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Dehydrins presence in xylem parenchyma cells enhances hydraulic conductivity and physiological performance in *Nothofagus dombeyi*

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ABSTRACT

Background: Nothofagus dombeyi is an evergreen tree that grows mainly in "Lahares," sites formed by volcanic scoria and subject to high radiation and low nighttime temperatures. It grows from sea level to the treeline, exploiting sites where freezing temperatures are frequent. In addition, this species is capable to acclimate to cold, maintaining high rates of physiological performance even at low temperatures.
Methods: We assessed the electron transport rate (ETR), xylem conductivity, embolism of vessels, and dehydrins (DHNs) accumulation in the xylem of *N. dombeyi* individuals subject to low temperatures in both cold-acclimated and non-acclimated plants. We hypothesize that *N. dombeyi* accumulates dehydrins in the xylem in response to cold; these dehydrins could be involved in avoiding the damages induced by freezing.
Results: An anti-DHN antibody recognized three cold-induced proteins between 15 and 40 kDa in the xylem of *N. dombeyi*. The hydraulic conductivity of xylem and electron transport rate (ETR) was higher in cold-acclimated plants than in non-acclimated plants. Contrarily, the percentage of embolized vessels was higher in non-acclimated plants than in cold-acclimated plants, being these responses consistent with the presence of dehydrins in the xylem.
Conclusions: We conclude that dehydrins could be participating in a mechanism to avoid the effects of cold-induced embolism conserving xylem functionality and help to explain the altitudinal upper distribution for *N. dombeyi*.

1. Introduction

Exposure to low temperatures can cause several negative effects to plant species such as: changes in the spatial organization of biological membranes, slowing of biochemical processes, and decreased cell water potential (Wise and Tunacliffe, 2004; Rorat, 2006). During freezing, water supply from roots to leaves is limited because soil and stems are frozen (Tranquillini, 1980; Hacke and Sperry, 2001). When xylem is frozen under tension, extensive embolism develops after thaw (Sperry and Sullivan, 1992). Embolism occurs as air bubbles, forced out of the solution during freezing, expand and provoke cavitation during thaw (Sperry and Sullivan, 1992; Langan et al., 1997). Embolized xylem vessels can be recovered, but only during periods of substantial positive xylem tension. Although the mechanisms involved in embolism-recovering are unclear, most hypotheses suggest an active role for the xylem parenchyma in water movement into embolized vessels (Améglio et al., 2001; Ewers et al., 2001).

Dehydrins (DHNs), also known as LEA D-11 or LEA II (late embryogenesis abundant), are proteins whose expression is induced by various environmental factors that cause cells dehydration (Ingram and Bartels, 1996; Kumar and Bhatla, 2006). Among these factors, drought and freezing are among the most notable (Rampino et al., 2006; Wahid and Close, 2007). DHNs are hydrophilic and form amphipathic α-helices that stabilize membranes during dehydration induced by freezing temperatures (Janská et al., 2010; Theocharis et al., 2012), suggesting their role in freezing tolerance (Trischuk et al., 2014). Presence of DHNs has been reported in parenchyma ray cells in the xylem of *Prunus persica* (Wisniewski et al., 1999; Kosova et al., 2007) and bark tissues in many species of woody plant (Wisniewsky et al., 1996). The presence of DHNs in living xylem cells suggests a role of DHNs in water uptake and/or transport under stress condition (Rorat, 2006). These proteins have also been suggested as been involved in membrane stabilization (Koag et al., 2003, Koag et al., 2009, Eriksson et al., 2011).

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Nothofagus dombeyi (Mirb.) Oerst. is an evergreen pioneer tree that grows mainly in "Lahares," sites formed by volcanic scoria and subjected to high radiation and low night-time temperature. It grows from sea level to the treeline, exploiting sites where low freezing temperatures commonly occur (McQueen, 1977). Even at low temperatures, *N. dombeyi* has a relatively high photosynthetic rate (Reyes et al., 2005), suggesting the presence of cold-tolerance mechanisms. In addition, electron transport rate (ETR) has been shown to correlate with photosynthesis (Lambers et al., 2008; Ploschuk et al., 2014) and they are considered a good proxy of physiological performance (Lambers et al., 2008; Molina-Montenegro et al., 2011). Thus, a positive correlation between hydraulic conductivity and ETR could suggest a carbon gain through photosynthesis (Hubbard et al., 2001; Brodribb et al., 2002). Also, a constant water supply is required to maintain a high photosynthetic rate, avoiding stomatal closure. Thus, if *N. dombeyi* individuals maintain their physiological performance even under cold-stress conditions, they must also have mechanism to maintain their hydraulic conductance.

In this study, we evaluated the xylem conductivity, electron transport rate, and presence of dehydrins in the xylem of *N. dombeyi* subject to low temperatures. We tested this on cold-acclimated and non-acclimated plants. Our general hypothesis is that *N. dombeyi* accumulates dehydrins in the xylem in response to cold; these dehydrins could be involved in avoiding the damages induced by freezing. Also, cold-acclimated plants should perform better at low temperatures and accumulate more DHNs compared with non-acclimated plants.

2. Materials and methods

2.1. Plant material

Thirty individuals, 2-years-old, of *N. dombeyi* from Aysén, XI region of Chile, located near the southern-most part of their distribution were collected and transported to the laboratory to be used in all experiments. They were divided into three groups of 10 plants each, which were then subjected to different temperature treatments in walk-in growth chambers (20 m^2). All groups were maintained at 16 h/8 h light/dark photoperiod. Two groups were kept under control conditions (18 °C/15 °C day/night; 400 μmol m^{-2} s^{-1} PPFD); the third group was cold-acclimated (A) at 4 °C/2 °C day/night for 30 days under the same light conditions. After 30 days, one group from control conditions (hereafter: non-acclimated, NA), and all plants from A were kept under cold/frost conditions (8 °C/-4 °C day/night) for 3 days (F treatment for now on). The last group of plants from NA remained at the same temperature (18 °C/15 °C day/night) as control. Overall, we had the following experimental setup: NA, NA-F, and A-F with $n = 10$ plants per treatment. After freezing treatment, hydraulic conductivity, electron transport rate, and DHNs content were measured on all individuals from the three groups.

2.2. Xylem conductivity and tissue printing

Hydraulic conductivity was measured using segments excised from the distal ends of branches in all individuals. The size of excised segments was standardized so that all diameters fell within the range of 10–12 mm with the bark removed, and with lengths ranging between 15 and 20 cm. Collected branches were cut under water in order to avoid embolisms. Hydraulic conductance (Kh) was measured gravimetrically following the methodology by Zimmerman (1978). Part of each excised stem (the same used in Kh measurements) was used for tissue printing, where a transversal section of stem was pressed on a nitrocellulose membrane and expose to antibodies as described in the next section. Finally the relative amount of proteins was calculated densitometrically using SigmaScan 5 software (SPSS Inc., Chicago, Ill, USA). The area covered by dehydrins was also measured in tissue printing of barks using digital images and Sigma-Scan 5 software (SPSS Inc., Chicago, Ill, USA).

The stem tissue was sectioned and stained with Saffranin-O, mounted in a 1:1 solution of glycerol and 50% ethanol. Transverse sections were then photographed at 40× magnification with a Nikon Eclipse 50i microscope (Nikon Japan.) using a digital camera (data no shown). To determine vessel diameter, we analyzed the cross sections images using the software Image-J (version 1.48). The "Analyze Particles" tool was used to identify each vessel and determine the lumen area in μm^2. Using this information we calculated the percentage of the total number of vessels that were embolized at each treatment. Using the vessel lumen diameter data we then approximated the volumetric flow rate (J_v) through the stems based on the remaining functional vessels using the Hagen–Poiseuille equation and following the methods of Lewis and Boose (1995):

$$Jv = \left(\pi D^4/128\mu\right) * (\Delta p/\Delta x')$$

where D is the conduit diameter, μ is the viscosity of water, and $\Delta p/\Delta x'$ is the pressure gradient.

2.3. Protein extraction and western-blot assays

Proteins from xylem were extracted in phosphate buffer (100 mM sodium phosphate, 5 mM ascorbate, 0.3 mM triton x-100, 1 mM EDTA and 3% PVP, pH 7.0), centrifuged at 12,000 *g* at 4 °C for 30 min and the supernatant collected and assayed for protein content using a RC DC™ protein assay kit (Bio-Rad Laboratories, Mississauga, ON, Canada). Proteins were resolved by sodium dodecyl sulfate–polyacrylamide gel electrophoresis or SDS–PAGE (Laemmli, 1970) using a Miniprotean III electrophoresis cell (Bio-Rad, Hercules, Calif., USA). Twenty micrograms of proteins was loaded in each well, and then transferred onto a nitrocellulose membrane (GE Osmonics, Minnetonka, Minn., USA). After blocking with 3% non-fat dried milk at 4 °C overnight, the membrane was then incubated for 2 h with an anti-dehydrin rabbit polyclonal antiserum, against a consensus peptide (TGEKKGIMDKIKEKLPGQH) diluted 1:1000. Primary antiserum was detected with alkaline phosphatase for tissue printings and HRP conjugated anti-rabbit IgG for western-blot. Following incubation, membranes were washed and ultimately developed. The specificity of dehydrin detection was confirmed by blocking the primary antiserum for 30 min with an equal volume of the K segment peptide salt (5 mg/ml) prior to the first incubation of the membranes.

2.4. Chlorophyll fluorescence parameters (ETR)

We measured electron transport rate (ETR) using a mini-PAM portable fluorometer (Waltz, Effeltrich, Germany). Measurements were carried out in full-expanded leaves every 5 days during 30 days; saturation pulses of light were applied for 0.8 s at an intensity of 3500 μmol m^{-2} s^{-1}. Electron transport rate (ETR) was calculated using the next equation:

$$\mathrm{ETR} = \phi_{\mathrm{PSII}} I \alpha / 2$$

where ϕ_{PSII} is the quantum yield of photosystem II, I is the incident PPFD (in the 400–700 nm wavelength); α is the leaf absorbance, taken here as 0.84 (Björkman and Demmig, 1987); and the factor of 2 accounts for the fact that two photons are required per electron passed through PSII, assuming linear electron flow and even distribution of absorbed quanta between PSII and PSI. The units of ETR are μmol electrons m^{-2} s^{-1}.

2.5. Data analysis

The effects of simulated freezing on xylem conductivity, percentage of embolized vessels, and fluorescence variables (ETR) were compared