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Research paper

Freezing resistance in Patagonian woody shrubs: the role of cell wall elasticity and stem vessel size

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Freezing resistance through avoidance or tolerance of extracellular ice nucleation is important for plant survival in habitats with frequent subzero temperatures. However, the role of cell walls in leaf freezing resistance and the coordination between leaf and stem physiological processes under subzero temperatures are not well understood. We studied leaf and stem responses to freezing temperatures, leaf and stem supercooling, leaf bulk elastic modulus and stem xylem vessel size of six Patagonian shrub species from two sites (plateau and low elevation sites) with different elevation and minimum temperatures. Ice seeding was initiated in the stem and quickly spread to leaves, but two species from the plateau site had barriers against rapid spread of ice. Shrubs with xylem vessels smaller in diameter had greater stem supercooling capacity, i.e., ice nucleated at lower subzero temperatures. Only one species with the lowest ice nucleation temperature among all species studied exhibited freezing avoidance by substantial supercooling, while the rest were able to tolerate extracellular freezing from −11.3 to −20 °C. Leaves of species with more rigid cell walls (higher bulk elastic modulus) could survive freezing to lower subzero temperatures, suggesting that rigid cell walls potentially reduce the degree of physical injury to cell membranes during the extracellular freezing and/or thaw processes. In conclusion, our results reveal the temporal–spatial ice spreading pattern (from stem to leaves) in Patagonian shrubs, and indicate the role of xylem vessel size in determining supercooling capacity and the role of cell wall elasticity in determining leaf tolerance of extracellular ice formation.

Keywords: bulk elastic modulus, ice nucleation temperature, leaf lethal temperature, Patagonian steppe, pressure–volume relationship, supercooling.

Introduction

Freezing resistance is important for plant survival in habitats with frequent subzero temperatures, and consequently, the species-specific capacity to resist freezing injury limits the geographical distribution of plants in temperate and high elevation tropical regions (George et al. 1974, Sakai et al. 1981, Woodward 1987, Stuart et al. 2007). Freezing resistance of plants from the southern hemisphere is poorly understood compared with that of the northern hemisphere plants (Sakai et al. 1981, Feild and Brodribb 2001). The southern hemisphere generally has weaker seasonality in temperature compared with the continental northern hemisphere due to a different ratio of land versus ocean masses and thus a more maritime climate, which may result in differences in plant freezing adaptations in

southern versus northern hemispheres. Nocturnal freezing temperatures frequently occur in winter in the Argentinian Patagonian steppe (Bucci et al. 2009), one of the largest cold deserts in the world. Freezing resistance has to be of importance for the survival of Patagonian shrubs that maintain physiologically active leaves during the winter. Patagonian steppe with relatively low species diversity is a good model system to study plant freezing resistance mechanisms, which can be easily extrapolated to the ecosystem level.

Freezing resistance is defined as the ability of plants to withstand freezing temperatures. Vascular plants can either avoid or tolerate freezing injury, with strategies including thermal insulation (Goldstein and Meinzer 1983), supercooling to avoid extracellular ice formation (Levitt 1972, George et al. 1974) and extracellular freezing tolerance (Goldstein et al. 1985, Azocar et al. 1988, Lipp et al. 1994). All physical and biological systems supercool, i.e., prevent ice formation below the tissue-specific equilibrium freezing temperature, to some degree (Larcher 1982, Goldstein et al. 1985). However, there are few organisms whose ice nucleation temperatures (INTs) are low enough to allow them to survive subzero temperatures in their natural habitats without freezing (permanent supercooling). Supercooling in plants is not fully understood but appears to be related to structural properties of tissues (Wisniewski and Fuller 1999, Melcher et al. 2000), low apoplastic water content (Goldstein et al. 1985, Melcher et al. 2000) and antifreeze chemical compounds (Alonso-Amelot 2008). Freezing injury of plant tissues usually results from dehydration of the cytoplasm during formation of extracellular ice crystals, the mechanical disruption of cell membranes by growing extracellular ice and lethal intracellular ice formation (for reviews, see Pearce 2001, Smallwood and Bowles 2002). For species that tolerate extracellular ice formation, freezing tolerance capacity appears to be related to membrane properties, cryoprotective proteins and cell wall structure (Smallwood and Bowles 2002).

Cell walls contribute to the extracellular freezing tolerance of cell membranes, perhaps as barriers against the propagation of extracellular ice (Wisniewski et al. 1991, Smallwood and Bowles 2002, Yamada et al. 2002), but some results are inconsistent with this conceptual model. Extracellular freezing tolerance in isolated suspended protoplast (with no cell wall) is similar to that in intact cells (Siminovitch et al. 1976, Singh 1979). Isolated suspended protoplasts may even have higher extracellular freezing tolerance than intact cells (Tao et al. 1983, Murai and Yoshida 1998), suggesting negative effects of cell walls on extracellular freezing tolerance of the cell membrane. To help clarify this controversy, we investigated the relationship between cell wall elasticity and leaf freezing resistance in Patagonian woody shrubs. Cell wall elasticity is estimated in this study by the modulus of elasticity (ε), which is obtained from the relationship between cell turgor pressure and cell water content. High ε values indicate low cell wall elasticities.

The objective of this study was to characterize physiological traits that contribute to either avoidance of or tolerance to extracellular ice formation in Patagonian shrubs. In addition, tissue-level traits related to stem and leaf ice nucleation were evaluated. In particular, the relationship between cell wall elasticity and leaf freezing processes was studied. Temperature regimes that plants experience are an important factor determining plant freezing resistance (Goldstein et al. 1985, Sierra-Almeida et al. 2009, Sierra-Almeida and Cavieres 2010). Therefore, plants from two habitats with different minimum temperatures were studied here to test the effects of habitat temperature regime on plant freezing adaptations. The experimental design included the study of dominant Patagonian shrubs from two adjacent sites characterized by different minimum temperatures: a higher elevation plateau site and a lower elevation site. Only one species is common to both sites and no congeneric species has been found.

Materials and methods

Field sites

This research was performed at a low elevation site (close to sea level) and a relatively high elevation site on a plateau near Comodoro Rivadavia city (45°51′S; 67°28′W), which is close to the Patagonian coast. The plateaus in this area are dissected by narrow canyons with altitudinal differences of 670 m. The distance between two study sites is ~15 km. Precipitation falls mainly in the autumn and winter, with an annual total of 245 mm. The mean annual temperature of Comodoro Rivadavia is 12.9 °C and daily mean temperatures range from 20 °C in January (summer) to 7 °C in July (winter). The minimum air temperature in winter could be as low as −15.1 °C in Comodoro Rivadavia, while −20 °C is common in more continental locations within Patagonia at the same latitude during the winter. Mean annual wind speed is 27 km h^{-1} with maximum values during spring and summer of ~43 km h^{-1}.

The vegetation in this area is typical for Patagonian steppes characterized by tussock grasses and shrubs (Soriano 1956, Bucci et al. 2009). The shrubs on plateaus are prone to high winds and cold fronts, while those in canyons (low elevation sites) are partially protected by the surrounding plateaus. Daily minimum temperature at the canyon site during the winter was −5 °C, while it reached −12 °C on the plateau (Figure 1). Typical daily maximum temperature at the canyon site was 15 °C during the winter, while it reached 10 °C on the plateau. Soils in the canyon contain a larger percentage of sand than the typical soils of the plateau, which have relatively coarse sand within a matrix of gravel. Average soil water potentials at both sites during the study period (winter) and a few months before the experiment were close to 0 MPa. The higher elevation plateau site and the canyon site will be referred in this study as 'plateau' and 'low elevation' sites, respectively.

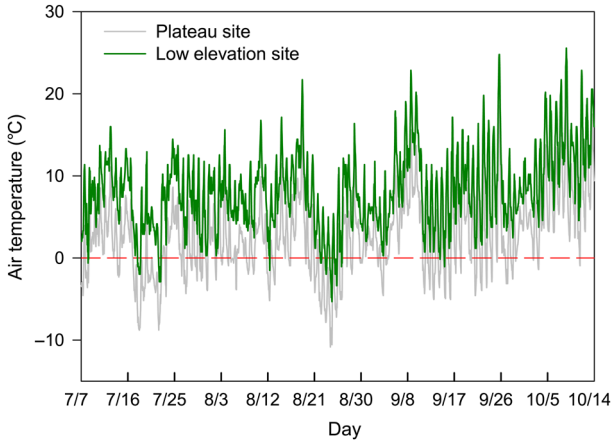

Figure 1. Winter air temperatures at the low elevation site (solid line) and the plateau site (gray line) in 2013 winter. The broken line indicates 0 °C.

Four shrub species from the low elevation site and three shrub species from the plateau site were studied (Table 1). All of these species maintain leaves during the winter season, and almost all evergreen shrub species suitable for physiological measurements were included in the present study. One deciduous species (*Lycium chilense* Miers ex Bertero) that only maintains active leaves during a few weeks of the winter season was also included in the present study. Leaves of this deciduous species may be at a senescent stage. However, because it is still metabolically active during this period, its leaves have to develop freezing resistance characteristics and thus were included in this study. *Berberis microphylla* Hort. ex K.Koch is the only species that is found at both sites. The measurements were performed during the middle of the winter (from June to August) of 2007 or 2008 when all the plants were cold-acclimated. The bulk elastic modulus was also measured in the summer season (February) for three species to assess whether there was cold acclimation for this trait. Sampling and measurements were done on uniformly sunny days.

Leaf bulk elastic modulus and vessel diameter

Pressure–volume analysis (Scholander et al. 1965, Tyree and Hammel 1972) was used to determine leaf bulk elastic modulus. Small shoots were obtained from sun-exposed branches cut in the field in the early morning, recut immediately under water and covered with black plastic bags with the cut end in water for ~2 h until measurements began in the laboratory. Pressure–volume curves were determined for three to six fully developed small shoots per species (including *B. microphylla* at the low elevation site and the plateau site separately) from different individuals. Leaf water potentials (Ψ_L) of the shoot samples were determined with a pressure chamber (PMS Instrument Company, Albany, OR, USA) and their fresh weights were measured immediately. Shoot samples were allowed to dehydrate slowly on the laboratory bench. Water potentials and fresh weight measurements were repeated frequently until the water potentials exceeded the measurement range of the pressure chamber (−6 MPa). Saturated weight of each sample, used to calculate relative water content (RWC), was extrapolated as the weight at zero Ψ_L via a linear regression between sample fresh weight and Ψ_L above the turgor loss point (Meinzer and Moore 1988).

Table 1. Low elevation and plateau shrub species selected for the present study and their life form and leaf phenology.

Species	Family	Life form	Leaf phenology
Low elevation site			
Colliguaja integerrima Gillies and Hook	Euphorbiaceae	Tall shrub	Evergreen
Berberis microphylla Hort. ex K.Koch	Berberidaceae	Tall shrub	Evergreen
Lycium chilense Miers ex Bertero	Solanaceae	Tall shrub	Deciduous
Acantholippia seriphioides (A.Gray) Moldenke	Verbenaceae	Small shrub	Evergreen
Plateau site			
Senecio filaginoides DC	Asteraceae	Small shrub	Evergreen
Berberis microphylla Hort. ex K.Koch	Berberidaceae	Tall shrub	Evergreen
Nardophyllum obtusifolium Hook and Arn	Asteraceae	Small shrub	Evergreen

The pressure–volume curves were obtained by plotting $-1/\Psi_L$ against relative water deficit (RWD = 1 − RWC). Bulk elastic modulus was calculated as $\Delta P/\Delta$RWC according to Kubiske and Abrams (1991), where ΔP is the change in turgor pressure and ΔRWC is the change in RWC. Bulk elastic modulus over the full range of turgor was used in this study to better represent tissue elastic properties across the full range of turgor values including the wilting point (Kubiske and Abrams 1991). Although bulk elastic modulus was not possible to measure after turgor loss with this method, it is used to represent cell wall elasticity even after turgor loss because it is strongly correlated to Young's modulus of the cell wall and mainly determined by cell wall physical properties (Saito et al. 2006), which would change little during turgor loss. Vessel diameters of stem xylem of all the species and sites were measured under a microscope with an ocular micrometer (n = 80–226 vessels).

Ice nucleation temperatures of leaves and stems

Thermal analyses were conducted on stem tissues and on leaves attached and detached from the stem to determine ice nucleation temperatures (INT). Sun-exposed mature stem and leaf samples were collected in the early morning and transported rapidly to the laboratory in a car for thermal analyses (n = 3–9). Detached leaves were placed in zip bags, while for leaves attached to the stem, a small branch was used. The temperature of the freezer was lowered at a rate of 7 °C h^{-1} from ambient to −20 °C. This rate is similar to those used in other studies (e.g., Goldstein and Nobel 1994, Lipp et al. 1994, Rada et al. 2001,

Arias et al. 2015), and it reflects the maximum rate of temperature decrease in the field during the late afternoon in the study area. The temperature of the leaf or stem was monitored with copper–constantan thermocouples placed in close contact with the leaf or stem tissue and held in place using small pieces of surgical tape. The thermocouples were connected to a datalogger (CR10X, Campbell Scientific, Logan, UT, USA), and leaf or stem temperatures were recorded at 4-s intervals. The leaf or stem INTs were obtained from the leaf or stem tissue thermal analysis. A rapid increase in temperature (exotherm) indicates heat release from water during extracellular ice nucleation. Only one exotherm was observed; thus, we were unable to detect intracellular ice nucleation with this method. This may have resulted from low liquid water contents in intracellular spaces for these species, and/or it was a gradual process compared with extracellular ice formation.

Leaf membrane leakage measurement

The membrane electrolyte leakage method was used to assess the influence of low temperatures on leaves (Wilner 1960, Lipp et al. 1994). Sun-exposed mature leaves were collected from the field in the early morning, kept in plastic bags with wet paper towels to reduce water loss and then transported to the laboratory within 2 h for measurements. Leaf RWC determined with the oven-dry method ranged from 13 to 54% across species. Leaf samples with several leaves (~0.2 g) were placed into small tubes and incubated in a freezer. Several leaves were used because the leaves were very small (0.1–1.1 cm^2). The temperature in the freezer was cooled at the same rate used for thermal analyses (7 °C h^{-1}) to reach the following temperature targets: 25/room temperature, 15, 7, 2, 0, –2, –4, –6, –8, –10, –13, –15 and –19 °C. After maintaining the samples at a particular target temperature for 15 min (see Lipp et al. 1994), a group of leaf samples were removed from the freezer and thawed for 2 h at 4 °C, and then 10 ml of deionized water was added to each tube. The solutions with leaf samples were held at 4 °C for 24 h with occasional mixing and shaking. Electrical conductivity (EC) of the solution was then measured with an electrical conductance/resistance meter (Hanna HI 98311, Hanna Instruments, Woonsocket, RI, USA). After EC measurements, the tubes were enclosed in a boiling water bath to obtain the EC with complete electrolyte leakage. Electrical conductivity of the solution with leaf samples was measured again after 24 h with occasional mixing and shaking. Boiling leaves to achieve complete electrolyte leakage is a conventional procedure for determining leaf freezing resistance with the membrane leakage method (e.g., Wilner 1960, Arora et al. 1992, Renaut et al. 2004, Scholz et al. 2012, Zhang et al. 2014, Arias et al. 2015). Other possibilities are freezing leaves at –70 °C (see Lipp et al. 1994, Melcher et al. 2000) or using the EC at –19 °C (the minimum treatment temperature) as the reference EC with complete electrolyte leakage (Figure 2a). For leaves with high freezing resistance, however, –19 °C or even lower temperatures may not be able to completely damage the membrane

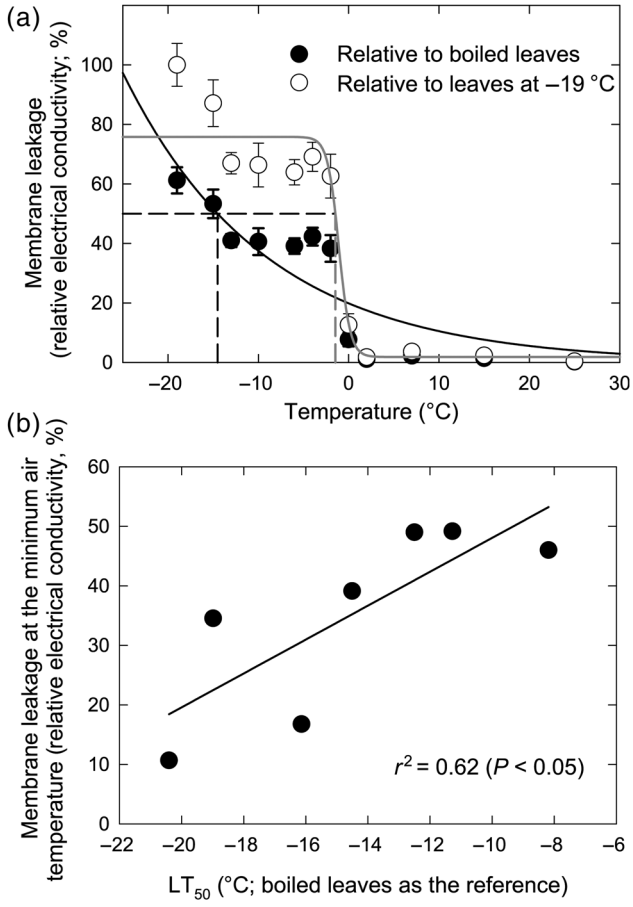

Figure 2. (a) A comparison of using different methodologies for LT_{50} (the temperature at which 50% of membrane leakage occurred) estimation for *B. microphylla* from the low elevation site. Filled points are leaf relative EC (%; indicating percent cell membrane leakage) calculated using EC of boiled leaves as the reference EC (complete electrolyte leakage), while open points are those using the EC at –19 °C as the reference EC. The solid line denotes an exponential function, while the gray line a sigmoid function fitted to the data. Dashed lines indicate LT_{50}. (b) The relationship between leaf relative EC (%; indicating percent cell membrane leakage) at minimum air temperatures of the study sites and LT_{50} estimated using EC of boiled leaves as the reference.

to get the maximum EC, and consequently would underestimate their freezing resistance. For example, using the EC at –19 °C as the maximum EC for *B. microphylla* resulted in a >60% membrane damage value at –2 °C (Figure 2a), which is not possible as *B. microphylla* leaves apparently survive through temperatures lower than –2 °C in winter (Figure 1). Using the EC at –19 °C as the maximum EC for *Senecio filaginoides* DC from the plateau could result in a relative EC value of ~90% at –12 °C, which indicates complete leaf damage. However, no leaf permanent damage or dieback was observed for *S. filaginoides* in the field, which experiences –12 °C at the plateau site (Figure 1). Additionally, employing the most commonly used procedure (boiling leaf samples) makes comparison with the literature more appropriate. The relative EC, an indicator of membrane electrolyte leakage, was calculated for each sample as a percentage:

$$\text{Relative EC} = \frac{\text{EC after the temperature treatment}}{\text{EC after boiling}} \times 100 \quad (1)$$

The temperature at 50% relative EC is defined as the leaf lethal temperature of the species (LT_{50}), which could be associated with membrane structural transitions (Rajashekar et al. 1979). Leaf lethal temperature at 50% relative EC is widely used as an indicator of freezing resistance (e.g., Lipp et al. 1994, Melcher et al. 2000, Cavender-Bares et al. 2005, Sierra-Almeida et al. 2009, Sierra-Almeida and Cavieres 2010, Zhang et al. 2014, Arias et al. 2015). Samples were not inoculated on a regular basis with ice to stimulate ice seeding and to prevent supercooling in the present study because: (i) we wanted to simulate natural conditions and to avoid artificial effects of ice inoculation as ice formation on the leaf surface or wet leaves was not observed during days with subzero air temperatures (Arias et al. 2015); (ii) ice inoculation will prevent supercooling and make it impossible to distinguish between freezing resistance mechanisms: freezing avoidance through supercooling or freezing tolerance to extracellular ice formation; and (iii) ice inoculation avoids the extent of substantial supercooling and may underestimate the freezing resistance of those plants that resist freezing through substantial supercooling but not extracellular freezing tolerance. A test was performed with two of the studied species tolerant to extracellular ice formation (*B. microphylla* and *Nardophyllum obtusifolium* Hook and Arn), and showed that ice inoculation had no effect on membrane leakage and freezing resistance assessment (Figure 3). Cell membrane leakage measurements without ice inoculation are widely used to assess freezing resistance and to distinguish freezing resistance mechanisms (e.g., Goldstein et al. 1985, Rada et al. 1987, Lipp et al. 1994, Melcher et al. 2000, Feild and Brodribb 2001, Rada et al. 2001, Cavender-Bares et al. 2005, Sierra-Almeida et al. 2009, Sierra-Almeida and Cavieres 2010, Zhang et al. 2014, Arias et al. 2015).

Freezing resistance mechanism

The freezing resistance mechanism was determined by comparing LT_{50} and leaf INT (Squeo et al. 1991, Bravo et al. 2001, Sierra-Almeida et al. 2009). Species that exhibited LT_{50} within the range of INT were categorized as freezing-avoidant plants, while species with LT_{50} being sustainably lower than INT were categorized as freezing-tolerant plants (Squeo et al. 1991, Bravo et al. 2001, Sierra-Almeida et al. 2009).

Data analysis

Differences in LT_{50}, stem and leaf INT between low elevation and plateau shrubs and between *B. microphylla* from different sites were examined using Mann–Whitney *U*-tests. Linear regressions were used to test the dependence of LT_{50} on ε, and stem INT on xylem vessel diameter. Sigmoid curves were fitted to the relationship between relative EC and temperature for all the species except *B. microphylla*. Exponential functions were fitted to

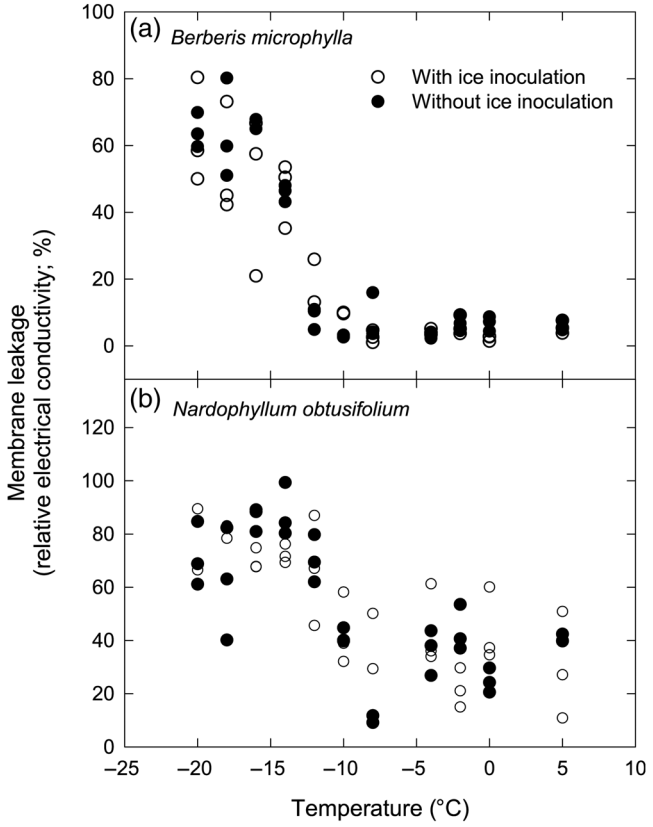

Figure 3. Membrane leakage (relative electrolyte conductivity) of ice-inoculated leaves and of noninoculated leaves as a function of treatment temperature in *B. microphylla* (a) and *N. obtusifolium* (b). Open points in (a) and (b) are leaves treated with ice inoculation, while filled points those without ice inoculation.

B. microphylla because the sigmoid function generated a maximum relative EC below 40%, which ignored the increasing trend at −19 °C and made extrapolation of the relationship to 50% impossible. In addition, fitting the relative EC data calculated using the EC at −19 °C as the reference with sigmoid function generated a LT_{50} of −1.48 °C for *B. microphylla* (Figure 2a), which is physically impossible because no leaf tissue death or lethal damage was observed in the field during the winter. The LT_{50} were then derived from the regression equations. Leaf lethal temperature at 50% relative EC derived as described above were closely associated with membrane leakage at the minimum air temperatures of the study sites across species (Figure 2b), suggesting that the LT_{50} calculated with the present method is a good indicator of freezing resistance. The differences in slope and intercept between linear regressions among stem INT, attached leaf INT and detached leaf INT and 1 : 1 relationship were tested with analysis of covariance.

Results

Species and site-specific leaf freezing resistance varied greatly; temperatures at which 50% of membrane leakage occurred

(LT_{50}) ranged from –8.2 to –20.4 °C among studied Patagonian shrubs (Table 2). Leaf and stem supercooling also varied largely among species, as leaf extracellular INT ranged from –3.5 to –8.4 °C, and that of the stems ranged from –1.6 to –8.1 °C (Table 2). No significant differences were found in LT_{50} between low elevation and plateau shrubs (Table 2). Leaf and stem INTs were ~2 °C higher in the plants growing at the plateau site than those growing at the low elevation site although significant differences were found only for stem INT (Table 2). *Berberis microphylla*, the only shrub species growing at both sites, exhibited lower leaf LT_{50} on the plateau than at the low elevation site (–19 and –14.5 °C, respectively; Table 2).

Table 2. Extracellular ice nucleation temperature (INT) of leaf (attached to stem) and stem, and leaf lethal temperature (LT_{50}; the temperature at which 50% membrane leakage occurred) of low elevation and plateau shrubs. Values are means ± SEs. Values for *B. microphylla* followed by the same lowercase letter did not differ significantly between low elevation and plateau individuals ($P > 0.05$). The same uppercase letter between low elevation and plateau sites indicates that these variables did not differ significantly ($P > 0.05$).

Species	Leaf LT_{50} (°C)	Leaf INT (°C)	Stem INT (°C)
Low elevation site			
C. integerrima	–20.4	–5.5 ± 0.2	–4.1 ± 0.17
B. microphylla	–14.5a	–5.4 ± 0.4a	–5.5 ± 0.22a
L. chilense	–16.1	–6.0 ± 0.4	–6.4 ± 0.04
A. seriphioides	–8.2	–8.4 ± 0.7	–8.1 ± 0.03
Low elevation mean	–14.8 ± 2.5A	–6.3 ± 0.7A	–6.0 ± 0.84A
Plateau			
S. filaginoides	–12.5	–3.5 ± 0.3	–2.7 ± 0.07
B. microphylla	–19.0b	–4.7 ± 0.3a	–2.7 ± 0.01b
N. obtusifolium	–11.3	–5.4 ± 0.5	–1.6 ± 0.01
Plateau mean	–14.3 ± 2.4A	–4.5 ± 0.6A	–2.3 ± 0.37B

Ice nucleation temperatures of stems and detached leaves (Figure 4a), stems and attached leaves (Figure 4b) as well as those of attached leaves and detached leaves (Figure 4c) were significantly related. Across species, the intercept of the linear relationship between INT of detached leaf and stem INT differed significantly from the 1 : 1 relationship (Figure 4a; $P < 0.05$), suggesting that ice nucleation of stems occurred at higher temperatures than that of detached leaves. When leaves were attached to the stem, most species showed leaf INT closer to stem INT. However, leaves of two plateau shrubs had much lower leaf INT than stem INT even when they were attached (Table 2; Figure 4b). The slope of the linear line fitted to the relationship between INT of attached leaves and INT of detached leaves differed significantly from the 1 : 1 relationship (Figure 4c;

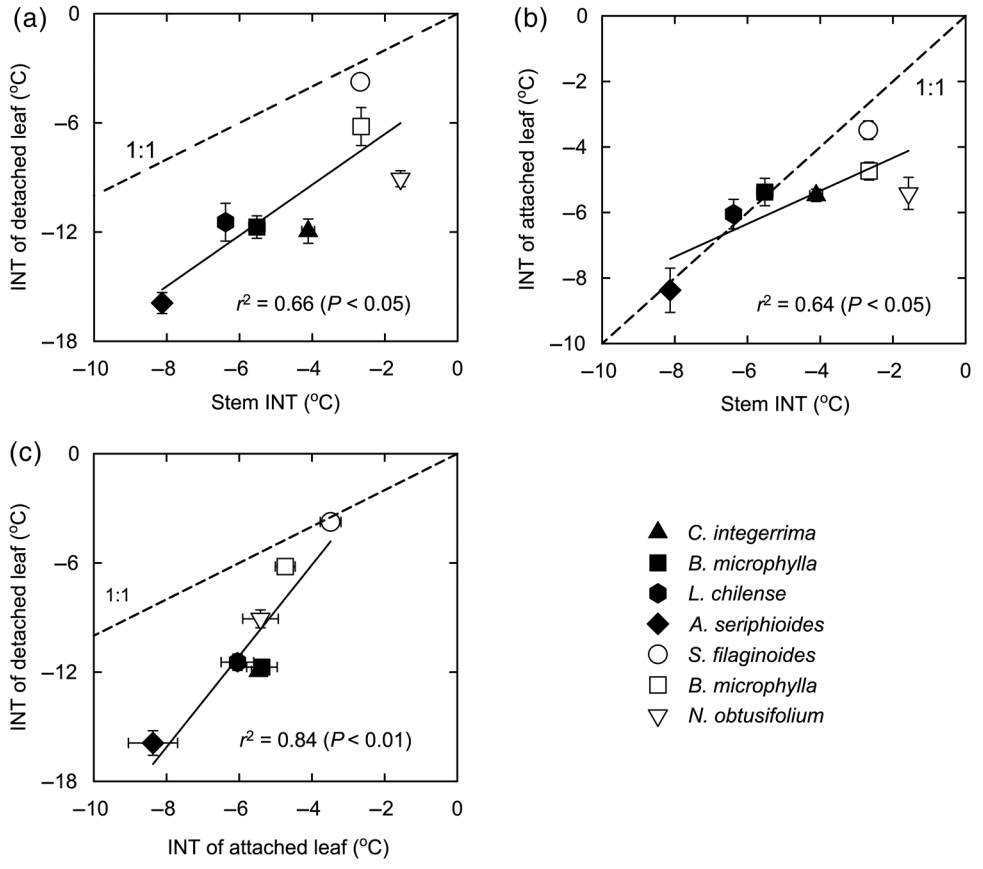

Figure 4. Ice nucleation temperatures (INT) of detached leaves (a) and leaves attached to stems (b) in relation to the stem INT, and the relationship between INT of detached leaves and attached leaves (c). Symbols denote means ± SEs, open symbols for plateau shrubs and filled symbols for species from the low elevation site. The dashed line is the 1 : 1 relationship.

$P < 0.05$) because most species showed substantially lower INT in detached leaves than in attached leaves, with the greater differences observed in low elevation plants. Therefore, the supercooling capacity of some species would be overestimated when using detached leaves.

Xylem conduit size differed between shrubs from the plateau site and low elevation site (Figure 5). Shrubs at the plateau site had xylem vessels with larger diameters compared with those growing at the low elevation site except for *Acantoliphia seriphioides* (A.Gray) Moldenke from the plateau site, which had the largest vessels among all the species studied. Stem INT was positively associated with xylem vessel diameter when *A. seriphioides* was not included in the analysis (Figure 5).

Relative EC of leaves (a measure of membrane leakage) increased as temperature decreased (Figure 6). In most species, the increase in relative EC coincided with the ice nucleation (INT; Figure 6). In two species (Figure 6d and g), a small amount of leakage was observed before the INT. Membrane leakage before the INT has also been reported in high elevation Hawaiian plants as a result of changes in membrane properties below 0 °C but before INT (Lipp et al. 1994). This could also be an artifact of using many small leaves for each individual and because the INTs reported here were the averages of several individuals. Some leaves may initiate ice seeding sooner than others (see the range of INT in Figure 6). Additionally, several small leaves were used for the freezing injury test, and consequently, the relative EC represented an average electrolyte leakage of all leaves.

Leaf INTs of attached leaves in all low elevation shrubs were below the typical daily minimum air temperatures at the low elevation site (Figures 1 and 6), while those of all the plateau shrubs were above the typical daily minimum air temperatures (Figures 1 and 6). Consequently, freezing occurs frequently in the plateau shrubs but less frequently in the low elevation shrubs. One low elevation species with the lowest INT among all the shrubs (*A. seriphioides*) had an LT_{50} within the range of leaf INT, while the LT_{50} of all the other shrubs were substantially more negative than leaf INT (Figure 6).

The LT_{50} as well as the difference between LT_{50} and leaf INT (attached leaves) were negatively related to bulk elastic modulus across all species (Figure 7). Bulk elastic moduli of the Patagonian shrubs were significantly higher in winter than in summer (Figure 8).

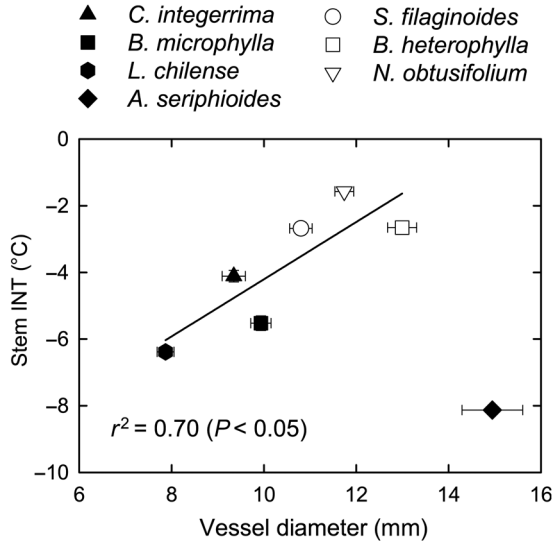

Figure 5. Stem ice nucleation temperature (INT) in relation to xylem vessel diameter. The line indicates the linear regression fitted to the data with *A. seriphioides* not included. Symbols denote means ± standard errors: open symbols for plateau shrubs and closed symbols for species from the low elevation site.

Discussion

Our results show that Patagonian shrubs varied considerably in leaf supercooling (as indicated by INT that ranged from −3.5 to −8.4 °C) and in leaf freezing resistance (as indicated by LT_{50} that ranged from −8.2 to −20.4 °C). We also revealed the mechanisms used by Patagonian shrubs to survive subzero temperatures; most species resist freezing through tolerating extracellular ice formation, while one species does so through permanent supercooling. We also found a novel relationship between cell wall elasticity and leaf tolerance to extracellular ice formation and a relationship between xylem vessel diameter and stem INT across species. Even though the association between freezing resistance and cell wall elasticity does not necessarily imply causation, it suggests an important role of cell wall properties in determining leaf freezing resistance.

Leaf freezing resistance mechanisms and ice nucleation in stem and leaf

Only one species (*A. seriphioides*) with the lowest leaf INT among the Patagonian shrubs exhibited freezing avoidance by supercooling; its LT_{50} (the temperature causing 50% membrane leakage) was within the range of the INT. The rest of the species tolerate extracellular freezing, with their LT_{50} being substantially lower than the leaf INT. Interestingly, leaves of shrubs from the plateau site exposed to lower subzero air temperatures tended to freeze at high temperatures (INTs were closer to the equilibrium freezing temperatures) compared with the lower elevation plants. Ice nucleation occurs at higher temperatures in plants exposed to lower subzero temperatures because once extracellular ice is formed, the dehydration process with water moving from the symplastic to the apoplastic compartments will be more gradual and less damaging (Goldstein and Nobel 1991, Lipp et al. 1994). Therefore, despite the absence of an overall difference in freezing resistance between shrubs from the plateau and low elevation sites, different temperature regimes influence physiological mechanisms and structural adaptations (e.g., vessel size) related to freezing resistance. In addition, more gradual

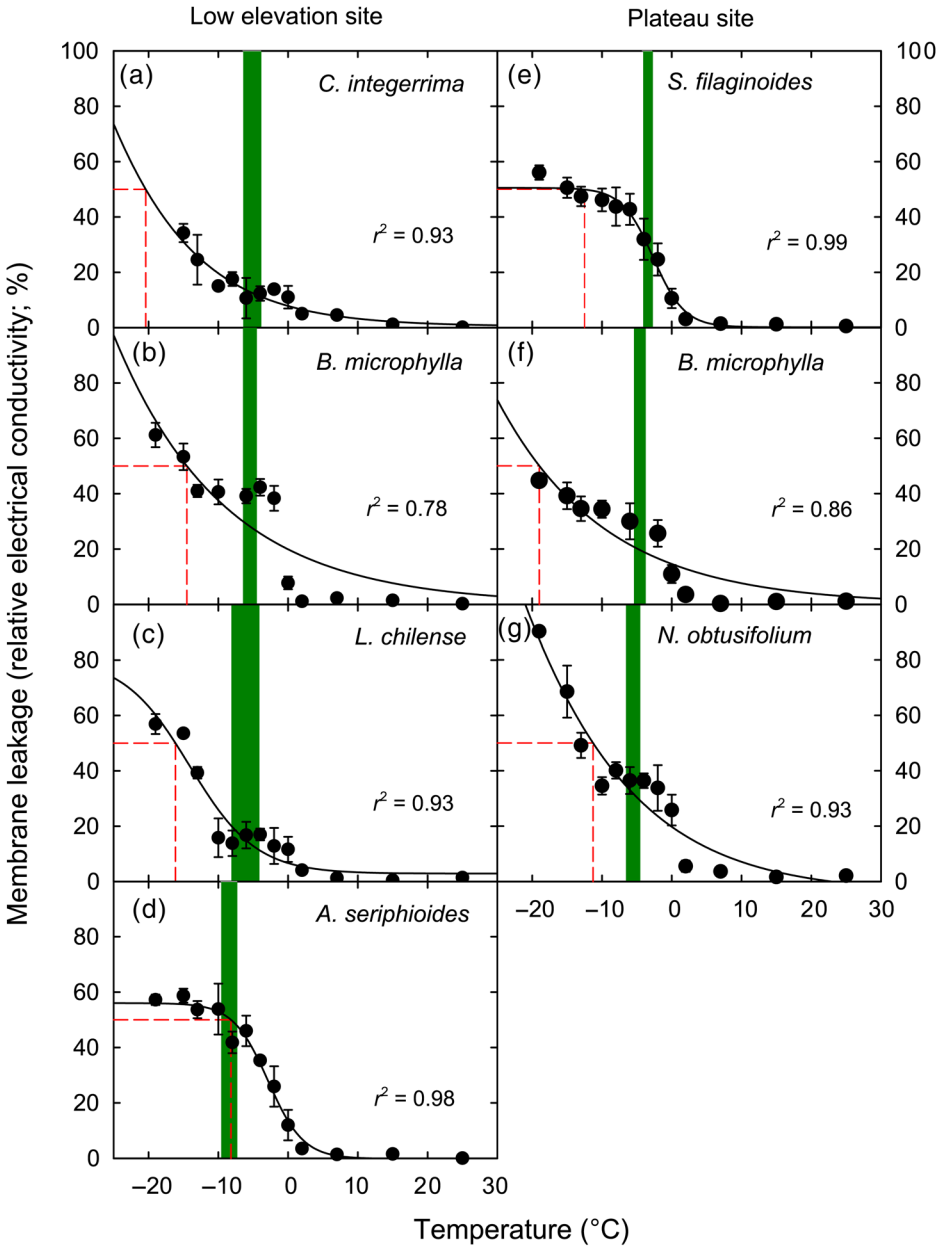

Figure 6. Leaf relative EC (%; indicating percent cell membrane leakage) as a function of leaf temperature. Shaded areas indicate the range of ice nucleation temperatures (INTs), while broken lines indicate the leaf lethal temperature at which 50% of membrane leakage occurred (LT_{50}). Sigmoid (a, c, d, e and g) or exponential (b and f) functions were fitted to the data, and $P < 0.001$ for all the regressions.

water movement from the symplastic to the apoplastic compartments after extracellular ice nucleation could be responsible for the slower changes in EC after INT (ice nucleation) in some species (Figure 6a–c, f and g).

We revealed the dynamics of ice nucleation across tissues in Patagonian shrubs; ice seeding initiates in the stem and then propagates into the leaves. This temporal–spatial pattern is similar to other evergreen woody species (Wisniewski et al. 1997, Wisniewski and Fuller 1999). Additionally, leaves that were attached to the stems in species from low elevation site exhibited INTs that were similar to that of the stem, indicating that the spreading of ice from stem to leaf is fast. Ice nucleation in plants is assumed to initiate in large xylem vessels that have a large volume of water (Sakai and Larcher 1987, Ball et al. 2002, 2006, Hacker and Neuner 2007), because the probability that an ice nucleation agent can seed ice increases with water volume size (Wilson et al. 2003). Notably, leaves of two shrub species from the plateau site had 2–4 °C lower INTs than that of the stem, suggesting the presence of a barrier against ice spreading from stem to leaves. Barriers against ice spreading to leaves have also been found in other plants (Neuner et al. 1997, Hacker and Neuner 2007). The delay in ice spreading in these

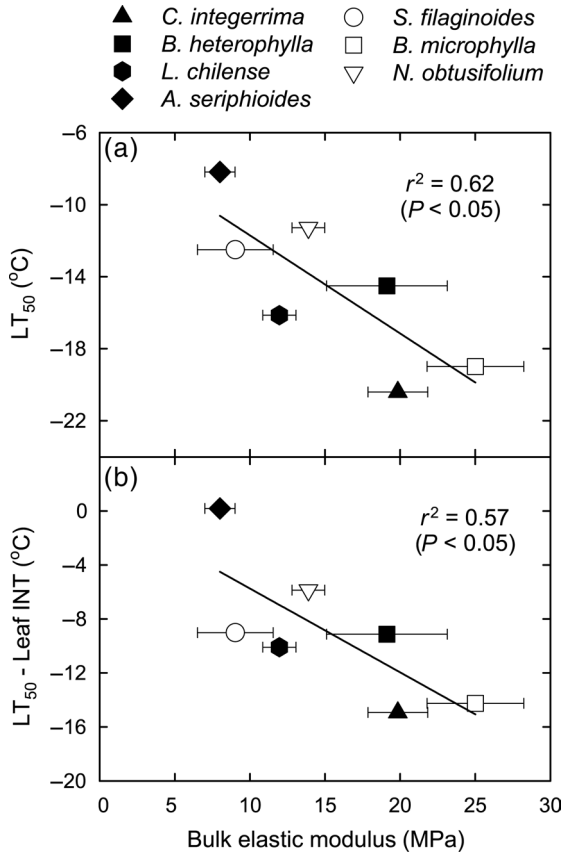

Figure 7. (a) Leaf lethal temperature at which 50% of membrane leakage occurred (LT_{50}), and (b) LT_{50}-ice nucleation temperature (INT) of attached leaves in relation to leaf elastic modulus (ε) derived from pressure–volume relationships. Symbols denote means ± SEs, open symbols are species at the plateau site and filled symbols for species from the low elevation site. Linear regressions were fitted to the data.

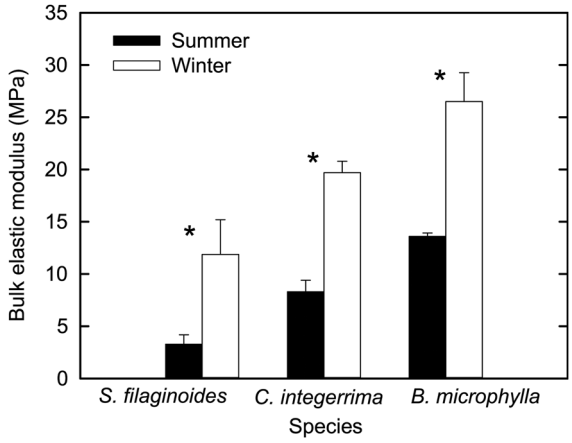

Figure 8. Seasonal variation in leaf bulk elastic modulus of three of the studied Patagonian shrub species. Bars are means + SE. Solid bars for summer, and open bars for winter. Significant differences between summer and winter values are indicated with an asterisk (P < 0.05, one-way analysis of variance).

two species also suggests a weak hydraulic connection between stem and leaves. This is consistent with the hydraulic segmentation between stem and leaves observed for these species (Bucci et al. 2013) and could be related to a high degree of freeze–thaw-induced cavitation in petioles.

We found that Patagonian shrubs with smaller xylem vessels tended to have lower stem INTs. This is probably a consequence of their lower water volume and lower probability of presence of ice nucleation agents. The species with the lowest INT among Patagonian shrubs (*A. seriphioides*) did not follow the relationship between vessel diameter and stem INT. For *A. seriphioides*, other factors related to supercooling may separate it from the relationship observed in other species. *Acantoliphia seriphioides* is an aromatic species with essential oils containing thymol and carvacrol (Fuselli et al. 2005), which are used by some plants to enhance supercooling (Alonso-Amelot 2008). Notably, plateau shrubs with higher stem INT had larger vessels compared with low elevation shrubs (except *A. seriphioides*). Relatively large vessels and consequently large water volume may be involved to achieve high stem INT to reduce dehydration damages related to substantial supercooling (Goldstein et al. 1985), since extracellular ice nucleation is unavoidable in Plateau shrubs exposed to lower air temperatures.

The role of cell wall elasticity in leaf freezing resistance

We revealed a strong association of leaf freezing resistance (LT_{50}) with leaf bulk elastic modulus in Patagonian shrubs, which is related to cell wall mechanical strength and cell wall structure (Tyree and Hammel 1972, Tyree et al. 1978, Saito et al. 2006). This relationship suggests that species with more rigid cell walls were able to tolerate lower subzero temperatures after extracellular freezing. This pattern agrees with a previous study showing that species with more rigid cell walls exhibited less leaf tissue damage at –20 °C (Scholz et al. 2012). Cell wall properties, particularly the size of micropores, have been shown to be related to leaf extracellular freezing tolerance (Wisniewski et al. 1991, Smallwood and Bowles 2002). In this study, we suggest that the cell wall mechanical strength (elasticity or rigidity) is also related to extracellular freezing tolerances of cell membranes. Ice seeding in the air space between cell walls and cell membranes is unlikely to occur since nucleating agents are mostly found in apoplastic spaces between adjacent cell walls (Goldstein and Nobel 1991). Therefore, during extracellular freezing, the cell walls and plasma membranes remain in tight contact (Siminovitch and Scarth 1938, Griffith and Antikienen 1996). Consequently, the growing extracellular ice could impose more mechanical pressure on cell membranes attached with elastic cell walls, which would cause more physical disruption to cell membranes, compared with cells with more rigid walls.

Rigid cell walls may also prevent or reduce cell membrane damage during the thaw process. When the cell wall returns to its original shape quickly during the thaw process, the instantaneous shape change in the cell wall may cause physical damages to

membranes that could not adjust as fast as cell walls during the volume change (Iljin 1933). Therefore, a rigid cell wall that experiences a smaller shape change during extracellular freezing will decrease the degree of physical injury to the cell membrane during the thaw process.

The effect of the cell wall rigidity on extracellular ice formation tolerance agrees with the increased cell wall rigidity in winter compared with the summer season (this study and Scholz et al. 2012). Increased cell wall thickness and mechanical strength during cold acclimation were also found in several other species (e.g., Rajashekar and Lafta 1996, Kubacka-Zebalska and Kacperska 1999, Stefanowska et al. 1999, Arias et al. 2015). In contrast, cell walls were not shown to contribute to extracellular freezing tolerance of cell membranes in a few studies, which reported that isolated protoplasts (with no cell walls) had similar or higher freezing tolerances compared with intact cells (Siminovitch et al. 1976, Singh 1979, Tao et al. 1983, Murai and Yoshida 1998). It is possible that cell walls in intact tissues do impose more mechanical pressure during extracellular freezing compared with the isolated protoplasts in solution, but mechanical pressure on cell membranes is unavoidable in intact plant tissues, and the rigid cell wall would transfer less mechanical pressure to the cell membrane and cause less membrane damage. In this perspective, our results expand rather than contradict the studies comparing isolated protoplasts and intact cells.

In contrast to rigid cell walls resulting in increased freezing tolerance, some studies in herbaceous plants (McCully et al. 2004), snow gum (Ball et al. 2004), a moss species (Lenné et al. 2010) and a pine species (Roden et al. 2009) showed that cells with elastic cell walls can undergo reversible shrinkage (i.e., cytorrhysis) during freezing, consequently lowering the risk of intracellular freezing compared with cells with rigid walls. Strong freezing tolerance in cells with elastic walls adds complexities to the role of cell walls during freezing. It is possible that species or cell types that are able to substantially supercool their apoplastic solution and form glassy states during extracellular freezing (e.g., ray cells in snow gum; Ball et al. 2004) will be facilitated by a rigid cell wall in resisting physical pressure of extracellular ice, while species or cell types that are vulnerable to intracellular freezing and cell cavitation (e.g., living fibers and mesophyll cells in snow gum; Ball et al. 2004) will benefit from elastic cell walls that facilitate reversible cytorrhysis. The role of cell walls in extracellular freezing still deserves further studies integrating physical and physiological measurements, as well as analyses of interspecific relationships among different plant groups. Monitoring the change in cell wall elasticity of different types of cells and simultaneously the bulk leaf elastic modulus during cold acclimation will also help to better understand the role of cell walls in freezing resistance.

In conclusion, we found that all studied Patagonian shrubs resist freezing temperatures through tolerating extracellular ice formation, with the exception of one species exhibiting freezing avoidance by substantial (permanent) supercooling. In all but two species, ice formation initiated in the stem vessels and then propagated rapidly to the leaves. The two additional species from the plateau site had barriers against ice spreading from the stem to the leaves. We also revealed the important role of cell wall elasticity in determining the tolerance to extracellular ice formation and the role of vessel size in determining supercooling capacity.

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Conflict of interest

None declared.

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