Plant Physiol. (1996) 111: 605–612

Cell-Wall Changes and Cell Tension in Response to Cold Acclimation and Exogenous Abscisic Acid in Leaves and Cell Cultures[1]

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Freeze-induced cell tensions were determined by cell water relations in leaves of broadleaf evergreen species and cell cultures of grapes (*Vitis* spp.) and apple (*Malus domestica*). Cell tensions increased in response to cold acclimation in leaves of broadleaf evergreen species during extracellular freezing, indicating a higher resistance to cell volume changes during freezing in cold-hardened leaves than in unhardened leaves. Unhardened leaves, typically, did not develop tension greater than 3.67 MPa, whereas cold-hardened leaves attained tensions up to 12 MPa. With further freezing there was a rapid decline and a loss of tension in unhardened leaves of all the broadleaf evergreen species studied. Also, similar results were observed in cold-hardened leaves of all of the species except in those of inkberry (*Ilex glabra*) and *Euonymus fortunei*, in which negative pressures persisted below −40°C. Abscisic acid treatment of inkberry and *Euonymus kiautschovica* resulted in increases in freeze-induced tensions in leaves, suggesting that both cold acclimation and abscisic acid have similar effects on freezing behavior—specifically on the ability of cell walls to undergo deformation. Decreases in peak tensions were generally associated with lethal freezing injury and may suggest cavitation of cellular water. However, in suspension-cultured cells of grapes and apple, no cell tension was observed during freezing. Cold acclimation of these cells resulted in an increase in the cell-wall strength and a decrease in the limiting cell-wall pore size from 35 to 22 Å in grape cells and from 29 to 22 Å in apple cells.

Plants generally survive low temperatures by extracellular freezing. Typically during freezing of plant tissues, cellular water migrates to extracellular ice, causing cell dehydration and cell collapse (Levitt, 1980; Singh and Miller, 1985; Pearce, 1988; Pearce and Ashworth, 1992). In fact, for cell dehydration to occur during freezing, cells should be able to undergo cell volume reduction or cell deformation, which is generally dependent on the mechanical properties of cell walls. Rigid cell walls may not allow for free collapse of cells during extracellular freezing, resulting in the development of negative pressures in the cells (Rajashekar and Burke, 1982). Indeed, occurrence of negative pressure has been proposed in many plant species (Rajashekar and Burke, 1982; Anderson et al., 1983; Hansen and Beck, 1988; Zhu and Beck, 1991), and direct measurements of negative pressures have been made in an artificial cell during freezing (Zhu et al., 1989). Cell resistance to collapse can lead to reduction in cell dehydration during freezing. Although dehydration has long been suggested to be one of the factors that may cause freezing injury (Levitt, 1980), its role in plants' survival against low temperature is not clear. However, the development of negative pressures in plant cells may lead to cavitation (Tyree and Dixon, 1986; Young, 1989).

Cavitation involves development of bubbles of vapor or gas that subsequently collapse, resulting in the rupture of protoplasm and perhaps even in intracellular ice formation, which can lead to cell death (Young, 1989). Cavitation has been observed in woody stems during freezing and is suggested to be a possible cause of freezing injury (Weiser and Wallner, 1988). Cell walls also play an important role in excluding ice from the cells; thus, cell-wall pore characteristics determine whether the cell wall could be an effective barrier against ice entry into the cells (Burke et al., 1976). Cell-wall pores also have a direct role in the movement of water from cells during freezing (Wisniewski et al., 1987) and in the transport of various substances into the cells (Carpita et al., 1979). Thus, cell-wall mechanical behavior as well as its pore characteristics can influence the nature of freezing in plant tissues.

Cold acclimation in plants is associated with a number of cellular changes, including those in the cell walls (Levitt, 1980; Huner et al., 1981; Wallner et al., 1986; Weiser et al., 1990; Ristic and Ashworth, 1993). However, it is not known how these cell-wall changes affect the physical properties of cell walls in relation to their ability to undergo deformation during freezing. Also, cold acclimation is known to be associated with the accumulation of ABA, which is believed to be responsible for induction of changes associated with cold acclimation in plants (Chen et al., 1983; Reaney and Gusta, 1987; Robertson et al., 1987).

In this paper we report changes in physical characteristics of cell walls and development of negative pressures during freezing in response to cold acclimation and exogenous ABA application in broadleaf evergreen species and cultured cells of grapes (*Vitis* spp.) and apple (*Malus domestica*). The cell-wall changes in these species were examined in relation to their effects on freezing behavior.

[1] Supported partially by the U.S. Department of Agriculture grant no. CRCR-1-2065 and Kansas Agricultural Experiment Station (contribution no. 94-495-J).

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MATERIALS AND METHODS

Plant Materials

Cold-hardened leaves of broadleaf evergreen species were collected in December and January, 1988 to 1989, from plants on the Kansas State University campus (Manhattan). Samples were sealed with moist paper towels in plastic bags and stored at −2°C until use. Unhardened leaves were collected in the spring and used immediately without storage. Plants of inkberry (*Ilex glabra*) and *Euonymus kiautschovica* were grown in pots containing a mixture of peat and perlite in a greenhouse for at least 10 weeks before use. Sections of actively growing stem of grapes (*Vitis* spp. cv Venus) and apple (*Malus domestica* cv Golden Delicious) fruits were collected from the Ashland Horticultural Research Farm (Manhattan, KS) to initiate cell cultures.

Cell Culture

Fruit discs (1 cm in diameter) from mature Golden Delicious apple fruits were used for cell culture. The discs were sterilized with 2.25% sodium hypochlorite for 10 min and rinsed in distilled water. The explants were grown on a modified solid Murashige-Skoog medium as described by Pech et al. (1979) to initiate callus. Subsequently, the callus was transferred to the above-described medium without agar and agitated on an orbital shaker at 100 cycles/min. The suspension-cultured cells were maintained by subculturing at 10-d intervals. Grape stem cuttings were used to initiate callus. Sterile explants were grown on Shenk-Hildebrandt solid medium (Shenk and Hildebrandt, 1972) containing 3.7 mg/L naphthalene acetic acid and 1.08 mg/L kinetin. Suspension cultures were started from 5- to 7-week-old callus using Shenk-Hildebrandt liquid medium. The cultures were maintained by subculturing at 10-d intervals on an orbital shaker set at 90 cycles/min.

Cold Acclimation

Plants of inkberry and *E. kiautschovica*, grown in greenhouse for at least 10 weeks, were transferred to a cold chamber set at 3°C and a 10-h photoperiod, where they were grown for at least 6 weeks for cold acclimation. Dehardening was accomplished by transferring plants to growth chambers set at 20°C and a 12-h photoperiod and holding them for at least 1 week. In the case of cell cultures, freshly subcultured cells of grapes and apple were grown at 4°C with agitation at 100 cycles/min for 3 to 5 weeks.

ABA Treatment

Plants of inkberry and *E. kiautschovica* grown for at least 10 weeks in the greenhouse were sprayed with 0.1 mM (±)ABA (Sigma) in 0.05% Tween 20 until the foliage was fully covered with the solution once every day for 3 d. Similarly, control plants received sprays of 0.05% Tween 20 without ABA. Plants were transferred to a growth chamber set at 20°C with a 10-h photoperiod. Leaf samples were collected after 6 d of ABA application to determine freezing curves.

Determination of Unfrozen Water

Unfrozen water content of tissues during freezing was determined using pulse NMR ^{1}H-NMR (20 MHz) methods as described elsewhere (Rajashekar and Burke, 1986). The free induction decay following a 90° pulse after 20 μs was used to monitor the proton signal. After the contribution from the solid protons was subtracted, the signals with Boltzmann temperature correction were used to compute the unfrozen water content. Strips of 1.5-cm-wide leaves were rolled into a tight wad and placed into a sample tube. Aggregates of grape cell-suspension cultures were harvested by centrifugation and blotted dry with filter paper before loading them into a sample tube. Samples were nucleated between −2 and −2.5°C and cooled in approximately 1°C steps and held at test temperatures for at least 1 h before measuring the amount of unfrozen water during extracellular freezing. Following ice nucleation, samples were allowed to reach equilibrium for up to 3 h as determined by a steady free induction signal by sample protons.

To measure the unfrozen water content during intracellular freezing, samples were thawed completely to room temperature and rapidly frozen in liquid nitrogen for 10 min. To confirm the presence of intracellular ice in leaves, leaf epidermal layers were frozen in liquid nitrogen and observed at −10°C under a phase-contrast microscope. The leaf samples frozen in liquid nitrogen were warmed back to −2.5°C without allowing them to completely thaw. The measurements of unfrozen water were made on these samples as described above. Water potential of ice and osmotic potentials were determined as described elsewhere (Rajashekar and Burke, 1982). Pressure potentials were computed based on cell water relations during extracellular and intracellular freezing. Pressure potential was obtained by subtracting cell osmotic pressure during intracellular freezing from that during extracellular freezing at a given temperature. The osmolality of expressed cell sap was estimated using an osmometer (Wescor, Logan, UT) or by using NMR methods to determine the melting point depression in intracellularly frozen samples (Gusta et al., 1975).

To ascertain whether the samples had reached equilibrium during extracellular freezing, leaves of inkberry and *E. kiautschovica* were nucleated at −2°C and cooled at 2°C/h to −4°C. The amount of unfrozen water was determined periodically while the samples were held at this temperature for up to 12 h in the NMR spectrometer.

Freezing Injury

Killing temperatures for leaves were determined based on the electrolyte leakage test (Sukumaran and Weiser, 1972). Leaves were cooled at 5°C/h with ice inoculation at approximately −2°C and thawed at 4°C for 12 h before measuring the electrolyte leakage. Total electrolytes in leaves were measured on samples that had been subjected to 80°C for 30 min.

Limiting Cell-Wall Pore Size and Cell-Wall Strength

The solute exclusion method was used to determine the limiting cell-wall pore size in cell cultures (Carpita et al.,

1979). Various solutes with different molecular sizes were used as osmotic agents to cause either plasmolysis or cell cytorrhysis. The osmotic agents included mannitol, Suc, PEG 400, PEG 600, and PEG 1000. The cells were harvested by centrifugation at 2000 rpm for 3 min and transferred to a 0.2 M solution (mannitol and Suc) or a 10% (v/v) aqueous solution of PEG and equilibrated for at least 30 min. The concentration of osmotic agents was increased stepwise by 0.2 M or 10% increments to a final concentration of 1 M or 30% (v/v). Cells were equilibrated at each concentration for 30 to 40 min. Cells were observed under a phase-contrast microscope for plasmolysis or cytorrhysis and counted using a hemocytometer.

The mechanical strength of cell walls of suspension-cultured cells was estimated by nitrogen decompression technique (Carpita, 1985). Samples of suspension culture (10–15 mL) contained in a beaker were placed in a stainless steel decompression chamber connected to nitrogen gas. Cells were subjected to increasing pressures with nitrogen gas and were decompressed rapidly to the ambient pressure by allowing the cells to exit the chamber through a side valve. The decompressed cells were observed for cell rupture as described before. The pressure required to cause 50% cell rupture was considered to be the breaking pressure.

RESULTS AND DISCUSSION

Cell-Wall Effects on Freezing Behavior and Negative Pressures

Differences in the fraction of unfrozen water were observed between extracellular freezing and intracellular freezing in the leaves of inkberry (Fig. 1). There was more unfrozen water in the leaves during extracellular freezing than during intracellular freezing. Furthermore, much larger differences in the fraction of unfrozen water between extracellular freezing and intracellular freezing were observed in cold-hardened leaves than in unhardened leaves. A similar response due to cold-acclimation treatment was also observed in the leaves of *E. kiautschovica* (Fig. 2). However, the fractions of unfrozen water were similar during extracellular freezing and intracellular freezing in the unhardened leaves of *E. kiautschovica*. When the unfrozen water is measured in plant tissues, it is particularly important to allow for equilibrium freezing. To confirm this, the leaves of inkberry and *E. kiautschovica* were held at −4°C for up to 12 h in the NMR spectrometer prior to the measurement of free induction decay signals. The results showed that the equilibrium had been reached within 1 h and no additional changes in the fraction of unfrozen water was observed after this time period.

Since extracellular freezing in plant tissues is accompanied by cell dehydration, any resistance to cell volume changes can reduce the extent of dehydration, resulting in the development of negative pressures (Rajashekar and Burke, 1982; Anderson et al., 1983). On the other hand, intracellular freezing does not involve cell volume reduction and is primarily governed by osmotic considerations alone. Thus, properties of cell walls that can allow for cell

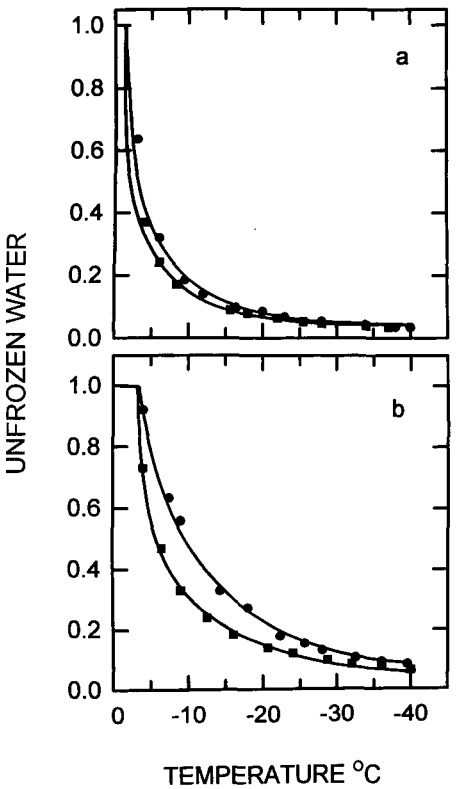

Figure 1. Fraction of unfrozen water in leaves of unhardened and cold-hardened leaves of inkberry. Unfrozen water as a fraction of freezable water in unhardened (a) and cold-hardened (b) leaves during extracellular freezing (●) and intracellular freezing (■) is presented. Intracellular freezing was induced in leaves by rapidly cooling in liquid nitrogen.

volume changes are likely to have a significant impact on the freezing behavior (extracellular) of plant tissues. An increase in the cell resistance to volume changes in leaf cells during extracellular freezing was observed after cold-acclimation treatment in both species. In addition, when cold-hardened plants were dehardened at 20°C for 1 week, the cold-acclimation-induced changes in the freezing behavior could be reversed and the freezing characteristics of dehardened leaves were similar to those of the unhardened control plants (data not shown).

During extracellular freezing, unhardened leaf cells of inkberry developed tensions that reached a maximum (3.67 MPa) at −15.6°C, followed by a decline with a further decrease in temperature (Fig. 3). In fact, below −25°C there was a complete loss of tension in unhardened leaves. On the other hand, unhardened leaf cells of *E. kiautschovica* did not develop any tension during extracellular freezing. However, in response to cold-acclimation treatment, leaves of both species developed higher tensions during extracellular freezing than unhardened leaves. In cold-hardened inkberry leaves, a sharp increase in tension was observed with decreasing temperature, reaching a plateau of approximately 10 MPa below −24°C. Even higher tension (12 MPa) was observed in the cold-hardened leaves of *E. kiautschovica* during extracellular freezing at −18°C. However, a further decrease in temperature

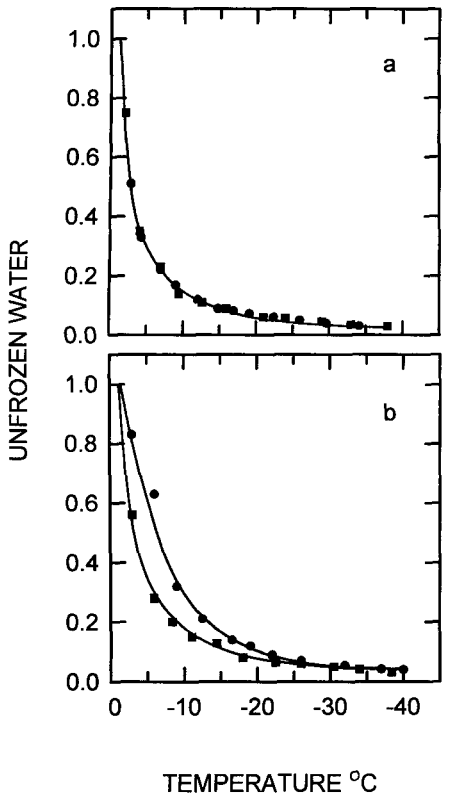

Figure 2. Fraction of unfrozen water in leaves of unhardened and cold-hardened leaves of *E. kiautschovica*. Unfrozen water as a function of freezable water in unhardened (a) and cold-hardened (b) leaves during extracellular freezing (●) and intracellular freezing (■) is presented. Intracellular freezing was induced by rapidly cooling in liquid nitrogen.

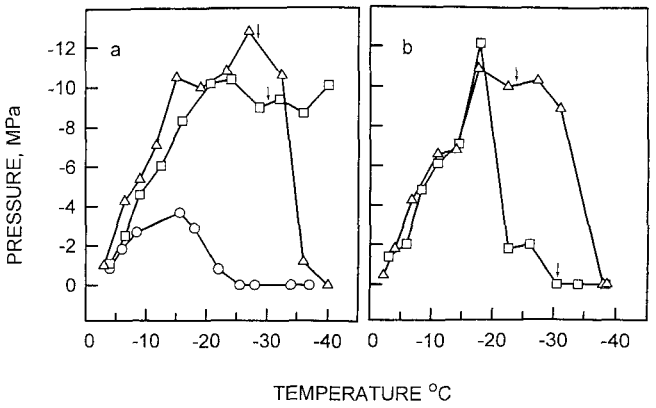

Figure 3. Predicted negative pressures in leaves during extracellular freezing. Negative pressures in leaves of unhardened (○), cold-hardened (□), and ABA-treated (△) plants of inkberry (a) and *E. kiautschovica* (b) during extracellular freezing are shown. Killing temperatures of leaves are indicated by arrows on the pressure curves.

resulted in a rapid decline in tension of leaf cells. Lethal freezing injury occurred at −31 and −30°C in cold-hardened leaves of inkberry and *E. kiautschovica*, respectively, as cell tension began to decrease after reaching a peak (Fig. 3). In addition, development of tension in leaf cells during extracellular freezing was observed in many other broadleaf evergreen species (Table I). The cold-hardened leaf cells attained maximum tensions ranging from 2.7 to 8.9 MPa, whereas unhardened leaf cells did not reach above 3 MPa. Unhardened leaves and cold-hardened leaves of all the broadleaf evergreen, with the exception of inkberry and *Euonymus fortunei*, showed subsequent loss of tension with progressive freezing.

Our results show that there is an increase in cell resistance to collapse during freezing in leaves of all the broadleaf evergreen species as a result of cold acclimation. Huner et al. (1981) noted an increase in cell-wall thickness, along with many morphological changes associated with cold hardening in rye leaves. We found that there was less freeze-induced dehydration in cold-hardened leaves than would be expected if cells did not develop tension during extracellular freezing.

We observed that both unhardened and cold-hardened leaves could develop tensions during freezing, although cold-hardened leaves have a greater tendency to do so, perhaps due to their rigid cell walls. Other studies have also shown that tissues can develop negative pressures as a result of cell-wall rigidity during freezing in citrus, winter barley, wheat, broadleaf evergreens, and shagbark hickory (Rajashekar and Burke, 1982; Anderson et al., 1983; Zhu and Beck, 1991). However, one concern has been whether cellular water can withstand development of huge negative pressures during freezing. Using thermodynamic considerations to study the stability of liquids, Benson and Gerjuoy (1949) and others (Henderson and Speedy, 1987) suggested that water can indeed withstand large tensions. Using microscopic inclusion of water in quartz matrices, Zheng et al. (1991) demonstrated that water can withstand tensions up to 140 MPa, and it is interesting that they found that with isochoric (i.e. constant volume) cooling, water can be cooled to −40°C under tension. However, cell tension

Table I. *Maximum tension in leaves of broadleaf evergreen species during freezing*

Cold-hardened leaves were collected from the field in December and January, 1988 to 1989. Unhardened leaves were collected in May, 1989. Data show mean values ± SD for three to four experiments.

Species	Unhardened	Cold-Hardened	
		Pressure	Water content
	MPa	*MPa*	*g/g dry wt*
Buxus microphylla Seibold & Zucc.	−1.1 ± 0.3	−2.7 ± 1.0	1.27 ± 0.4
Buxus sempervirens L.	−2.0 ± 0.6	−4.6 ± 1.4	1.05 ± 0.1
E. fortunei (Turcz.) Hand.-Mazz.	−2.9 ± 1.0	−8.9 ± 1.2[a]	1.53 ± 0.1
Hedera helix L.	−2.2 ± 1.1	−4.8 ± 1.1	1.14 ± 0.2
Ilex meserveae S. Y. Hu.	−2.8 ± 0.7	−8.0 ± 1.2	1.43 ± 0.3
Kamila latifolia L.	−0.8 ± 0.3	−2.7 ± 0.9	1.23 ± 0.2
Rhododendron sp.	−2.9 ± 0.4	−3.7 ± 0.3	1.30 ± 0.1

[a] Tension persisted below −40°C.

can induce cavitation. At room temperature even small tensions (1 MPa) are known to cause cavitation in plants and in artificial osmotic cell (Tyree and Dixon, 1983; Steudle and Heydt, 1988). Furthermore, the chances of cavitation are greatly increased by the presence of nucleation sources such as gas bubbles or dissolved gases in water (Knapp et al., 1970). Cavitation in cells resulting from tensions involves the formation of bubbles in the protoplasm, which can grow and subsequently collapse with explosive pressures, causing damage to the protoplasm. From acoustic emission data it has been learned that cavitation may occur in stem cells in both supercooling and nonsupercooling species during freezing (Weiser and Wallner, 1988; Raschi et al., 1989).

In the present study, the loss of tension in leaf cells during freezing may be attributed to one of two possible causes: (a) intracellular ice formation due to either intracellular ice nucleation or ice growth into the cells from extracellular sites, or (b) cavitation in cellular water. In light of the fact that these leaf cells develop considerable tension and that the water under tension is in a metastable state, there is a strong possibility for cavitation during extracellular freezing. Decline in cell tension, which could result from cavitation, was associated with lethal injury to leaves of inkberry and *E. kiautschovica*. It is possible that cavitation alone can inflict lethal injury to plant tissues during freezing even in the absence of intracellular ice formation. Our results indicate that cell walls are more rigid after cold acclimation, and thus, cells are more prone to develop negative pressures and subsequent cavitation. This seems inconsistent with the fact that cold-hardened cells are more cold tolerant than unhardened cells. However, one must take into account the effects of cellular changes associated with cold acclimation on the tensile strength of cellular water. It has been shown that interaction of water with hydrophilic materials and increases in viscosity, which are known to occur in cold-hardened cells (Levitt, 1980), can increase the tensile strength and lower the chances of damaging cavitation. Green et al. (1990) estimated the mechanical stability of aqueous solution of NaCl under tension and found that an NaCl solution had much greater peak stability under tension (approximately 240 MPa) than did pure water. One can expect rather high tensions in viscous water, perhaps, on the order of gigapascals at glass transition temperatures (Zheng et al., 1991). In addition, it is likely that viscous protoplasm in cold-hardened cells can dampen the damaging effect of cavitation, since it can considerably decrease the size and the rate of growth and collapse of cavities in the protoplasmic water (Poritsky, 1952). Therefore, it is reasonable to assume that protoplasmic properties of cells may play an important role in reducing or avoiding injury that may result from cavitation, at least in cold-hardened tissues.

To simulate cell volume changes during extracellular freezing, the epidermal layer of leaves of *E. kiautschovica* and apple cells in suspension culture were subjected to a hypertonic solution of PEG (55%, w/v; molecular weight 3350). Cell dehydration occurred through cytorrhysis, but very few cells showed cell-wall buckling or concave cell walls, unlike the typical depiction of cell deformation during extracellular freezing (Levitt, 1980). Most leaf cells appear to undergo cell volume reduction via shear strain without cell-wall buckling (Hayden et al., 1965). Shear strain response was more pronounced in the peripheral cells of the sample. This is rather expected in a tissue that consists of a matrix of cells with cell walls of one cell abutting those of surrounding cells. Our observations are consistent with those made on leaves of cereal crops during freezing, where cells became flat as they lost water (Pearce, 1988). Indeed, cell walls are generally too thick to deform through elastic buckling or plastic collapse (Niklas, 1989). Even apple cells in suspension culture treated with hypertonic PEG did not show cell-wall buckling (Fig. 4), but they became flaccid and thin. Cells were often folded and twisted due to dehydration. The cell deformation was completely reversible when the tissue was transferred to a hypotonic solution of PEG. In addition, the cell deformation caused by PEG treatment did not result in tearing or damage to cell walls. This is not, however, unexpected, considering that groups or rows of cells undergo shear deformation together as a single unit and cell-wall buckling is not always observed. Shear deformation in cells involves angular displacement of one or more sides of cell walls causing cell collapse. Furthermore, the rate of change in cell volume during extracellular freezing is dependent not only on the initial cell water content and osmotic concentration (Gusta et al., 1975), but also on the cell-wall rigidity. Cold acclimation in plants is known to typically lower tissue water content and increase osmotic concentration by accumulation of new solutes (Levitt, 1980). Thus, cold-hardened cells are more likely to undergo smaller volume changes during freezing in contrast to hydrated, unhardened tissues.

ABA and Negative Pressures

When unhardened plants of inkberry and *E. kiautschovica* were treated with ABA, there was an increase in cell resistance to collapse during extracellular freezing (Fig. 5) leading to the development of tensions in leaf cells (Fig. 3). The ABA-treated leaves showed similar peak tensions (12.75 MPa for inkberry and 10.9 MPa for *E. kiautschovica*) as did the cold-hardened leaves. A rapid loss of tension occurred in leaves of both species with progressive freezing and was associated with the lethal freezing injury at −27.5°C for inkberry and −24°C for *E. kiautschovica*. These results demon-

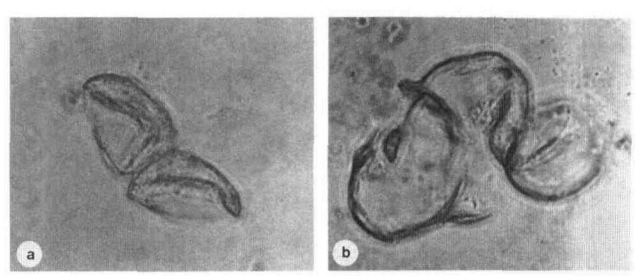

Figure 4. Cytorrhysis and cell rupture of apple cells in suspension culture. a, Cytorrhysis was induced in cells by a stepwise increase in concentration of PEG 1000. b, Cell rupture was caused by rapid decompression from a pressure exceeding cell-wall breaking pressure (3.17–4.13 MPa). Both panels are shown at the same magnification.

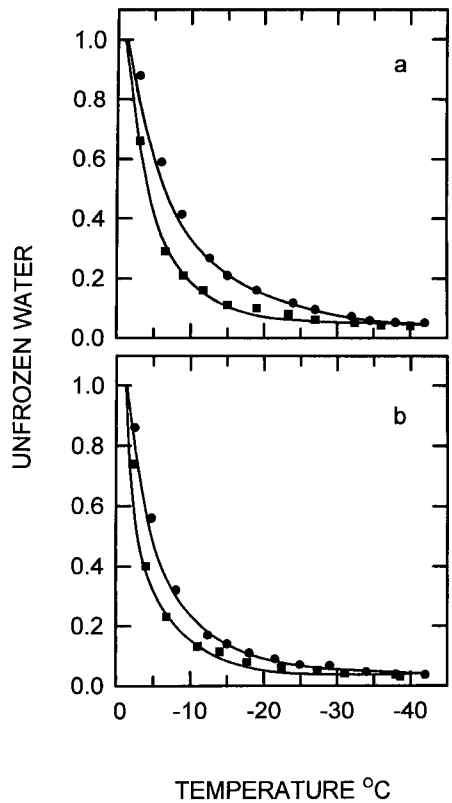

Figure 5. Fraction of unfrozen water in leaves of inkberry and *E. kiautschovica* treated with ABA. Unfrozen water as a fraction of freezable water during extracellular freezing (●) and intracellular freezing (■) in leaves of inkberry (a) and *E. kiautschovica* (b) plants treated with ABA.

strate that a decrease in cell tension possibly due to cavitation is related to lethal injury, and cold acclimation has effects on cell-wall rigidity similar to those of exogenous application of ABA. ABA is known to accumulate during cold acclimation of plants (Chen et al., 1983). Also, there have been many studies that suggest that ABA may be a mediator of cold tolerance in plants (Robertson et al., 1987; Thomashow, 1990). In addition, exogenous application of ABA has been shown to increase cold tolerance in whole plants and cell cultures, to substitute the cold-acclimation treatment, and to cause the expression of proteins associated with cold acclimation (Chen and Gusta, 1983; Robertson et al., 1987). Therefore, it is possible that ABA can induce changes in cell walls similar to those observed during cold acclimation. It is well documented that cells undergo many changes, including the ones in cell walls in response to cold acclimation (Levitt, 1980; Wallner et al., 1986; Weiser et al., 1990). An increase in cell-wall rigidity in cold-hardened and ABA-treated leaves is rather expected when we consider some of the changes in cell walls associated with cold acclimation. Cold acclimation in plants is known to induce the accumulation of a number of cell-wall components, including callose, cellulose, soluble polysaccharides, and pectic polysaccharides (Wallner et al., 1986). Furthermore, cold acclimation can result in deposition of extensin, a hydroxy-rich glycoprotein, on cell walls, which is known to impart greater structural rigidity to cell walls (Weiser et al., 1990).

Limiting Cell-Wall Pore Size in Cell Culture

We used suspension-cultured cells of apple and grapes to determine bulk modulus and other cell-wall characteristics, such as cell-wall pore sizes and cell-wall strength, since these properties can be more readily determined in cultured cells than in intact tissues. The changes in unfrozen water during extracellular freezing and intracellular freezing were similar in cultured cells, indicating little cell-wall resistance to collapse in both unhardened controls and cold-hardened cells. These results suggest that the freezing behavior in cell cultures is perhaps different from that in intact tissues, in that cell organization and interconnected matrix of cells in a tissue may be needed to impart greater resistance to cell collapse.

The limiting cell-wall pore sizes for unhardened cells of both apple and grapes were approximately 29 and 35 Å, respectively (Fig. 6). The limiting cell-wall pore size refers to the largest cell-wall pores, and solutes larger than these pores cannot pass through the cell walls, causing cytorrhysis. The cell-wall pore sizes observed for apple and grapes are consistent with those reported for suspension-cultured cells and tissues of many plant species (Carpita et al., 1979). Cold-acclimation treatment resulted in the reduction of not only limiting cell-wall pore sizes but also the percentage of plasmolysis with all the solutes (Fig. 6). Cell-wall pores play an important role in excluding ice from the cell. In woody xylem parenchyma cells, cell-wall porosity, particularly that of pit membranes, was suggested to be critical for the movement of water in woody xylem parenchyma cells during freezing (Wisniewski et al., 1987). In addition,

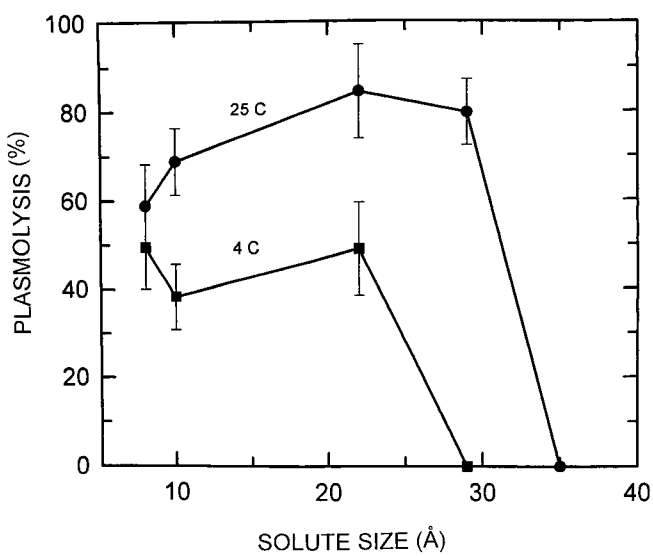

Figure 6. Plasmolysis of unhardened and cold-hardened apple cells in suspension culture. Cells grown at 25 and 4°C were plasmolyzed by a stepwise increase in concentration of solutes of varying molecular sizes (mannitol, Suc, PEG 400, PEG 600, and PEG 1000). Data are mean percentages ± SE of plasmolysis of three experiments (sample size, 150–200 cells).

Table II. *Properties of cell walls of cells of grapes and apple*

Limiting cell-wall pore sizes were determined by solute exclusion technique (sample size, 150–200 cells). Cell-wall breaking pressures were estimated by decompression. Mean pressures for 50% cell rupture with SD are presented (n = 3; sample size, 150–200 cells).

Property	Grape		Apple	
	Unhardened	Cold hardened	Unhardened	Cold hardened
Limiting cell-wall pore size (Å)	35	22	29	22
Cell-wall breaking pressure (MPa)	2.16 ± 0.16	3.37 ± 13	3.17 ± 0.12	3.56 ± 0.05

cell-wall pore size can determine the movement of many large molecules such as proteins, nucleic acids, and toxins across the cell walls (Carpita et al., 1979). To examine whether changes in cell-wall pore sizes have any impact on the intracellular ice formation in cells, both unhardened and cold-hardened grape cells were cooled at 2°C/h to −10°C with ice inoculation at −2°C. The cells were observed under a phase-contrast microscope at −10°C for the presence of intracellular ice. The results showed that a greater percentage of unhardened cells (37.6 ± 3.4%) had intracellular ice than did cold-hardened cells (4.3 ± 1.5%). These observations show the impact of cell-wall characteristics on the freezing behavior and support the conclusion that reduced cell-wall pore size, in response to cold acclimation, may have a favorable effect on lowering the formation of intracellular ice.

Cell-Wall Strength in Cell Culture

Studies of cell-wall strength in apple and grape cells showed that cold-hardened cells had greater cell-wall strength than did unhardened control cells (Table II). The cell-wall breaking pressure increased approximately by 54% in grape cells and by 12% in apple cells in response to cold acclimation. Typically, cell rupture occurs when the combined cell turgor pressure and applied pressure exceeds the cell-wall strength (Fig. 4). The breaking pressures for apple and grape cells are comparable to those reported for tobacco and carrot cells (Carpita, 1985; Iraki et al., 1989). With increasing accumulation of cell-wall materials during cold acclimation, it is likely that cell-wall strength is enhanced. Although cell-wall strength can play an important role in maintaining cell turgor, cell elongation, and growth (Cosgrove, 1987), its effect on cell collapse and on intracellular ice formation is unclear. Iraki et al. (1989) observed that salt-adapted tobacco cells had weaker cell walls than did control cells. The unadapted cells accumulated Hyp-rich proteins in contrast to adapted cells. They attributed cell-wall strength to the cellulose-extensin matrix containing isodityrosine linkages, which provide a rigid network in cell walls. Weiser et al. (1990) showed that extensin and other cell-wall structural components accumulate during cold acclimation. Thus, it is reasonable to speculate that extensin in cold-hardened tissues may provide structural strength and may even influence the ability of cells to collapse during freezing.

In summary, cold acclimation can induce cell-wall rigidity leading to greater cell tensions during extracellular freezing in the leaves of broadleaf evergreen species. Loss of tension in leaves during freezing has been attributed to cavitation in cellular water and is linked to freezing injury. A similar response was observed in leaves by exogenous application of ABA to two broadleaf evergreen species. Negative pressures were not detected during freezing in either unhardened or cold-hardened grape cells in cell culture. In cultured cells of apple and grapes, limiting cell-wall pore sizes were reduced, whereas cell-wall breaking pressures increased in response to cold acclimation.

Received October 11, 1995; accepted March 26, 1996.

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