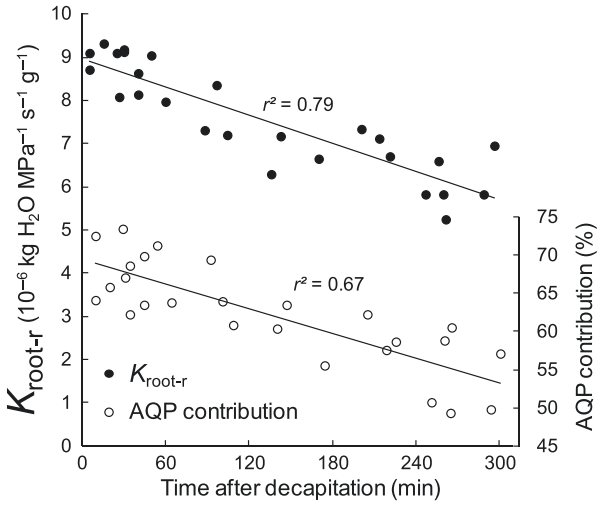

FIGURE 4 The impact of shoot decapitation on root-specific root hydraulic conductance (K_{root-r}; black circles) and aquaporin contribution to K_{root} (AQP contribution; white circles) of well-watered plants of clone 15 of *Pinus radiata*

for all the plants and independent of the time between plant decapitation and connection to the HCFM demonstrating the effectiveness of the radicals to inhibit the aquaporin activity. Therefore, the decline in K_{root-r}, throughout the 3 hours, was matched by a reduction in the AQP contribution (from 69.2% to 53.1%) to K_{root} (Figure 4).

At the early stages of the water stress treatment (beginning of Week 2), the aquaporin contribution to K_{root} was significantly reduced ($P < 0.001$) in all three clones under water stress conditions when compared with the control (Figure 5). This reduction varied among the clones, with the largest reduction (68.0%) in Clone 48 and the lowest (32.5%) in Clone 15.

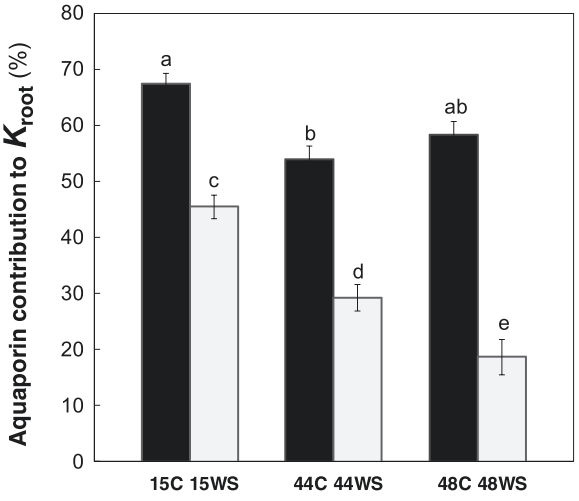

FIGURE 5 Aquaporin contribution to K_{root} of Clones 15, 44, and 48 of *Pinus radiata* for control plants (15C, 44C, and 48C; black bars) and water-stressed plants (15WS, 44WS, and 48WS; grey bars) 2 weeks after the beginning of the imposed water stress. Values are means ± SE ($n = 6$). Different letters indicate statistically significant differences ($P < 0.05$)

3.4 | Whole-plant transpiration and soil water content

Under control conditions, Clone 15 had the greatest ($P < 0.01$) transpiration rates (T_p) throughout the experiment with an average value of 371 gH_2O $week^{-1}$, whereas Clones 44 and 48 had an average T_p of 335 and 324 gH_2O $week^{-1}$ (Figure 6a). On a leaf area basis, those values corresponded to 150.1 ± 4.6, 138.8 ± 1.2, and 134.9 ± 2.1 kgH_2O $week^{-1}$ m^{-2} for Clones 15, 44, and 78, respectively. Average weekly T_p did not differ between clones under water stress conditions ($P > 0.05$). T_p decreased significantly in response to water stress to reach, as average, 16 ± 1 gH_2O $week^{-1}$ (or 6.8 ± 0.4 kgH_2O $week^{-1}$ m^{-2} on a leaf area basis) by the sixth week of the imposed drought (Figure 6a). Over the measurement period, volumetric soil water content of the control plants was 22% (close to full saturation for the potting mix we used; Figure 6b). The drop in soil water content in stressed plants was similar ($P > 0.05$) among the three clones, resulting in soil water contents of 9% in Week 2 and 4% in Week 7.

3.5 | Stomatal conductance and leaf water potential

Instantaneous stomatal conductance (g_s) decreased strongly after the first week of stress in all three water-stressed clones (Figure 7a [relative values] and Figure S2 [absolute values]). However, the effect of drought on g_s differed between clones throughout the experiment ($P < 0.001$) with Clones 15 and 48 experiencing small and large stomatal closure, respectively. The different stomatal closure experienced by the clones under water stress throughout the experiment was linearly associated with the decrease in $K_{plant-l}$ (Figure 7b). A slope analysis of these responses of g_s to $K_{plant-l}$ indicated that stomatal sensitivity to $K_{plant-l}$ decreased ($P < 0.02$) by more than two-fold as drought became more severe.

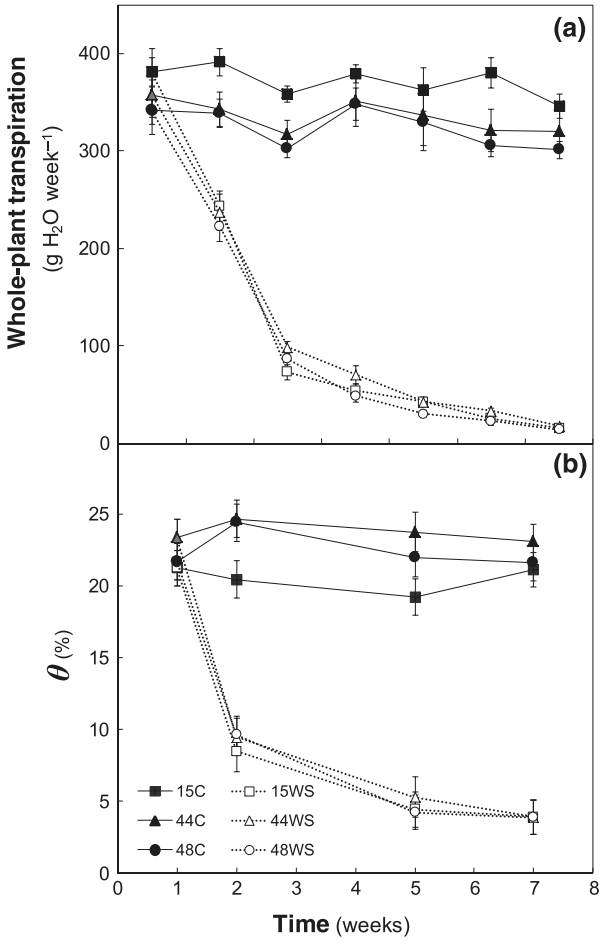

FIGURE 6 Time courses of (a) whole-plant transpiration and (b) volumetric soil water content (θ) of Clones 15, 44, and 48 of *Pinus radiata* in control plants (15C [black squares], 44C [black triangles], and 48C [black circles]) and water-stressed plants (15WS [white squares], 44WS [white triangles], and 48WS [white circles]). Values are means ± SE (n = 6–10)

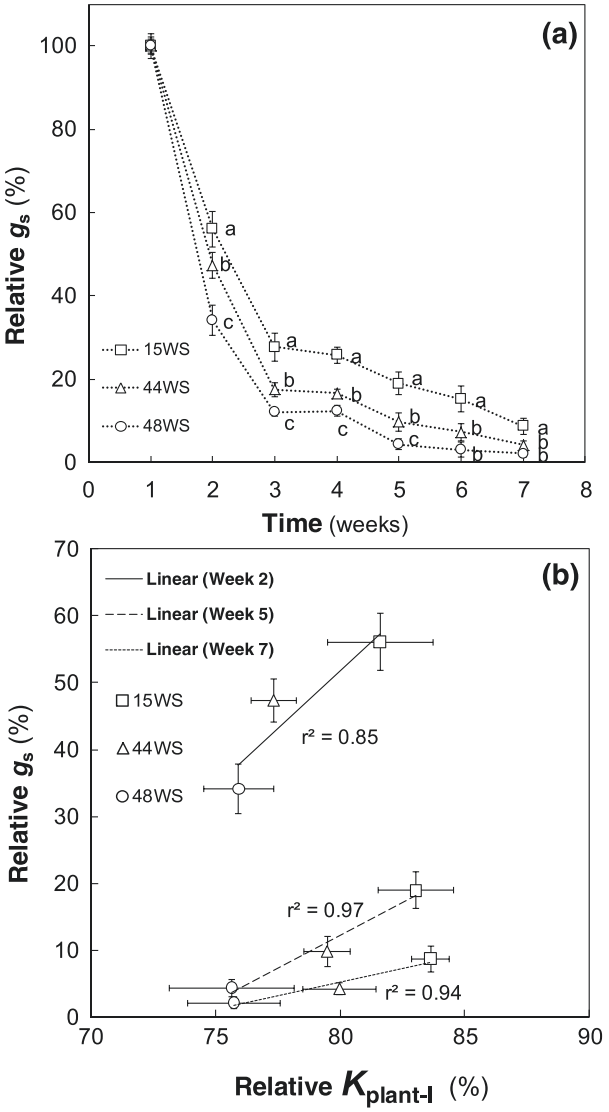

FIGURE 7 (a) Time courses of relative stomatal conductance (relative to control plants, in %) in water-stressed plants of Clones 15, 44, and 48 of *Pinus radiata* (15WS [white squares], 44WS [white triangles], and 48WS [white circles]). Values are means ± SE (n = 6). Different letters indicate statistically significant differences between clones for each sampling time (P < 0.05). (b) Relationship between decreased leaf-specific whole-plant hydraulic conductance (relative $K_{plant\text{-}l}$, in %) and relative stomatal conductance (in %) for Clones 15, 44, and 48 of *Pinus radiata* after 2, 5, and 7 weeks after the beginning of the imposed water stress

Leaf midday water potential (Ψ_{leaf}) of the control plants did not vary throughout the experiment and were stable across clones at −0.69 ± 0.02 MPa (Figure 8a). However, Ψ_{leaf} of water-stressed plants was significantly reduced (P < 0.001) from the third week of the experiment (Figure 8a). Plants with higher $K_{plant\text{-}l}$ (15WS) had a slower decline in Ψ_{leaf} and reached less negative values of Ψ_{leaf} at the end of the experiment. Among clones, there was a common non-linear negative relationship between g_s and Ψ_{leaf} (Figure 8b). Approximately, 50% of the reduction in g_s occurred before Ψ_{leaf} fell below −1 MPa, progressing to 85% at −2 MPa.

3.6 | Dynamics during recovery

When 15WS plants were rewatered after 1 week of water stress (15WS-R), $K_{root\text{-}r}$ increased progressively in the 5 hr after rewatering, reaching similar values to those of control plants (P > 0.05; Figure 9a). The increase in $K_{root\text{-}r}$ was paralleled by an increase in root aquaporin activity (Figure 9a) and a reopening of stomata in rewatered plants (Figure 9b). Rewatered plants did not completely open the stomata until the following day after rewatering. The next day after rewatering, stomatal conductance did not differ between control and rewatered plants (P > 0.05).

4 | DISCUSSION

4.1 | Coordination between the plant hydraulic system, leaf water potential, and stomatal conductance

This study showed that changes in root biomass, root specific conductance, and root aquaporin activity under water stress are integrated when whole plant is measured. As opposed to measuring only individual

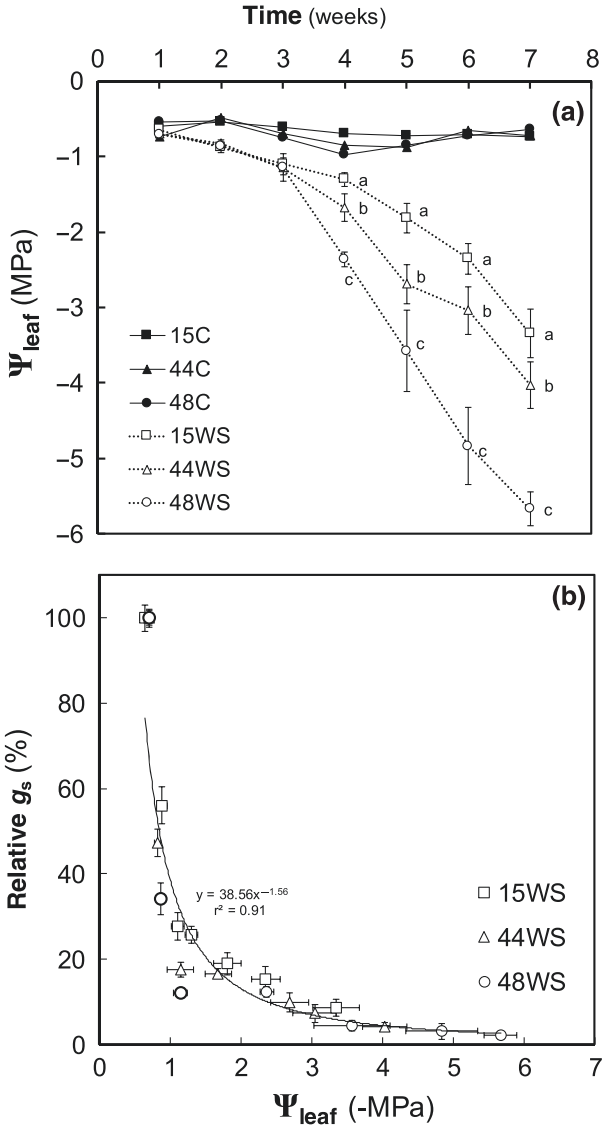

FIGURE 8 (a) Time courses of leaf midday water potentials (Ψ_{leaf}) in control (15C, 44C, and 48C) and water-stressed *Pinus radiata* clones (15WS, 44WS, and 48WS). Values are means ± SE (n = 6). Different letters for the water-stressed treatment indicate statistically significant differences between clones for each sampling time (P < 0.05). There was no difference in Ψ_{leaf} for any of the weeks in the control treatment. (b) Trajectories of percentage of stomatal closure to declining Ψ_{leaf} during imposed water stress in Clones 15, 44, and 48 of *P. radiata*

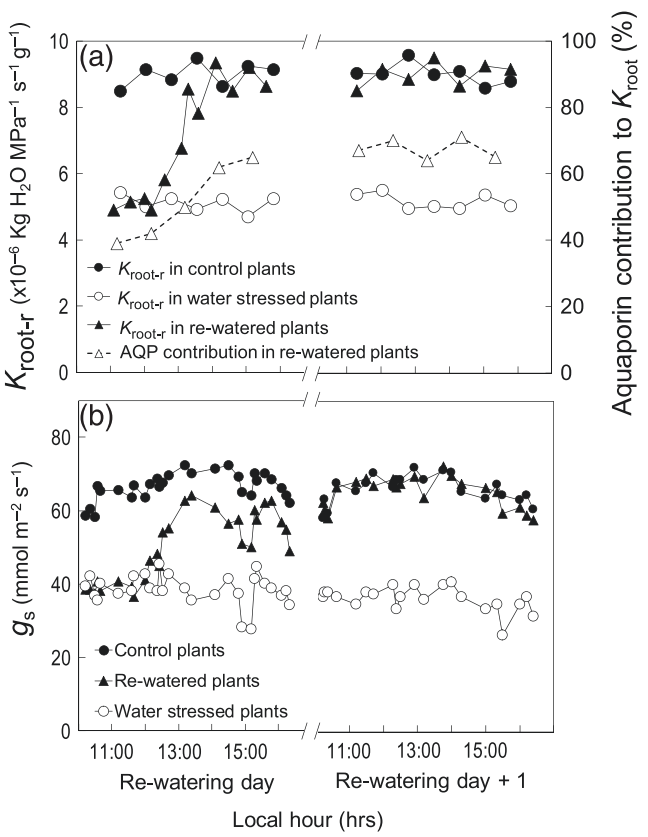

FIGURE 9 Trajectories after a rewatering event of (a) K_{root-r} in control (black circles), water-stressed (white circles) and rewatered plants (black triangles) and AQP contribution to K_{root}in rewatered plants (white triangles) and (b) stomatal conductance in control (black circles), water-stressed (white circles), and rewatered plants (black triangles) of Clone 15 of *P. radiata*. Each point represents an individual measurement of each parameter

roots, the hydraulic conductance of the whole root system represents whole-plant water uptake. We expected variation across clones in growth rates and that this variability would be linked to traits that reflect differences in key structural and hydraulic traits such that rapid growth rates under sufficient water availability would be associated with increased K_{root} and K_{shoot} and decreased tolerance to water stress. Whole-plant hydraulic conductance was down-regulated in response to drought stress through changes in K_{root-r}. The different magnitude of regulation of hydraulic conductance between clones under water stress conditions resulted in Clone 15, the fastest growing clone, having the greatest ability to supply water to the needles (represented by $K_{plant-l}$) and Clone 48 the lowest (Figure 2b). Soil volumetric water content was similar for stressed plants of all three clones throughout the experiment; thus, each clone was exposed to similar soil water potential (Figure 6). However, the clones presented different rates of stomatal closure and decreases in Ψ_{leaf} in response to the imposed water stress over time (Figures 7a and 8a), thus revealing a hydraulic effect on the regulation of g_s and Ψ_{leaf}. Clone 15, with the highest $K_{plant-l}$ under water stress, had the slowest stomatal closure and the slowest decline in Ψ_{leaf}. This clone had the highest growth rate. Conversely, Clone 48, which had the lowest value of $K_{plant-l}$, had the fastest decline in Ψ_{leaf} and the highest stomatal closure.

The relationship between stomatal closure and Ψ_{leaf} (Figure 8b) was similar for the three clones and demonstrated the expected strong stomatal regulation of an isohydric species to drought stress. At the beginning of the imposed water shortage, stressed plants experienced a large decline in g_s (Figure 7a). A more than 50% decline in g_s occurred before Ψ_{leaf} reached −1 MPa, before any potential loss of xylem conductivity due to embolism occurred in either stem of needles (Figure S1; Bouche et al., 2014; Brodribb & Cochard, 2009). Sharp reductions in g_s together with maintaining constant Ψ_{leaf} would require a feed-forward control of g_s with respect to Ψ_{leaf} via feedback sensing of water status in some other portion of the plant such as the roots (Meinzer, 2002). Hubbard, Ryan, Stiller, and Sperry (2001) and, more recently, Attia, Domec, Oren, Way, and Moshelion (2015) and Chaumont and Tyerman (2014) proposed that stomatal isohydric

behaviour is regulated by $K_{plant\text{-}l}$ to maintain more constant Ψ_{leaf}. This hypothesis was supported by the relationship between stomatal closure and the decreased $K_{plant\text{-}l}$ found in this study (Figure 7b), which demonstrated that $K_{plant\text{-}l}$ acted in concert with stomata to limit water loss under conditions of low soil water content (Domec & Johnson, 2012; Meinzer, 2002). However, despite results showing strong coordination between stomatal closure and $K_{plant\text{-}l}$ until the end of the experiment (Figure 7b), stressed plants had no further reduction in $K_{plant\text{-}l}$ after Week 3 (Figure 2a) that can explain the slower but continued decline in g_s (Figure 7a). This suggests that additional factors could be involved in the regulation of g_s (Franks, Drake, & Froend, 2007). After the initial stomatal closure, g_s could be more dependent on Ψ_{leaf} (Brodribb & McAdam, 2013), which in turn, in our experiment, was modulated by $K_{plant\text{-}l}$, that is, a higher $K_{plant\text{-}l}$ resulted in a less negative Ψ_{leaf}. As the relationship between stomatal closure and Ψ_{leaf} was similar for all three clones (Figure 8b), the less negative Ψ_{leaf} (mediated by higher $K_{plant\text{-}l}$) allowed the stomata to remain more open. Therefore, as water stress progressed, Ψ_{leaf} related to the balance between g_s (which controls water losses) and hydraulic conductance (which controls water supply to the leaves; Tardieu, Simonneau, & Parent, 2015).

To our knowledge, this is the first study of *P. radiata* hydraulics undertaken by using different genotypes of this species. Our findings agree with results reported recently for other *Pinus* species. Corcuera et al. (2012) observed reductions in $K_{plant\text{-}l}$ under drought stress for different populations of *Pinus pinaster* adapted to mesic or xeric areas, and demonstrated that more xeric-adapted populations had higher $K_{plant\text{-}l}$ under drought stress conditions. Martínez-Vilalta et al. (2009) studied the branch-level hydraulic properties of *Pinus sylvestris* populations along a climatic gradient and found that higher leaf-specific stem hydraulic conductivities enabled the maintenance of lower xylem water potential gradients with increasing climate dryness.

4.2 | Hydraulic function and coordination of roots and shoots in drying soil

The down-regulation of $K_{root\text{-}r}$ was the leading cause of changes in $K_{plant\text{-}l}$ (Figure 2) and appeared to be an important aspect of drought sensing. In addition, as the soil volumetric water content decreased during the experiment, it is likely that drought-induced stomatal closure became linked to an interaction between both chemical and hydraulic information (De Diego et al., 2012; Tardieu & Davies, 1993). Stomatal conductance has been shown to decline even in the absence of reduction in Ψ_{leaf} (Zhang & Davies, 1987). Brodribb and McAdam (2013) showed for *P. radiata* that foliar accumulation of abscisic acid needed 5 weeks to rise to maximum levels after the beginning of an imposed drought, or occurred when Ψ_{leaf} was below −2.5 MPa. Five weeks of imposed drought were also the time required to reach this Ψ_{leaf} in two of the clones studied (15 and 44), indicating that a hormonal signal and Ψ_{leaf} could potentially have had an additive effect on stomatal closure at very high water stress. Apparently, when water stress started, abscisic acid was not the signal that triggered stomatal closure in *P. radiata* (Brodribb & McAdam, 2013). In our study, the sharp decline in $K_{root\text{-}r}$ when water stress started (Figure 3 e) and the strong relationships between hydraulics and stomatal closure (Figure 7b) suggest that the down-regulation of $K_{root\text{-}r}$ provided the hydraulic signal that caused the stomatal closure. Conversely, we did not find reductions in $K_{shoot\text{-}l}$ in response to drought stress (Figure 2c,d). Additionally, the parallel recovery of $K_{root\text{-}r}$ and g_s demonstrates strong coordination between them (Figure 9a). Rewatered plants recovered to the same values of $K_{root\text{-}r}$ as the control plants within a few hours of rewatering. However, the total recovery of g_s was not observed until the following day after watering, which has previously been observed for *P. radiata* (Brodribb & McAdam, 2013). This could be due to the possible residual effect of accumulated abscisic acid.

Our results suggest that the aquaporin activity under water stress contributed strongly to the decline and recovery of $K_{root\text{-}r}$ (Figures 5 and 9a; Johnson et al., 2014). Thus, aquaporin activity is an inherent component of the hydraulic system. Two studies have demonstrated the dynamics of root hydraulics involving aquaporins were linked to an isohydric stomatal behaviour in a cultivar of *Vitis vinifera* L. (Vandeleur et al., 2009) and in poplar (Laur & Hacke, 2013). Because a molecular analysis of aquaporins was not performed in this study, the interpretation of the present data in terms of aquaporin activity reduction may be questioned. However, several experimental observations support our interpretation. First, the rapid recovery of $K_{root\text{-}r}$ after rewatering (Figure 9a) demonstrated that the earlier reduction in $K_{root\text{-}r}$ was not caused by anatomical modifications. Second, the increasing AQP contribution, as measured using hydroxyl radicals, paralleled the increase in $K_{root\text{-}r}$ (Figure 9a). Third, the decapitation experiment revealed a coupled decline in $K_{root\text{-}r}$ and AQP contribution (Figure 4) within the 4 hr of shoot removal; an effect also observed by Vandeleur et al. (2014). Finally, $K_{root\text{-}inh}$ was similar for all the plants when hydroxyl radicals were perfused through the root system and independent of the time between plant decapitation and connection to the HCFM. These observations also demonstrated that the use of hydroxyl radicals was suitable for estimating the aquaporin contribution to K_{root}.

Based on measured and published (Bouche et al., 2014) vulnerability curves for *P. radiata* seedlings, xylem embolism was probably small or absent in our drying experiment (Figure S1). It has been suggested that for conifers, the evolution of hydraulic transport efficiency was independent of the evolution of hydraulic resistance to drought-induced cavitation (Corcuera et al., 2012; Martínez-Vilalta et al., 2009; Piñol & Sala, 2000). However, partitioning of hydraulic conductance supports the concept that hydraulic architecture is coordinated across the plant and is an adaptive trait related to the trade-off between transport water efficiency and hydraulic safety at the scale of the whole plant (Drake, Price, Poot, & Veneklaas, 2015). For *P. radiata*, the occurrence of a K_{root}/K_{shoot} ratio greater than one under normal conditions and the maintenance of this ratio even under water stress (Figure 3) could be a mechanism to limit stomatal closure. If K_{shoot} was greater than K_{root} and leaf water demand was high, the roots could not provide all the water required by the aerial component, even with optimal soil water availability. Therefore, embolism formation would be more likely and stomata would have to close to adjust water uptake.

5 | CONCLUSIONS

We conclude that exposure of *P. radiata* to drying soil results in a fast down-regulation of root hydraulic conductance, likely aquaporin mediated. This leads to a decrease in leaf-specific whole-plant hydraulic conductance ($K_{plant\text{-}l}$) and is coupled with the stomatal closure that maintains Ψ_{leaf} (isohydric stomatal behaviour). As water stress becomes more severe, higher $K_{plant\text{-}l}$ is associated with a slowed decline in Ψ_{leaf} and g_s. Therefore, maintaining higher $K_{plant\text{-}l}$ under drought conditions may provide a competitive advantage and could be considered a drought stress resistance mechanism for this species. Further, this work shows the need for integration of whole-plant hydraulic response when attempting to understand plant responses to water stress; it also highlights the high sensitivity of stomata and, consequently, net CO_2 assimilation, of *P. radiata* as soil dries and the crucial role of the hydraulic system in controlling the stomatal response. Plantations of this species are often exposed to drought; therefore, a good understanding of its hydraulic traits should have a major impact on forest productivity.

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ORCID

Juan Rodríguez-Gamir http://orcid.org/0000-0002-0874-0463
Jean-Christophe Domec http://orcid.org/0000-0003-0478-2559

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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Supplementary Information. Table S1. The results of multifactor analysis of variance (MANOVA) for the parameters: root hydraulic conductance (K_{root}), shoot hydraulic conductance (K_{shoot}), whole plant hydraulic conductance (K_{plant}), leaf-specific plant hydraulic conductance ($K_{plant\text{-}l}$), root-specific root hydraulic conductance ($K_{root\text{-}r}$), leaf-specific shoot hydraulic conductance ($K_{shoot\text{-}l}$) and K_{shoot}/K_{root} ratio, using *clone*, *treatment* and *week of sampling* as factors. (n=6 for each *clone* x *treatment* x *week* combination).

Factors:	**clone**	**week**	**treatment**	**clone × week**	**clone × treatment**	**week × treatment**	**clone × week × treatment**	**Residuals**
Df:	2	3	1	6	2	2	4	105
Parameter:	F value	F value	F value	F value	F value	F value	F value	F value
K_{root}	77.31 ***	4.54 **	331.20 ***	0.20 n.s.	10.11 ***	1.81 n.s.	0.94 n.s.	
K_{shoot}	162.87 ***	4.31 **	13.10 ***	1.13 n.s.	0.33 n.s.	0.54 n.s.	0.36 n.s.	
K_{plant}	165.03 ***	5.56 **	113.32 ***	0.96 n.s.	2.84 n.s.	0.97 n.s.	0.38 n.s.	
$K_{plant\text{-}l}$	61.08 **	0.36 n.s.	71.61 ***	0.15 n.s.	0.48 n.s.	0.16 n.s.	0.08 n.s.	
$K_{root\text{-}r}$	101.70 ***	0.79 n.s.	298.65 ***	0.33 n.s.	9.67 ***	0.19 n.s.	0.96 n.s.	
$K_{shoot\text{-}l}$	84.55 ***	0.56 n.s.	2.14 n.s.	0.34 n.s.	1.33 n.s.	0.30 n.s.	0.30 n.s.	
K_{root}/K_{shoot}	89.68 ***	1.77 n.s.	183.16 ***	0.37 n.s.	6.99 **	0.37 n.s.	1.66 n.s.	

Signification codes:

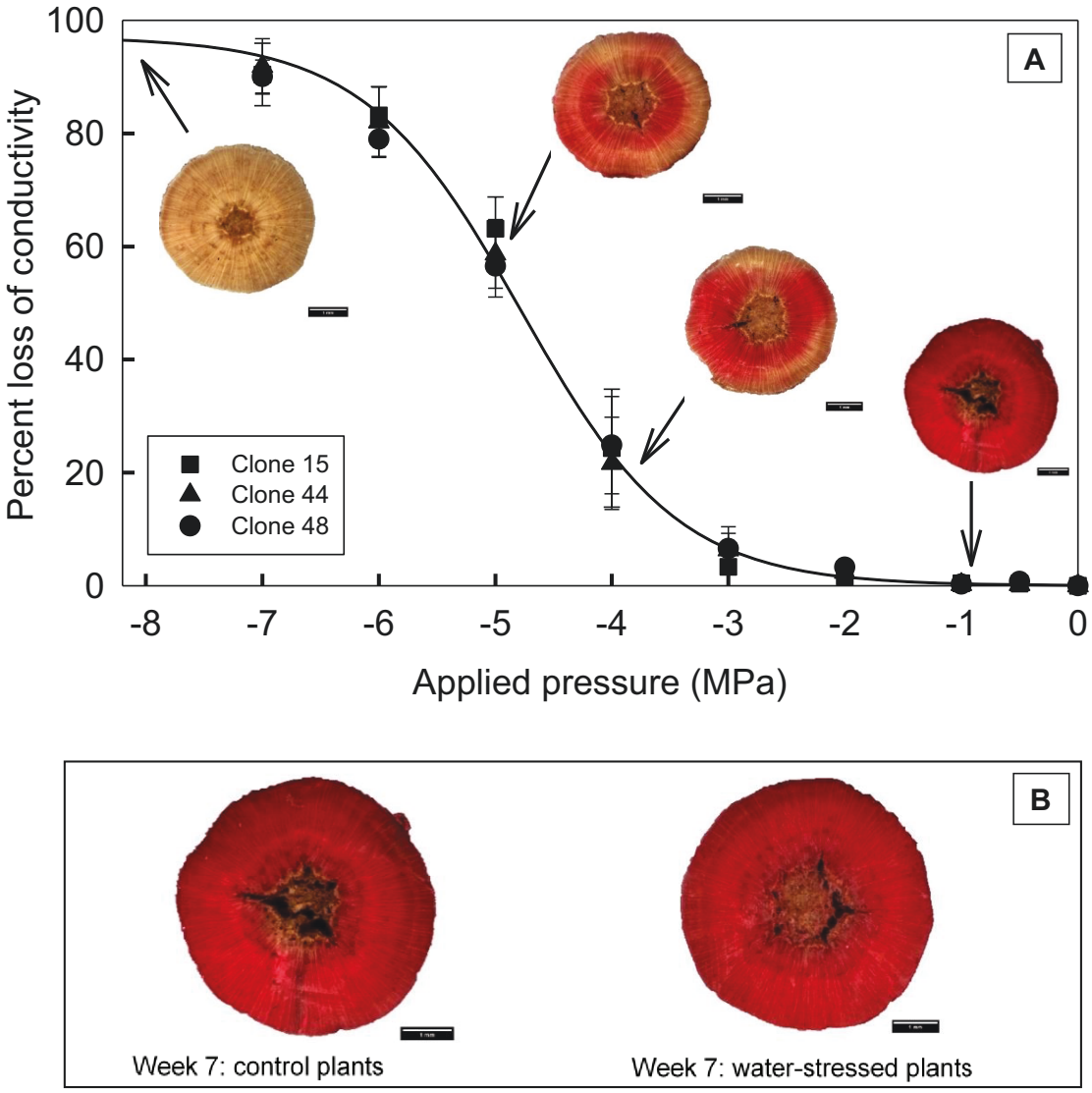
*** P<0.001

**P<0.01

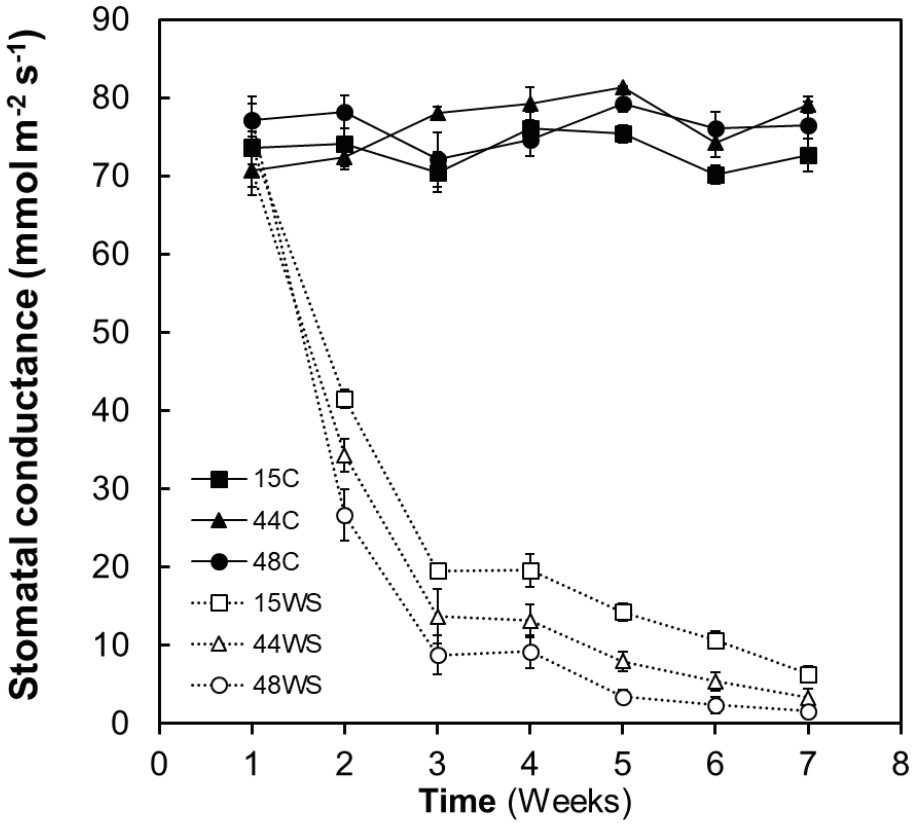
*P<0.05

.P<0.1

n.s., not-significant



Supplementary Information. Figure S1. (A) Vulnerability to embolism curve showing the percentage loss of stem hydraulic conductivity (PLC) in *Pinus radiata* seedlings for clones 15, 44 and 48. No difference in vulnerability curves was apparent between clones and therefore just one mean sigmoidal fitted curve is shown. The inserted images represent the stained stem cross sections of clone 48 at xylem pressures represented by the arrows. In **(B)** stained stem cross section of clone 48 at the end of the experiment in control (Ψ_{leaf} = -0.75 MPa) and water-stressed plants (Ψ_{leaf} = -5.6 MPa), indicating that no differences were found in the stained area between control and 7-weeks water stressed plants and that stem cavitation was no present during the drought period. Scale of each image represents 1 mm.



Supplementary Information. Figure S2. Time courses of stomatal conductance of clones 15, 44 and 48 of *P. radiata* for control plants (15C [black squares], 44C [black triangles] and 48C [black circles]) and water stressed plants (15WS [white squares], 44WS [white triangles] and 48WS [white circles]). Values are means ± SE (n=6).