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Exogenous Abscisic Acid Mimics Cold Acclimation for Cacti Differing in Freezing Tolerance[1]

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The responses to low temperature were determined for two species of cacti sensitive to freezing, *Ferocactus viridescens* and *Opuntia ficus-indica*, and a cold hardy species, *Opuntia fragilis*. Fourteen days after shifting the plants from day/night air temperatures of 30/20°C to 10/0°C, the chlorenchyma water content decreased only for *O. fragilis*. This temperature shift caused the freezing tolerance (measured by vital stain uptake) of chlorenchyma cells to be enhanced only by about 2.0°C for *F. viridescens* and *O. ficus-indica* but by 14.6°C for *O. fragilis*. Also, maintenance of high water content by injection of water into plants at 10/0°C reversed the acclimation. The endogenous abscisic acid (ABA) concentration was below 0.4 pmol g^{-1} fresh weight at 30/20°C, but after 14 d at 10/0°C it increased to 84 pmol g^{-1} fresh weight for *O. ficus-indica* and to 49 pmol g^{-1} fresh weight for *O. fragilis*. Four days after plants were sprayed with 7.5×10^{-5} M ABA at 30/20°C, freezing tolerance was enhanced by 0.5°C for *F. viridescens*, 4.1°C for *O. ficus-indica*, and 23.4°C for *O. fragilis*. Moreover, the time course for the change in freezing tolerance over 14 d was similar for plants shifted to low temperatures as for plants treated with exogenous ABA at moderate temperatures. Decreases in plant water content and increases in ABA concentration may be important for low-temperature acclimation by cacti, especially *O. fragilis*, which is widely distributed in Canada and the United States.

Cacti are generally native to hot, arid habitats, but most of the species native to regions north of Mexico experience subzero temperatures each year (Benson, 1982; Gibson and Nobel, 1986). Cacti that tolerate freezing temperatures include *Opuntia humifusa*, which grows as far north as southern Ontario, Canada, and can tolerate subzero temperatures of −24°C and *Opuntia fragilis*, which grows in northern Alberta, Canada, and can survive a 1-h treatment at −40°C (Nobel and Loik, 1990, 1993). In contrast, cacti that are native to the southwestern United States, such as *Ferocactus viridescens*, and others that are extensively cultivated for their fruits and cladodes, such as *Opuntia ficus-indica*, generally succumb to air temperatures of −6 to −10°C (Nobel, 1982; Russell and Felker, 1987).

The stem water content of *O. humifusa* in Iowa and *Opuntia erinacea* in Colorado decreases in the autumn (Koch and Kennedy, 1980; Littlejohn and Williams, 1983). Laboratory studies have shown that stem water loss does not greatly decrease the equilibrium freezing point (Loik and Nobel, 1991, 1993) and may be caused by a reduction in root water uptake at low soil temperatures (Lopez and Nobel, 1991). The reduction in water content of cacti during exposure to low temperatures is often accompanied by increasing amounts of sugars that can act as cryoprotectants (Goldstein and Nobel, 1991). In addition to providing some protection from low temperatures, loss of water may trigger other acclimation processes, such as production of ABA.

Many species exhibit an increase in endogenous ABA concentration when exposed to low temperatures (Irving, 1969; Chen and Gusta, 1983), and exogenous ABA can increase freezing tolerance by 6 to 23°C (Chen et al., 1983; Keith and McKersie, 1986; Xin and Li, 1992). Localized increases in ABA are generally followed by changes in gene expression (Johnson-Flanagan et al., 1991), which can lead to specific acclimation processes, such as the production of enzymes related to sugar metabolism and desiccation tolerance (Lee and Chen, 1993). Exogenous ABA shifts *Portulacaria afra* from C_3 to CAM (Ting, 1981) but does not enhance phospho*enol*pyruvate carboxylase gene expression in *Mesembryanthemum crystallinum* (Thomas et al., 1992). The relation of ABA to freezing tolerance for cacti, most of which are CAM plants, is unknown (Gibson and Nobel, 1986; Nobel, 1988; Loik and Nobel, 1993).

In the present study we examined the freezing tolerance of three species of cacti that differed in their ability to survive freezing temperatures: *F. viridescens*, which appears only in relatively warm habitats in coastal southern California (Benson, 1982), *O. ficus-indica*, which is cultivated in warm regions worldwide for fruits and stem segments (Russell and Felker, 1987), and *O. fragilis*, which apparently has the greatest freezing tolerance of any species of cactus (Loik and Nobel, 1993). *F. viridescens* and *O. ficus-indica* do not exhibit much water loss upon exposure to low temperatures, whereas *O. fragilis* exhibits appreciable water loss during low-temperature acclimation, leading to decreases in its water potential, relative water content, and tissue thickness (Loik and Nobel, 1993). Therefore, water content, ABA content, and freezing tolerance were determined for these species after acclimation to day/night air temperatures of 30/20°C or 10/0°C. ABA was applied to plants at the higher temperatures to determine whether it would mimic the effects of low temperature. Injections of water into plants maintained at the lower tem-

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Abbreviation: LT_{50}, the subzero temperature leading to half-maximal uptake of vital stain.

peratures were made to determine the importance of water loss to the acclimation process.

MATERIALS AND METHODS

Plant Material

Ferocactus viridescens (Nutt.) Britton and Rose (Cactaceae) was collected in San Diego County, CA. Cladodes (flattened stem segments) of *Opuntia ficus-indica* (L.) Miller were obtained from the Agricultural Experiment Station at the University of California, Riverside, CA. Cladodes of *Opuntia fragilis* (Nutt.) Haw. were collected from a population near Kaladar, Ontario, Canada. All plants were placed in soil collected from Agave Hill, near Palm Desert, CA (Nobel, 1976), and were maintained in a glasshouse at the University of California, Los Angeles, for at least 6 months. Conditions in the glasshouse included average day/night maximal/minimal air temperatures of 26/17°C, an average PPFD (400–700 nm) of 640 μmol m^{-2} s^{-1} (27 mol m^{-2} d^{-1}), and average maximal/minimal RH of 80/48%. Plants were watered two to three times per week so that the water potential in the root zone was greater than −0.3 MPa. Four weeks before experiments, plants were moved to environmental chambers with day/night air temperatures of 30/20°C, a PPFD of 600 μmol m^{-2} s^{-1} for 12 h d^{-1} (06:00–18:00 h), and maximal/minimal RH of 67/38%. To induce low-temperature acclimation, air temperatures were lowered to 10/0°C.

Freezing Tolerance

Freezing tolerance was assessed for chlorenchyma cooled in the dark at 10°C h^{-1}, similar to cooling rates that occur in the field during the winter (Nobel, 1981, 1988). Small sections (approximately 0.5 cm^2) of the tissue were removed with a razor blade (one from each of five plants), placed in 10-mL beakers, and cooled in a Revco ULT-80 ultralow-temperature freezer; the initial temperature was 20°C, and ice nucleation occurred at −4 to −6°C. The samples were maintained for 1 h at a particular air temperature, measured with a copper-constantan thermocouple (0.2 mm in diameter), and then warmed at 10°C h^{-1}.

After the low-temperature treatment, approximately 5 mL of 15 mM neutral red (3-amino-7-dimethylamino-2-methylphenazine [HCl]) were added. The beakers were stored overnight at 5°C to maximize uptake of the vital stain, which accumulates in the vacuoles of living cells only (Onwueme, 1979; Nobel and Loik, 1990). Freehand sections, approximately three cells thick, were examined at ×100 with an Olympus BH2 phase-contrast microscope. Stain uptake was determined for approximately 150 cells from each of five plants for each treatment temperature. Stain uptake was based on the dark red appearance of living cells, and stain intensity was constant at a particular temperature (intensity decreased below −30°C).

LT_{50} was quantified as the temperature leading to 50% stain uptake relative to samples treated for 1 h at 0°C (Onwueme, 1979), which led to 92% of cells taking up stain (Nobel and Loik, 1990). Such an LT_{50} is correlated with the survival of entire stems of cacti (Nobel, 1981, 1982). LT_{50} was determined graphically from separate experiments on five plants. Acclimation was quantified as the difference between LT_{50} for plants at day/night air temperatures of 30/20°C and LT_{50} for plants acclimated to 10/0°C for 14 d.

Water Relations

The water content of the chlorenchyma was measured for stem samples removed with a cork borer 16 mm in diameter. The chlorenchyma was separated from the water-storage parenchyma with a razor blade, and the cut surface was blotted with tissue paper to remove water from damaged cells. The samples were weighed and then dried in a forced-draft oven until no further weight loss occurred (generally 4 d). Fractional water content was calculated as (fresh weight − dry weight)/fresh weight. Water vapor conductance was measured using a Li-Cor LI-1200 steady-state porometer modified to accommodate the succulent stems.

To test for the effect of stem water content on freezing tolerance, five plants acclimated to 10/0°C were injected daily with degassed, distilled water. A syringe was used to inject the water into the water-storage parenchyma at a distance of 1 cm from the cladode margin using a needle 0.7 mm in diameter. Approximately 1 mL of water was injected into the relatively small stems of *O. fragilis* (about 20 g fresh weight), and about 30 mL were injected into the cladodes of *O. ficus-indica* (about 600 g fresh weight).

ABA

ABA (mixed isomers, Sigma) was dissolved in ethanol and then diluted with water to 7.5×10^{-5} M (Tanino et al., 1990). The solution was sprayed on five plants maintained at day/night air temperatures of 30/20°C approximately 3 h after the beginning of the dark period (controls were sprayed with distilled water). Freezing tolerance was assessed using vital stain uptake 4 d (Lee et al., 1991) after the plants were sprayed.

The chlorenchyma ABA concentration was determined for sprayed plants as well as for plants maintained at 30/20°C, 10/0°C, or at 10/0°C and injected with water. Samples were removed from each of five stems with the 16-mm-diameter cork borer, weighed, and ground in liquid nitrogen. They were extracted three times at 0°C in 80% methanol with 10 mg mL^{-1} of butylated hydroxytoluene and centrifuged at 2000*g* for 10 min (Smit et al., 1990). The supernatants from each extraction were combined and lyophilized. The extracts were resuspended in 10 mM Tris-HCl buffer (pH 6.5) to a final volume of 2 mL, purified using a DEAE-cellulose:DEAE-Sephadex (2:1) column, eluted with 0.5 M acetic acid, lyophilized, and then resuspended in 0.5 mL of PBS. [^{3}H]ABA was added before chromatography to correct for hormone losses.

ABA content was measured using a competitive binding immunoassay (Smit et al., 1990). Aliquots (100 μL each) of the purified extract, calf serum, [^{3}H]ABA, and rabbit anti-(+)ABA were mixed in a microcentrifuge tube and incubated for 1 h at 4°C. The proteins were precipitated with 90% saturated ammonium sulfate for 30 min, collected by centrifugation, and recentrifuged with 50% saturated ammonium sulfate. After the supernatant was removed by aspiration, the pellet was suspended in 0.15 mL of distilled water plus 1 mL

of scintillation fluid, and radioactivity was determined with a Beckman LS 1801 liquid scintillation counter.

Statistical Analyses

For all experiments, measurements were made on samples from five different plants (LT_{50} was determined graphically for about 750 cells at each treatment temperature). Means and SE were calculated using the statistical package CoStat (version 4.00; CoHort, Berkeley, CA) on an IBM PS/2, 30 MHz.

RESULTS

Water Relations

The water content of the chlorenchyma was examined for the three species following a shift from day/night air temperatures of 30/20°C to 10/0°C (Table I). The chlorenchyma water content was essentially unchanged for *F. viridescens* and *O. ficus-indica* ($P > 0.1$); exposure to day/night air temperatures of 10/0°C for 14 d caused the stems of *F. viridescens* to develop black necrotic spots leading to rot. On the other hand, chlorenchyma water content decreased by 7% for *O. fragilis* ($P < 0.001$) during 14 d at 10/0°C (Table I).

The water vapor conductance was measured to check patterns of stomatal opening. At 21:30 h, the water vapor conductance was 42 ± 2 mmol m^{-2} s^{-1} for *F. viridescens* (mean ± SE for five plants), 63 ± 8 mmol m^{-2} s^{-1} for *O. ficus-indica*, and 51 ± 2 mmol m^{-2} s^{-1} for *O. fragilis*. The water vapor conductance at 11:30 h averaged one-ninth as much as at 21:30 h for the three species.

ABA Content

At 30/20°C, the chlorenchyma ABA concentration was extremely low for *O. ficus-indica* and *O. fragilis* (Table II). Following 14 d at 10/0°C, the chlorenchyma ABA concentration averaged 66 pmol g^{-1} fresh weight for the two species. Plants at 30/20°C and sprayed with 7.5×10^{-5} M ABA had a chlorenchyma ABA concentration averaging 56 pmol g^{-1} fresh weight. The average chlorenchyma ABA concentration for plants at 10/0°C and injected daily with water was only 0.3 pmol g^{-1} fresh weight, similar to that for plants at 30/20°C (Table II).

Freezing Tolerance

Subzero air temperatures greatly reduced chlorenchyma cell viability, as indicated by the uptake of the vital stain neutral red (Fig. 1). For plants at day/night air temperatures of 30/20°C, LT_{50} was about −6°C. Fourteen days after a shift to day/night air temperatures of 10/0°C, LT_{50} decreased by 1.7°C for *F. viridescens* (Fig. 1A), 2.2°C for *O. ficus-indica* (Fig. 1B), and 14.6°C for *O. fragilis* (Fig. 1C).

After the application of ABA, the tolerance of low temperatures for plants at 30/20°C increased. Four days after plants were sprayed with 7.5×10^{-5} M ABA, LT_{50} decreased by 0.5°C for *F. viridescens* (Fig. 1A), 4.1°C for *O. ficus-indica* (Fig. 1B), and 23.4°C for *O. fragilis* (Fig. 1C).

Table I. *Chlorenchyma water content at day/night air temperatures of 30/20°C and 14 d after shifting to 10/0°C*

Data are means ± SE (*n* = five plants).

	Water Content	
	30/20°C	10/0°C
	% by weight	
F. viridescens	76 ± 2	75 ± 2
O. ficus-indica	90 ± 1	88 ± 1
O. fragilis	83 ± 0	76 ± 1

Table II. *ABA concentration in the chlorenchyma of O. ficus-indica and O. fragilis*

Plants were maintained for 14 d at day/night air temperatures of 30/20°C, 10/0°C, 30/20°C with exogenous ABA applied initially, and 10/0°C with daily water injection. Data are means ± SE (*n* = five plants).

Condition	ABA Concentration	
	O. ficus-indica	*O. fragilis*
	pmol g^{-1} fresh wt	
30/20°C	0.37 ± 0.04	0.02 ± 0.01
10/0°C	84 ± 27	49 ± 20
30/20°C + applied ABA	67 ± 29	42 ± 18
10/0°C + injected H_2O	0.28 ± 0.04	0.23 ± 0.12

Time Course and Response to ABA Concentration

The extent and the time course of the changes in LT_{50} over 14 d were similar when plants were shifted to 10/0°C or sprayed with 7.5×10^{-5} M ABA (Fig. 2). For *O. ficus-indica*, the decrease in LT_{50} was 2.6°C during 14 d at 10/0°C and 2.9°C for plants treated with ABA. LT_{50} for *O. fragilis* shifted to 10/0°C became 15°C lower at 6 d; following treatment with exogenous ABA at 30/20°C, LT_{50} became 22°C lower after 8 d (Fig. 2).

Increasing concentrations of ABA sprayed on plants maintained at 30/20°C decreased LT_{50} (Fig. 3). For *O. ficus-indica*, LT_{50} decreased from −5°C for plants sprayed with distilled water to about −10°C for plants sprayed with 1×10^{-4} M ABA (Fig. 3). Over the same range, LT_{50} decreased from −8 to −27°C for *O. fragilis*.

Water Injection and Freezing Tolerance

Injecting water into *O. fragilis* and *O. ficus-indica* for plants at 10/0°C increased LT_{50} (Fig. 4). When about 30 mL of water were injected daily into *O. ficus-indica*, LT_{50} was −2.3°C after 14 d. On the other hand, uninjected plants had an LT_{50} of −9.7°C after 14 d at 10/0°C. When about 1 mL of water was injected daily into the smaller stems of *O. fragilis*, LT_{50} became −3.5°C after 14 d. The LT_{50} for uninjected plants was −20.6°C after 14 d at 10/0°C. Most of the changes caused by injecting water were apparent 1 d after injection for both species (Fig. 4).

DISCUSSION

Stems of *F. viridescens*, *O. ficus-indica*, and *O. fragilis* exhibited different freezing tolerances when exposed to low

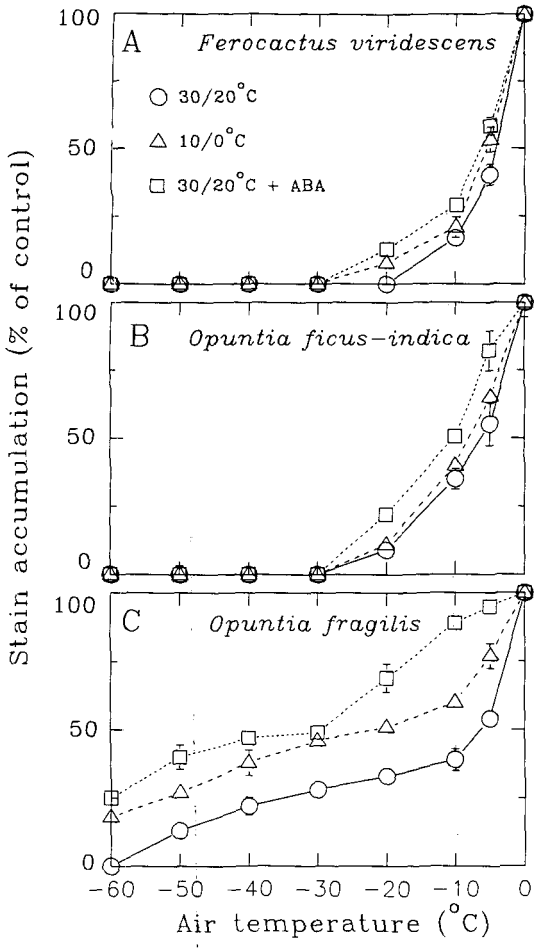

Figure 1. Viability of chlorenchyma cells of *F. viridescens* (A), *O. ficus-indica* (B), and *O. fragilis* (C) measured by stain accumulation at subzero temperatures. Uptake of a vital stain for stem samples treated for 1 h at the indicated temperature is expressed relative to stain uptake at 0°C. Plants were maintained at day/night air temperatures of 30/20°C (○), 14 d at 10/0°C (△), or 4 d at 30/20°C after spraying with 7.5×10^{-5} M ABA (□). Data are means ± SE (n = five plants). When bars are not shown, the SE was smaller than the symbol.

day/night air temperatures for 14 d. For *F. viridescens*, LT_{50} for chlorenchyma cells occurred at −6°C for plants maintained at 10/0°C. This species is the least tolerant of subzero temperatures of the four species of *Ferocactus* in the United States, restricting it to relatively warm habitats in coastal southern California (Nobel, 1980; Benson, 1982). The LT_{50} for *O. ficus-indica* at 10/0°C of −8°C is consistent with its limited survival during occasional freezing episodes in southern Texas, where it is used for cattle forage (Russell and Felker, 1987). On the other hand, *O. fragilis* had an LT_{50} at 10/0°C of −21°C and is one of the most freezing-tolerant species of cactus (Benson, 1982). Such low values of LT_{50} for *O. fragilis* are apparently due to its great cold acclimation ability (Loik and Nobel, 1993).

When plants were shifted from day/night air temperatures of 30/20°C to 10/0°C for 14 d, the tolerance of the lower temperatures was accompanied by water loss from the chlorenchyma. The change in water content of *O. fragilis* was about five times that for *F. viridescens* and *O. ficus-indica*. For *O. ficus-indica*, most of its 13% water loss during low-temperature acclimation occurs from the water-storage parenchyma (Goldstein and Nobel, 1991), as is also the case for desert cacti during drought (Barcikowski and Nobel, 1984). On the other hand, winter-hardy cacti, such as *O. humifusa*, can lose up to 42% of their chlorenchyma water during acclimation to low temperatures (Loik and Nobel, 1991), presumably inducing acclimation processes in that tissue.

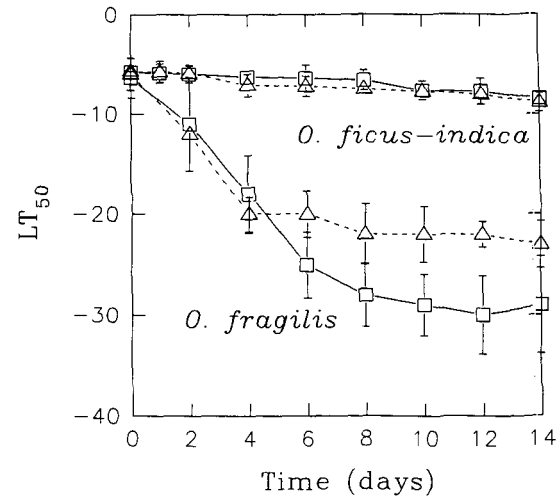

Figure 2. Time course of changes in freezing tolerance (LT_{50}) for *O. ficus-indica* and *O. fragilis*. Plants maintained at day/night air temperatures of 30/20°C were either shifted to 10/0°C (△) or sprayed with 7.5×10^{-5} M ABA (○). LT_{50} was determined from figures such as Figure 1. Data are means ± SE (n = five plants).

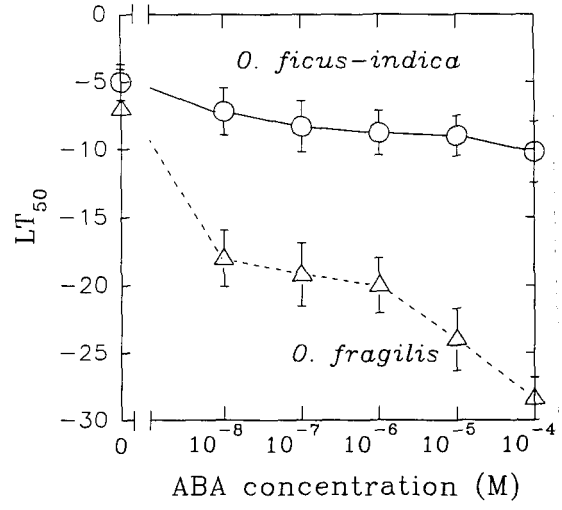

Figure 3. The change in freezing tolerance (LT_{50}) as a function of ABA concentration for *O. ficus-indica* (○) and *O. fragilis* (△). Viability of chlorenchyma cells was measured 4 d after ABA was sprayed on plants maintained at 30/20°C. LT_{50} was determined from figures such as Figure 1. Data at 0 ABA indicate LT_{50} for plants sprayed with distilled water. Data are means ± SE (n = five plants).

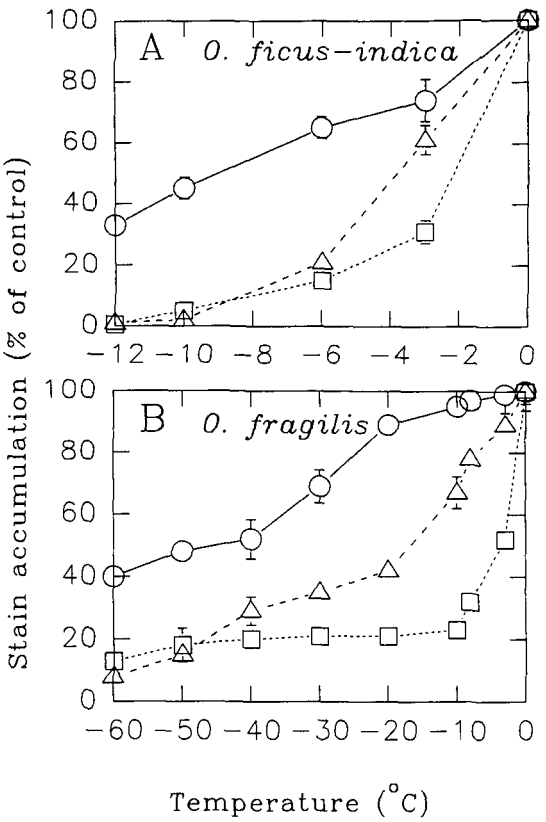

Figure 4. Viability of chlorenchyma cells of *O. ficus-indica* (A) and *O. fragilis* (B) for plants maintained at day/night air temperatures of 10/0°C (○), 10/0°C and injected daily for 14 d (□), and 10/0°C but injected only on d 14 (△). Plants were injected with 30 mL (*O. ficus-indica*) or 1 mL (*O. fragilis*) of degassed, distilled water. Data are means ± SE (n = five plants).

Reduced water content induced by low temperature is correlated with an increase in freezing tolerance for other species (Gusta and Fowler, 1976; Chen and Gusta, 1978), although some species acclimate to low temperature without changes in water content (Levitt, 1980). *O. ficus-indica* and *O. fragilis* at 10/0°C exhibited less tolerance of low temperatures after the stems were injected with water; in fact, LT_{50} was even higher than preacclimation levels. It is not clear whether freezing tolerance of injected plants was affected because of ABA content and its associated changes or by reduced mechanical damage during ice crystal formation (Olien, 1984; Steponkus, 1984).

All three cactus species exhibited increased freezing tolerance when stems maintained at 30/20°C were sprayed with ABA. The change in LT_{50} for *O. fragilis* was about 6-fold greater than for *F. viridescens* or *O. ficus-indica*. For all three species, the effect of ABA was slightly greater than that caused by a shift in air temperatures from 30/20°C to 10/0°C, consistent with results for cultured cells of *Bromus inermis, Daucus carota, Secale cereale,* and *Triticum aestivum* (Chen and Gusta, 1983). The time course for the change in LT_{50} for the two cactus species at 30/20°C sprayed with ABA for 14 d was similar to the time course after shifting to 10/0°C. For suspension-cultured cells of *Brassica napus* and *Zea mays,* addition of ABA causes a faster change in freezing tolerance than does decreasing the ambient temperature (Johnson-Flanagan et al., 1991; Xin and Li, 1992).

The greater change in LT_{50} in response to ABA application for *O. fragilis* than for *O. ficus-indica* suggests that either *O. fragilis* responds to ABA differently, more ABA reached its chlorenchyma, or it has a greater sensitivity to ABA than *O. ficus-indica*. Both species had similar water vapor conductance values; therefore, amounts of ABA entering through stomates should have been similar (the uptake of ABA by roots was not determined). Actually, the chlorenchyma ABA concentration averaged 37% lower for *O. fragilis* than for *O. ficus-indica*. Even though *O. fragilis* contained lower levels of endogenous and applied ABA, it had greater low-temperature acclimation and ABA-induced changes in freezing tolerance than did *O. ficus-indica*.

Many plant species exhibit an increase in endogenous ABA concentration in response to environmental stresses, including low temperature and drought (Davies and Zhang, 1991). Changes in protein synthesis, metabolism, and cell ultrastructure occur concomitantly with increases in ABA concentration, suggesting that ABA acts as a primary messenger that induces a cascade of acclimation responses. Moreover, ABA-deficient mutants of *Arabidopsis thaliana* lack low-temperature acclimation (Heino et al., 1990). Participation of other factors, such as kinetin and GA, may also be important for low-temperature acclimation (Reaney et al., 1989).

When air and soil temperatures decrease in the autumn, water uptake and stem water content decrease for cacti, whereas stomatal opening and CO_2 uptake continue (Nobel and Loik, 1990; Lopez and Nobel, 1991). The decrease in plant water content may trigger a general or local increase in ABA production or its release from storage (Davies and Zhang, 1991; Trewavas, 1992). Such increases in ABA content apparently are important for the low-temperature acclimation of *O. fragilis*, which is widely distributed in Canada and the United States, as well as for the agronomically useful species *O. ficus-indica*.

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