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RESEARCH PAPER

Characterization of antifreeze activity in Antarctic plants†

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Abstract

Deschampsia antarctica **and** ***Colobanthus quitensis*** **are the only vascular plants to have colonized the Maritime Antarctic, which is characterized by its permanently low temperature and frequent summer frosts. To understand how the plants survive freezing temperatures year-round, antifreeze activity was assayed in apoplastic extracts obtained from both non-acclimated and cold-acclimated Antarctic plants. By observing the shape of ice crystals grown in dilution series of the extracts, it was found that** ***D. antarctica*** **had antifreeze activity, but** ***C. quitensis*** **did not.** ***D. antarctica*** **exhibited antifreeze activity in the non-acclimated state and this activity increased after cold acclimation. The antifreeze activity in** ***D. antarctica*** **was labile to proteolysis and high temperature, active over a wide pH range, and associated with molecules greater than 10 kDa in molecular weight. These results show that** ***D. antarctica*** **produces antifreeze proteins that are secreted into the apoplast. When examined by SDS-PAGE, the apoplastic extracts from cold-acclimated** ***D. antarctica*** **exhibited 13 polypeptides. It is concluded that** ***D. antarctica*** **accumulates AFPs as part of its mechanism of freezing tolerance. Moreover, this is the first plant in which antifreeze activity has been observed to be constitutive.**

Key words: Antarctic plants, antifreeze activity, apoplastic proteins, cold acclimation, *Colobanthus quitensis*, *Deschampsia antarctica*, freezing tolerance, ice.

Introduction

Only two vascular plants, Antarctic hairgrass (*Deschampsia antarctica* of the Poaceae) and Antarctic pearlwort (*Colobanthus quitensis* of the Caryophyllaceae), have colonized the Maritime Antarctic (Lewis Smith, 2003). Because the environment of the Maritime Antarctic is permanently cold, these perennial plants must complete their life cycles under constant low temperature (Day *et al.*, 1999; Alberdi *et al.*, 2002; Lewis Smith, 2003). Although *D. antarctica* and *C. quitensis* are protected by deep snow for 6–7 months of the year, they are frequently exposed to freeze–thaw cycles during the summer when the daily temperature averages 4 °C (Day *et al.*, 1999; Lewis Smith, 2003). Both plants are able to grow in the summer because they maintain about 30% of the optimal net photosynthesis at 0 °C (Xiong *et al.*, 1999). During the long summer days, *D. antarctica* also accumulates the high levels of sucrose and proline needed for maintaining a high degree of stress tolerance (Bravo *et al.*, 2001). Surprisingly, *D. antarctica* and *C. quitensis* have very different responses to freezing temperatures. When grown under controlled conditions, non-acclimated *D. antarctica* exhibits an LT_{50} of −12 °C, and cold-acclimated plants survive −26 °C (Bravo *et al.*, 2001). By contrast, *C. quitensis* is not freezing-tolerant. It avoids freezing by supercooling and has an LT_{50} of about −5 °C, both before and after cold acclimation (Bravo *et al.*, 2001).

As winter approaches in temperate regions, perennial plants trigger a battery of structural and biochemical changes that increases their freezing resistance but also slows their growth. In contrast to plants from temperate regions, Antarctic plants are always in a cold-hardy state as shown by the fact that they exhibit the same LT_{50} in the field in the summer as plants cold-acclimated under laboratory conditions (Bravo *et al.*, 2001). These results show that Antarctic plants must grow and reproduce while maintaining a high level of resistance to abiotic stress. Therefore, it is of interest to understand not only the mechanism of freezing tolerance, but also the regulation of this process in Antarctic plants.

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† Dedicated to Marilyn Griffith (July 1953–February 2005).

In organisms that survive freezing, ice forms outside of the cells and its growth must be minimized to reduce both cellular dehydration and physical injury (Griffith and Antikainen, 1996). Many overwintering organisms, including insects, fish, bacteria, fungi, and plants, accumulate antifreeze proteins (AFPs) that bind to the faces of ice crystals during freezing and inhibit their growth (Duman and Olsen, 1993; Ewart *et al.*, 1999; Griffith and Yaish, 2004). The specific role of AFPs has not yet been elucidated in plants that survive freezing. AFPs are secreted into the apoplast where they enhance freezing survival by decreasing the freezing point of the tissues non-colligatively (Pihakaski-Maunsbach *et al.*, 2003). It has been suggested that AFPs slow down the rate of ice propagation through the tissues (Pearce and Fuller, 2001). AFPs also inhibit the recrystallization of ice (Knight and Duman, 1986; Doucet *et al.*, 2000). When ice recrystallizes, water molecules migrate from smaller crystals to larger ones, thus increasing both crystal size and the probability of injury to the tissues.

Both *D. antarctica* and *C. quitensis* have been reported to contain inhibitors of ice recrystallization in homogenates of above-ground tissues (Doucet *et al.*, 2000). These inhibitors of ice crystal growth may be AFPs; however, peptides such as melittin, glucagon, and analogues of type I AFPs that do not bind to ice can also inhibit ice recrystallization non-specifically by interfering with boundary migration between adjacent ice crystals when the system is completely frozen (Knight *et al.*, 1995). In order to ascertain whether AFPs are produced in Antarctic plants, it is important to assay antifreeze activity in a way that clarifies whether the proteins actually bind to the surface of ice. Therefore, the goal of this project was to assay antifreeze activity using the ice crystal modification assay that clearly distinguishes whether the two Antarctic vascular plant species produce proteins that bind to ice. In addition, the localization and regulation of the AFPs were examined.

Materials and methods

Plant materials

Deschampsia antarctica Desv. and *Colobanthus quitensis* (Kunth) Bartl. plants were collected from Robert Island (South Shetland Islands, Antarctica; 62°22′ S, 59°43′ W) and were propagated vegetatively in controlled environments using a peat-based potting soil (ProMix BX, Premier Horticulture Ltd, Rivière be Loup, PQ, Canada) mixed with Turface (3:1, v:v) in 250 ml pots. For non-acclimated plants, growth conditions were 15 °C at a photosynthetic photon flux of 200 μmol photons m^{-2} s^{-1} provided by a mixture of cool-white fluorescent and incandescent lamps with a 21 h daylength. Plants were cold-acclimated by transferring them to a growth chamber set at 4 °C, 200 μmol photons m^{-2} s^{-1} and a 16 h daylength for 3 weeks.

Apoplastic protein extraction

Apoplastic proteins were extracted from leaves as described by Hon *et al.* (1994). In brief, the leaves of *D. antarctica* were cut into 1.5 cm lengths and *C. quitensis* leaves were cut into 0.5–1.0 cm lengths, then they were rinsed several times with Milli-Q water and vacuum-infiltrated with a cold solution of 20 mM ascorbic acid and 20 mM $CaCl_2$. Leaf pieces were blotted dry and placed into a 20 ml syringe barrel, which was placed inside a 50 ml centrifuge tube and centrifuged at 4 °C for 30 min at 9000 *g* or 7000 *g* for *D. antarctica* and *C. quitensis,* respectively. Total apoplastic protein contents were measured in the extracts using Bradford's method (Bradford, 1976), as modified by Bio-Rad Laboratories (Mississauga, Ontario, Canada), with bovine serum albumin as the standard protein. Aliquots of the apoplastic extracts from both species were concentrated by ultrafiltration by using a Centricon YM10 with a 10 000 molecular weight cut-off (Millipore Inc., Bedford, MA, USA). All apoplastic extracts were stored at −20 °C until analysis.

Temperature, pH and protease treatments

To test temperature stability, apoplastic extracts from cold-acclimated plants of *D. antarctica* were aliquotted into microfuge tubes and incubated for 30 min at 20, 40, 60, or 100 °C, in a temperature-controlled water bath. Each tube was placed on ice and the antifreeze activity was assayed immediately. The effect of pH on antifreeze activity was analysed by adding 1 vol. of 4× concentrated apoplastic extract to 3 vol. of buffered solutions made using 50 mM Tris–HCl or Tris-base, depending on the pH. The final pH obtained was checked with colorpHast pH paper (EM-Science, Gibbstown, NJ, USA). Extracts were maintained at a given pH for 10 min at 20 °C, then placed on ice and antifreeze activity was assayed. Sensitivity to proteases was determined by adding Proteinase K or Pronase E (Sigma Chemical Co., St Louis, MO, USA) to apoplastic extracts at a final concentration of 1 mg protein ml^{-1}. The extracts were incubated at 20 °C and the antifreeze activity was assayed every 30 min until it was completely abolished.

Antifreeze assays

Antifreeze activity was assayed in 10 nl samples of apoplastic extracts by qualitatively observing the morphology of ice crystals grown in solution (DeVries, 1986; Hon *et al.*, 1994). The growth of a single ice crystal in each sample was controlled using the thermoelectric freezing stage of a nanolitre osmometer (Clifton Technology Physics, Hartford, NY, USA), and images of the ice crystals were captured using a phase-contrast photomicroscope (Olympus BHT, Tokyo, Japan) with a CCD TV camera (Elmo Canada Mfg. Corp., Brampton, ON, Canada), and Scion Image software (Scion Corp., Frederick, MD, USA). In this assay, ice crystals that are round when grown in solution indicate no antifreeze activity, whereas hexagonally shaped ice crystals indicate the presence of an inhibitor of the growth of ice. Thicker hexagonally shaped ice crystals indicate a higher concentration of ice-growth inhibitors.

SDS-polyacrylamide gel electrophoresis

Proteins were denatured and separated by SDS-PAGE according to the method of Laemmli (1970) with Bio-Rad's Mini Protean II system. Samples were mixed 4:1 with 5× sample buffer, boiled for 5 min and loaded on a 12% separating gel with a 4% stacking gel. Prestained protein molecular weight markers (MBI Fermentas, Burlington, ON, Canada) were used to determine apparent molecular masses of polypeptides in the gels. Electrophoresis was carried out at 100 V for about 2 h at 4 °C and the gel was stained with Coomassie blue colloidal staining (Neuhoff *et al.*, 1988).

Results

Apoplastic extracts from D. antarctica *and* C. quitensis

The leaves of both Antarctic plants are very small, which made it difficult to obtain high yields of apoplastic extracts.

D antarctica apparently has a strong leaf structure because it was possible to increase the relative centrifugal force up to 9000 *g* to collect the apoplastic fluid with little cellular damage, as shown by the colourless, clear extracts that were obtained. Leaves of *D. antarctica* rendered about 25 μl of apoplastic fluid g^{-1} of fresh material with a protein content of 0.15 μg protein μl^{-1} in non-acclimated and 0.28 μg protein μl^{-1} in cold-acclimated leaves. On the other hand, it was only possible to centrifuge *C. quitensis* leaves at 7000 *g* without causing damage. Just 10 μl of apoplastic fluid g^{-1} of fresh *C. quitensis* leaves was obtained and the protein concentration was similar in extracts from non-acclimated and cold-acclimated leaves (0.082 and 0.086 μg protein μl^{-1}, respectively). Therefore, the yield of apoplastic protein on a fresh tissue basis was 8-fold higher from cold-acclimated *D. antarctica* than cold-acclimated *C. quitensis*. By comparison, winter rye (*Secale cereale*) leaves can be centrifuged at only 800–2000 *g* to recover apoplastic fluids because higher centrifugal forces yield green fluids indicative of symplastic contamination (Hon *et al.*, 1994; Yu *et al.*, 2001).

D. antarctica *exhibits antifreeze activity*

Hexagonally shaped ice crystals, characteristic of ice growing in the presence of AFPs (DeVries, 1986), formed during freezing of apoplastic extracts of *D. antarctica*. Interestingly, the apoplastic fluid from leaves of both non-acclimated and cold-acclimated plants exhibited antifreeze activity (Fig. 1). After concentrating the *D. antarctica* extracts by ultrafiltration, no inhibition of the growth of ice was observed in the low molecular weight fraction of apoplastic extract of *D. antarctica* that passed through the ultrafiltration membrane with a 10 kDa molecular weight cut-off (Fig. 2A). However, the fraction greater than 10 kDa in molecular weight was concentrated 4-fold and exhibited higher antifreeze activity, as shown by more growth along the c-axis of the crystal (Fig. 2B). By contrast, the apoplastic extracts from non-acclimated and cold–acclimated C. *quitensis* leaves did not show any ice crystal growth inhibition, exhibiting just round, flat crystals (Fig. 1). After ultrafiltration, there was still no observable antifreeze activity from C. *quitensis* (Fig. 2C). These results show

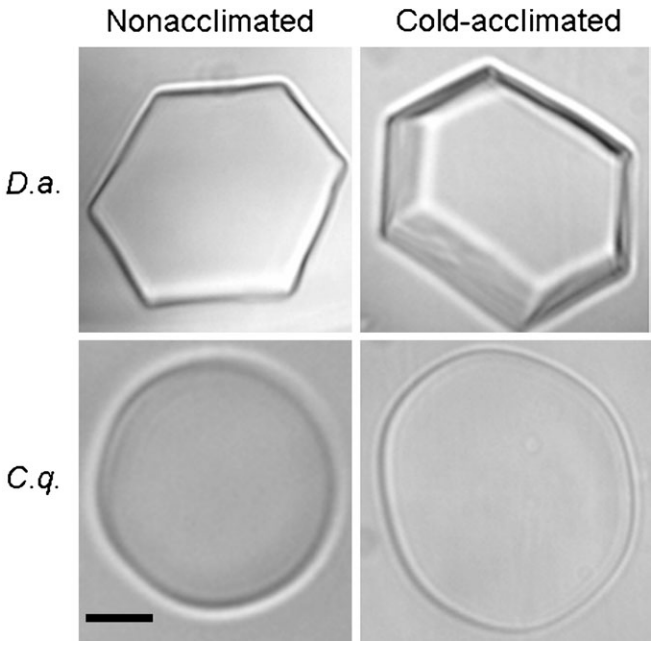

Fig. 1. Antifreeze activity in apoplastic extracts of Antarctic vascular plants. The growth of single ice crystals was examined in 10 nl samples of apoplastic extracts obtained from leaves of non-acclimated and cold-acclimated plants of *D. antarctica* (*D.a.*) and *C. quitensis* (*C.q.*). Each sample was flash-frozen at −40 °C to obtain small ice crystals, warmed until all but one ice crystal had melted, and then cooled to observe the shape of the single ice crystal as it grew. Normally, an ice crystal grows as a round disc. If an inhibitor of ice growth is present, then the crystal cannot grow homogeneously and hexagonal shapes are observed. Inhibition of the growth of ice was observed in apoplastic extracts of both non-acclimated and cold-acclimated *D. antarctica*, but no inhibition was seen in extracts from *C. quitensis.* Scale bar represents 10 μm.

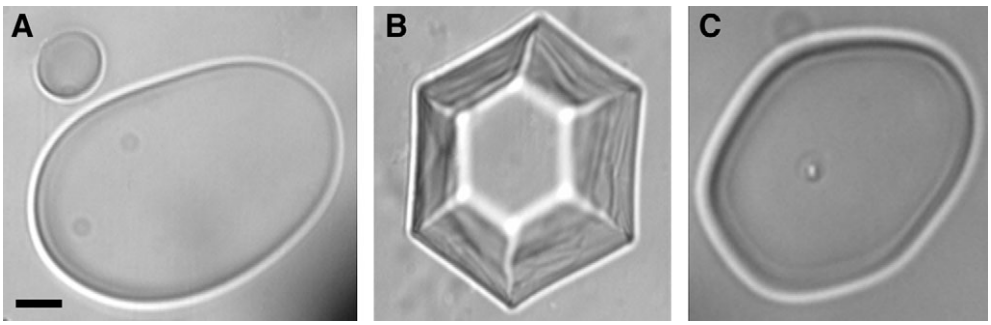

Fig. 2. Antifreeze activity in *D. antarctica* is associated with molecules greater than 10 kDa. Apolastic extracts of cold-acclimated *D. antarctica (D.a.)* and *C. quitensis (C.q.)* were concentrated by ultrafiltration until the volume was reduced by 4-fold. (A) The flow-through of *D. antarctica* extract lacked antifreeze activity, whereas (B) the concentrated extract exhibited higher antifreeze activity, indicating that the activity was associated with molecules greater than 10 kDa in molecular weight. (C) No antifreeze activity was observed in *C. quitensis* extracts that were concentrated 4-fold. Scale bar represents 10 μm.

that the antifreeze activity in *D. antarctica* was associated with a molecular weight fraction greater than 10 kDa.

The level of antifreeze activity was compared between extracts from non-acclimated and cold-acclimated *D. antarctica* plants by serial dilution. The non-acclimated extract lost its antifreeze activity completely at a 1:5 dilution, while the apoplastic extract from cold-acclimated leaves exhibited some antifreeze activity even at a 1:10 dilution (Fig. 3). Therefore, the antifreeze activity was 2–5 times higher in the apoplastic extracts of cold-acclimated compared with non-acclimated leaves.

Antifreeze activity in D. antarctica *is associated with proteins*

In order to demonstrate that antifreeze activity is associated with apoplastic proteins, a series of experiments to determine the protease sensitivity and the temperature and pH dependence of antifreeze activity were performed using apoplastic extracts from cold-acclimated *D. antarctica*. Antifreeze activity was completely eliminated after incubation of the extract with proteinase K for 6 h or with Pronase E for 30 min (Fig. 4A). In the temperature study, the antifreeze activity decreased at 40 °C and was completely abolished after 30 min of incubation at 60 °C (Fig. 4B). As shown by SDS-PAGE, Pronase E treatment of the apoplastic extract for 30 min effectively hydrolysed all polypeptides present (Fig. 5B). The loss of antifreeze activity by both protease treatment and heat denaturation indicated that the antifreeze activity in *D. antarctica* was associated with proteins present in the apoplastic extracts.

To test if a random protein could exhibit antifreeze activity, the shape of ice crystals grown in a solution containing 1 mg ml^{-1} of BSA as a negative control was examined. In all experiments, BSA exhibited round crystals (Fig. 4A), indicating no inhibition of ice crystal growth.

Antifreeze activity was stable throughout the pH range from 3 to 10 (Fig. 4C), although the apoplastic fluid was visibly turbid at pH 9 and 10. For this reason, a sample of apoplastic extract at pH 10 was centrifuged and the pellet and supernatant were assayed for antifreeze activity and examined by SDS–PAGE. No protein was recovered from the pellet (lane P, Fig. 5B); instead, the proteins remained in the supernatant (lane S, Fig. 5B), which exhibited antifreeze activity (Fig. 5C).

Apoplastic polypeptides in D. antarctica

The apoplastic extracts of non-acclimated leaves of *D. antarctica* contained 0.15 μg protein μl^{-1}, compared with 0.28 μg protein μl^{-1} in cold-acclimated leaves. When loaded on the basis of equal volumes and examined by SDS-PAGE, the apoplastic extract of non-acclimated plants exhibited only five easily detectable polypeptides of 36, 32, 30, 22,

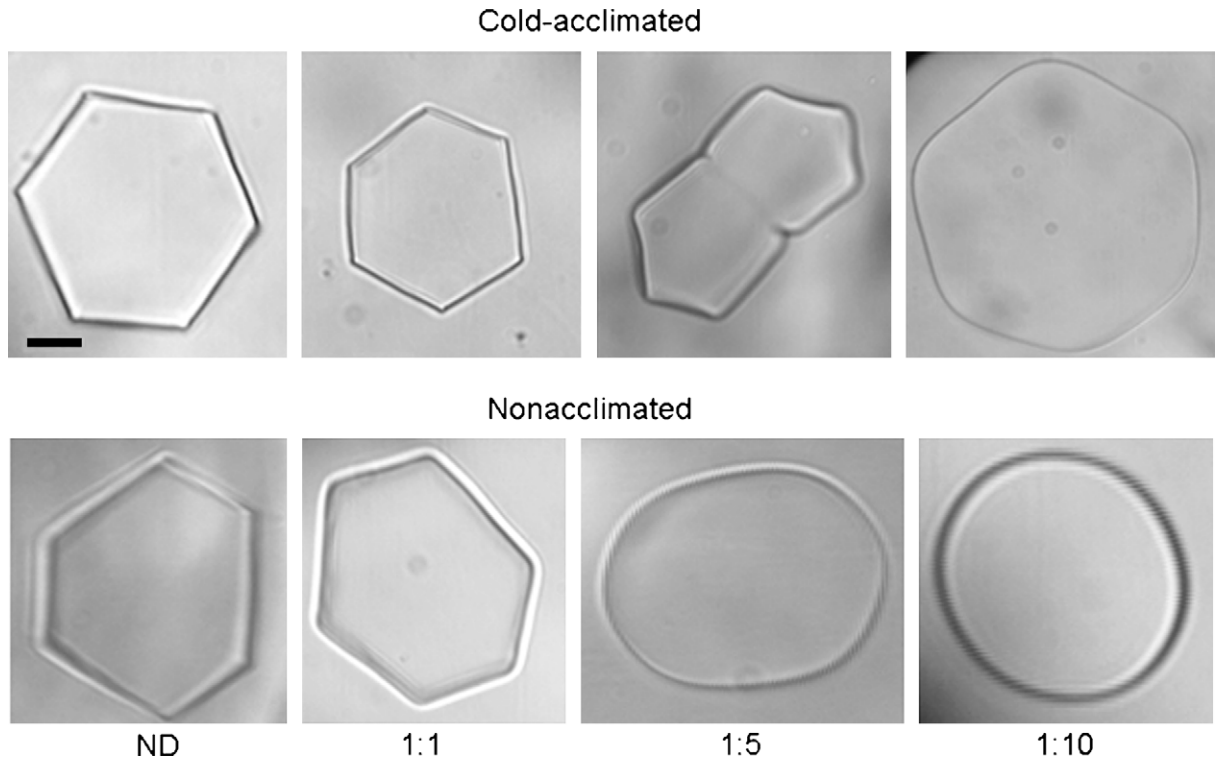

Fig. 3. Cold acclimation increases antifreeze activity in *D. antarctica.* Apoplastic extracts of non-acclimated and cold-acclimated *D. antarctica* were diluted by adding 1, 5 or 10 vols of ultrapure HPLC-grade water and then assayed for antifreeze activity. Cold-acclimated extracts diluted 10-fold still had very low activity, whereas activity was lost in non-acclimated samples diluted 5-fold. ND: not diluted. Scale bar represents 10 μm.

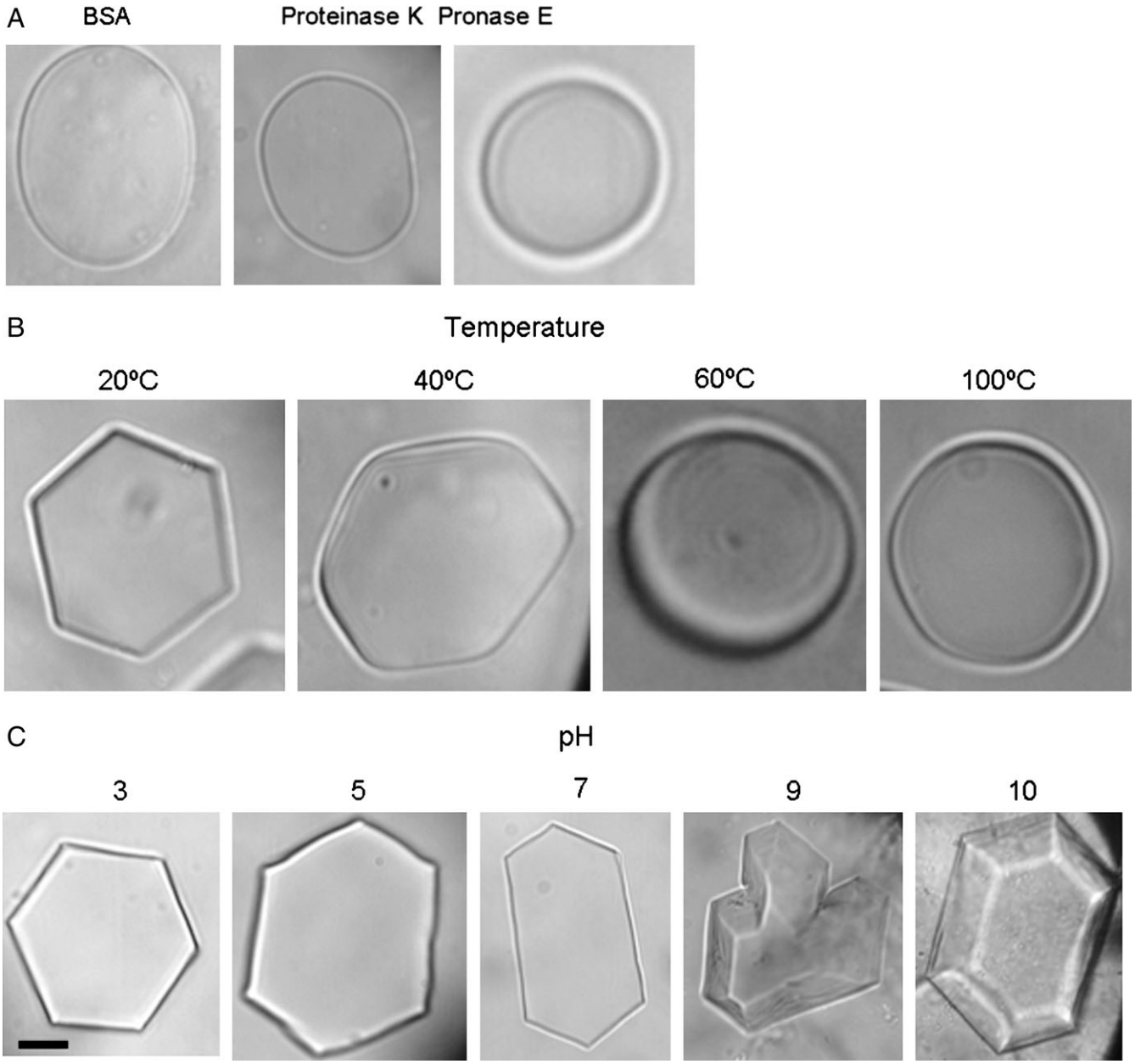

Fig. 4. Sensitivity of antifreeze activity to proteases, temperature and pH. (A) Sensitivity of apoplastic proteins to protease activity was determined by adding 1 mg ml^{-1} of proteinase K or Pronase E to apoplastic extracts from cold-acclimated plants of *D. antarctica* and incubating them at 20 °C for 6 h and 30 min, respectively. BSA was assayed as a control protein that lacks antifreeze activity. (B) Apoplastic extracts from cold-acclimated plants of *D. antarctica* were incubated for 30 min in a temperature-controlled water bath set at 20, 40, 60, and 100 °C. Antifreeze activity was lost at temperatures greater than 40 °C. (C) The effect of pH on antifreeze activity was analysed by adding 1 vol. of 4× concentrated apoplastic extract to 3 vols of buffered solutions made using TRIS–HCl or TRIS-base, depending on the pH. Antifreeze activity was present from pH 3.0 to pH 10.0 and was higher at alkaline pH. After each treatment, samples were assayed for antifreeze activity. Scale bar represents 10 μm.

and 10 kDa in apparent molecular mass. Eight additional polypeptides with apparent molecular masses ranging from 10 kDa to 74 kDa were detected in apoplastic extracts of cold-acclimated leaves of *D. antarctica* (Fig. 5A). Increasing the protein concentration by ultrafiltration resulted in higher antifreeze activity as indicated by greater c-axis growth of the ice crystal in the 4× concentrated apoplastic extract (Fig. 5C). Surprisingly, some of the polypeptides with high apparent molecular mass decreased in the concentrated extract, especially the 43 kDa polypeptide (compare lanes CA and 4× CA, Fig. 5A). This may be explained by irreversible binding of these polypeptides to the ultrafiltration membrane or by degradation of the polypeptides during ultrafiltration. Because of limited sample size, it was not possible to identify individual AFPs within the extracts at this time.

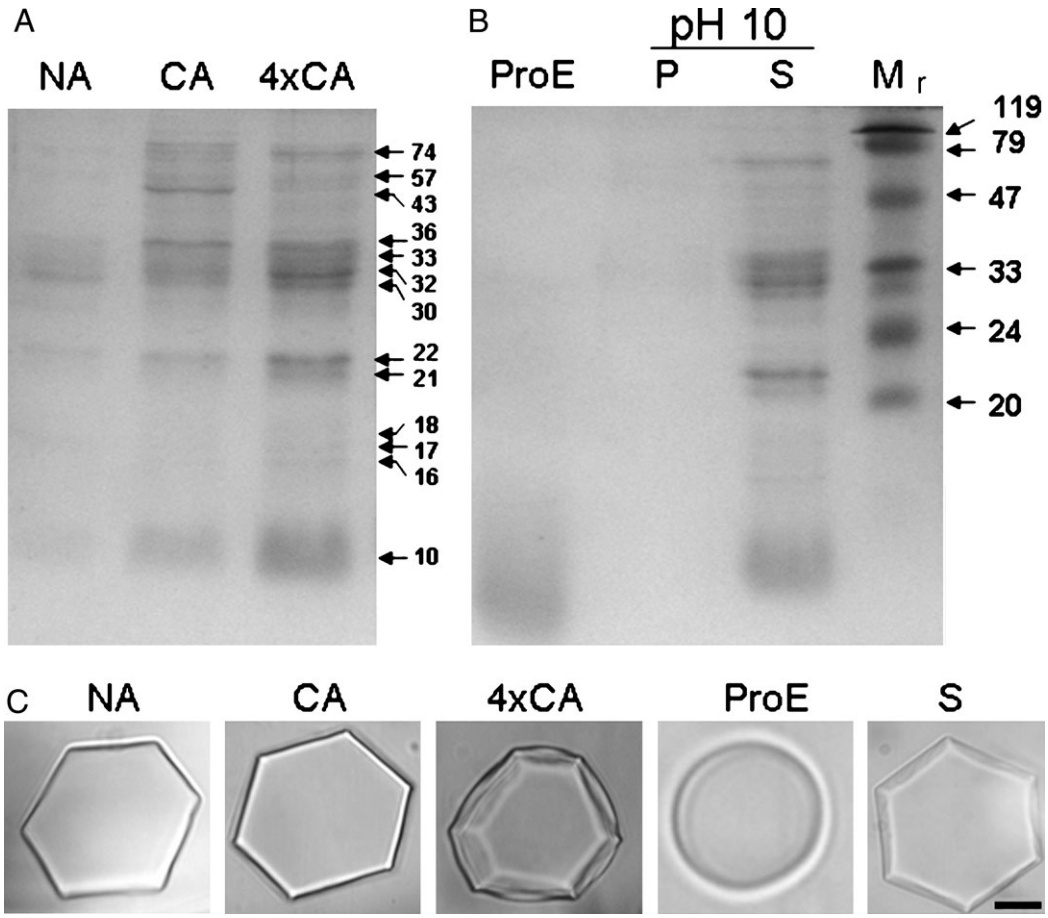

Fig. 5. Apoplastic proteins are responsible for antifreeze activity in *D. antarctica*. (A) Proteins from apoplastic extracts of non-acclimated (NA), cold-acclimated (CA), and 4× concentrated extract of CA *D. antarctica* (4×CA) were denatured and separated by SDS–PAGE. (B) The polypeptides in apoplastic extracts of *D. antarctica* were degraded by Pronase E (ProE) and were not affected by incubation at pH 10.0 (S, supernatant, and P, pellet, after centrifugation at 15 000 *g*). (C) The antifreeze activity observed in apoplastic extracts corresponding to treatments shown in (A) and (B). Scale bar represents 10 μm.

Discussion

Antifreeze activity in Antarctic plants

The unique vascular plant species that have successfully colonized Maritime Antarctica exhibited different mechanisms of freezing resistance in this study. Extracts from *D. antarctica* leaves exhibited antifreeze activity, whereas extracts from the leaves of *C. quitensis* did not, even when concentrated 4-fold (Fig. 1) to reach an apoplastic protein concentration similar to that exhibited by cold-acclimated *D. antarctica*.

Antarctic hairgrass antifreeze activity was not extraordinarily high. For instance, apoplastic extracts from cold-acclimated winter rye obtained by similar procedures were diluted 1:15 to 1:24 with water before losing antifreeze activity (Hon *et al.*, 1994; Stressmann *et al.*, 2004), whereas cold-acclimated *D. antarctica* extracts lost antifreeze activity at a dilution of about 1:10 (Fig. 3), which corresponded to about 0.03 mg apoplastic protein ml^{-1}. The presence of several polypeptides in SDS–PAGE analyses of apoplastic extracts of *D. antarctica* (Fig. 5), coupled with the sensitivity of the antifreeze activity to heat denaturation and protease degradation (Figs 4, 5), indicates that the activity is exerted by AFPs. These AFPs were surprisingly stable over a wide pH range, from pH 3 to 10 (Figs 4, 5).

These results are consistent with previous reports of antifreeze activity in plants. Only about half of the overwintering plants in temperate regions exhibit antifreeze activity (Duman and Olsen, 1993; Doucet *et al.*, 2000), which indicates that there is more than one mechanism among vascular plants for modifying the growth of ice during freezing. Antifreeze activity has been reported in many monocots, especially members of the Poaceae family that include cereals such as barley, wheat, oat, and rye (Antikainen and Griffith, 1997), and grasses (Duman and Olsen, 1993). On the other hand, many overwintering dicotyledonous plants lack antifreeze activity (Duman and Olsen, 1993; Antikainen and Griffith, 1997; Doucet *et al.*, 2000), so it was not so surprising to learn that there was no antifreeze activity in apoplastic extracts of *C. quitensis* (Figs 1, 2). The presence of antifreeze proteins in the apoplast of *D. antarctica* is consistent with previous studies

which showed that this species is highly freezing-tolerant: the LT_{50} of non-acclimated plants is about −12 °C while cold-acclimated plants reach −26 °C. On the other hand, *C. quitensis* does not tolerate the formation of ice within its tissues and does not increase its LT_{50} of about −5 °C during cold-acclimation. This species survives freezing temperatures by its moderate supercooling capability (Bravo *et al.*, 2001).

Doucet *et al.* (2000) reported that total soluble extracts from *D. antarctica* and *C. quitensis,* when combined with 30% sucrose, 50 mM Tris–HCl, pH 7.4, 20 mM ascorbate, and 10 mM EDTA, lost the ability to inhibit the recrystallization of ice when diluted to 0.05 and 0.1 mg protein ml^{-1}, respectively. The inhibitor of recrystallization from *D. antarctica* was stable to heating at 95 °C but had reduced activity after proteolytic degradation by Pronase, whereas the inhibitor from *C. quitensis* was stable to both heat and proteolytic treatment. Because these characteristics differ from those of the AFPs that were extracted from the leaf apoplast of *D. antarctica*, it is possible that the recrystallization inhibitors identified by Doucet and coworkers (2000) were polypeptides acting non-specifically (Knight *et al.*, 1995) or were heat-soluble proteins and solutes normally localized in the symplast. For example, Wisniewski *et al.* (1999) have shown that PCA60, a cold-induced, symplastic dehydrin found in the bark of peach trees, has the ability to bind to the surface of ice. An alternative explanation is that molecules released from the symplast were able to stabilize apoplastic proteins and prevent denaturation by heat.

Regulation of antifreeze activity

Apoplastic extracts from non-acclimated plants of *D. antarctica* showed a significant capacity to inhibit ice crystal growth (Fig. 3). As far as the authors know, this is the first report of constitutive antifreeze activity in plants. All previous studies have shown that antifreeze activity is only observed when plants are acclimated to cold temperatures and short days (Urrutia *et al.*, 1992; Marentes *et al.*, 1993; Duman and Olsen, 1993; Griffith and Yaish, 2004). One mechanism by which winter rye plants regulate antifreeze activity is to produce ethylene in response to cold and to drought, which, in turn, induces the accumulation of AFPs (Yu *et al.*, 2001). Once AFPs accumulate in the apoplast, their activities may be regulated by Ca^{2+} (Stressmann *et al.*, 2004).

As *D. antarctica* evolved under the selection pressure of constant low temperature on the Antarctic islands (Day *et al.*, 1999; Alberdi *et al.*, 2002; Lewis Smith, 2003), the species could have acquired regulatory elements for constitutive expression of AFP genes. However, *D. antarctica* also exhibits inducible antifreeze activity in response to low temperature and shorter daylengh (16 h d^{-1}) (Fig. 1), indicating that it still responds to cold even after generations of continuous growth at low temperature. Environmental temperature is not the only selective force responsible for changes in the regulation of antifreeze activity because *C. quitensis* evolved a different strategy for resisting freezing temperatures and still survives under the same environmental conditions.

Strategies of freezing resistance

Although AFPs alone may account for freezing resistance in polar fish that survive in ice-laden seawater at −1.8 °C (Marshall *et al*., 2004), they do not explain the capability of Antarctic plants to survive much colder temperatures. Instead, AFPs are likely to be just one component of the complex mechanism of freezing tolerance in Antarctic plants that includes the accumulation of a high amount of sucrose and non-structural carbohydrates, as well as stress-induced proteins such as dehydrins, during cold acclimation (Bravo *et al*., 2001; Zúñiga-Feest *et al.*, 2003; Olave-Concha *et al*., 2004). Dehydrins may be involved in cryoprotection or in preventing freeze-induced cell dehydration (Lin and Thomashow, 1992; Houde *et al.*, 1995; Bravo *et al.*, 2003). In addition, it has been shown that dehydrins may also have antifreeze activity (Wisniewski *et al.*, 1999; Griffith and Yaish, 2004). Altogether, these adaptations to freezing may account for the survival of *D. antarctica* and may partially explain why this unique grass species populates the Antarctic territories. The difference in antifreeze activity between *D. antarctica* and *C. quitensis* may reflect different strategies of avoiding freezing injury, both of which appear to be equally successful for plants colonizing the Maritime Antarctic.

Further studies are needed in order to address how antifreeze activity is maintained in non-acclimated *D. antarctica*. It would also be interesting to follow the antifreeze activity of *D. antarctica* in the field where populations of *D. antarctica* are increasing (Day *et al.*, 1999; Lewis Smith, 1994; Gerighausen *et al.*, 2003) as Antarctica is undergoing dramatic regional warming (Simpson, 2000; Karentz, 2003).

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