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ORIGINAL ARTICLE

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Extracellular freezing in leaves of freezing-sensitive species

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Abstract Low-temperature scanning-electron microscopy was used to study the freezing of leaves of five species that have no resistance to freezing: bean (*Phaseolus vulgaris* L.), tobacco (*Nicotiana tabacum* L.), tomato (*Lycopersicon esculentum* L.), cucumber (*Cucumis sativus* L.), and corn (*Zea mays* L.). In the leaves of the four dicotyledonous species, ice was extracellular and the cells of all tissues were collapsed. In contrast, in maize leaves ice was extracellular in the mesophyll, and these cells were collapsed, but the epidermal and bundle-sheath cells apparently retained their original shapes and volume. It is concluded that the leaves of the freezing-sensitive dicotyledonous species tested were killed by cellular dehydration induced by extracellular freezing, and not by intracellular freezing. Freezing injury in maize leaves apparently resulted from a combination of freezing-induced cellular dehydration of some cells and intracellular ice formation in epidermal and bundle-sheath cells.

Keywords Cell dehydration · Freezing sensitivity Ice formation (leaf) · *Nicotiana* (freezing sensitivity) *Phaseolus* (freezing sensitivity) · Zea (freezing sensitivity)

Abbreviation LTSEM: low-temperature scanning-electron microscopy

Introduction

Frost is a widespread environmental factor affecting the distribution of many crop and wild plants. Understanding how freezing damages plants is important for explaining their distribution or finding ways to improve their tolerance. Plant species vary widely in their capacity to survive freezing temperatures and an important question is whether freezing injury of species that tolerate some freezing and species that have no tolerance of freezing occurs in the same way or is different.

If, during a natural frost, freezing occurs within the cytoplasm, it will kill the cell. However freezing-tolerant plants generally survive freezing because ice forms outside the cells. The extracellular ice grows mainly by withdrawing water from within the cells, thus dehydrating them. The amount of water withdrawn, and the extent of intracellular dehydration, increases as the temperature falls (Mazur 1969; Gusta et al. 1975). Cells are killed when their tolerance of dehydration is exceeded. This is apparently due to failure of the structure of membranes (Steponkus 1984; Pearce and Willison 1985b).

Low-temperature scanning-electron microscopy (LTSEM) gives detailed images of the location of ice during freezing stress. This shows that in frost-stressed leaves of cold-acclimated wheat and rye, ice ramifies through the extracellular spaces, filling them, and the cells are collapsed due to the dehydration resulting from the growth of ice outside the cells (Pearce 1988; Pearce and Ashworth 1992). Ice is also extracellular, and the cells are dehydrated, in non-acclimated leaves of barley (Pearce 1988).

However, not all plant cells in frost-tolerant species are killed by extracellular ice formation. There are many frost-tolerant woody plants in which extracellular freezing occurs in the bark but the xylem parenchyma deep-supercools (Burke et al. 1976; Ashworth et al. 1988; Ashworth 1996) or in which bud organs deep-supercool (Graham and Mullin 1975; Ashworth 1990). In these, when the temperature limit to supercooling is reached, the cells freeze internally and are killed.

In contrast, the exact mechanisms by which the cells of plants that have no tolerance of freezing are killed,

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are less clear. However, knowledge of this process is essential for developing rational strategies for conferring freezing-tolerance on sensitive species. It was originally thought that damage to sensitive plants was due to intracellular freezing occurring a few degrees below zero Celsius (Burke et al. 1976). Generally, all kinds of organisms will freeze intracellularly if cooled sufficiently rapidly, but Levitt (1980) concluded that this was likely to be rare in plants in nature. On the other hand, even recently, it was suggested that freezing-tolerant plants differed from freezing-sensitive plants by having evolved mechanisms to avoid intracellular freezing (Griffith and Antikainen 1996). However, so far as we know, there is no direct, and no compelling indirect, evidence that susceptible species freeze extracellularly when cooled at rates found in nature.

We used LTSEM to test the hypothesis that in leaves of freezing-sensitive species ice forms extracellularly and the cells are dehydrated, i.e. that freezing occurs in a similar way to that in leaves of frost-tolerant species. If this is correct we should observe ice masses outside the cells and the cells should be collapsed. On the other hand, if the cells were to freeze intracellularly there would be no extracellular ice masses and the cell volume and shape would be similar to that in the non-stressed controls. We tested this in leaves because the previous LTSEM studies of freezing in cereal leaves (Pearce 1988; Pearce and Ashworth, 1992) provided a clear indication of the appearance to be expected. Important cold- and frost-susceptible crop species from three unrelated dicotyledonous families and one monocotyledonous family were chosen for testing: bean (Fabaceae), tobacco (Solanaceae), tomato (Solanaceae), cucumber (Cucurbitaceae) and maize (Poaceae).

We did our tests in the laboratory rather than the field, because of the logistical difficulties of the latter. Our tests reproduced the key features needed: intact plants rather than plant pieces, a cooling rate typical of the field (2 °C h^{-1}), and initiation of freezing at high sub-zero temperatures.

Following the initiation of freezing, we cooled our plants to –14 °C or below before freeze-fixing samples. Thus the specimens were examined after cooling to well below the temperature that would kill them. The rationale for this approach was as follows. In the laboratory it is difficult to take samples from whole plants at just a few degrees below 0 °C and freeze-fix them while ensuring appreciable warming does not occur during handling of the specimens. Moreover, ice-crystal sizes in whole-plant cells freeze-fixed with a high liquid water content are highly variable and can be large (Pearce and Willison 1985a). Consequently, it is difficult to use examination of material freeze-fixed at high sub-zero Celsius temperatures as a strategy to detect intracellular ice formation. However, fixation at a high sub-zero Celsius temperature is unnecessary for answering our question. The only phenomenon that could alter the distribution of ice once formed is re-crystallisation, the migration of water molecules from smaller to larger ice crystals. However, large ice crystals would be expected to form at the cooling rate used and these would re-crystallise only very slowly. Consequently, the location of the ice seen by LTSEM, extracellular or intracellular, would be the same at and below –14 °C, as at higher temperatures.

Thus LTSEM was used to view the location of ice and the shape of the cells of representative freezing-sensitive species during exposure to experimental freezing. The results showed that freezing is entirely extracellular in all except maize.

Materials and methods

Plant material and environmental conditions

Seeds of bean (*Phaseolus vulgaris* L.), tobacco (*Nicotiana tabacum* L.), tomato (*Lycopersicon esculentum* L.), cucumber (*Cucumis sativus* L.), and corn (*Zea mays* L.) were sown in 10-cm pots of a soil-less potting compost and grown with daily watering in a greenhouse at Purdue University at a minimum temperature of 18 °C. Seedlings of plants were used when two to four leaves were fully expanded.

Freezing treatment and freeze-fixation

The potted plants were placed in a polystyrene box with the root zone covered with a layer of vermiculite, sprayed with water to ensure nucleation of freezing, and placed in a controlled-environment chamber at a few degrees above 0 °C. Thermocouples (0.3-mm-diameter copper–constantan) were used to monitor air and leaf temperatures. Leaf freezing began at –2.5 °C (cucumber), –3.1 °C (tomato and maize), or –3.6 °C (tobacco). The leaves cooled at 2 °C h^{-1} to –14 °C (bean and maize) or –20 °C (other species). The box was then transferred to a walk-in freezer room at –14 °C (bean and maize) or –20 °C (other species). Transfer to the walk-in freezer took less than 5 min and during this time the temperature of the specimens rose less than 4 °C; the plants were then allowed to re-cool to –14 °C or –20 °C. Leaf pieces were cut from the plants using scalpels cooled to freezer-room temperature. The pieces were immediately placed in the specimen stubs, using Tissue-Tek (Miles Diagnostics, Elkhart, Ind., USA), mixed with carbon, as cryoadhesive, and freeze-fixed by plunging in melting Freon 12 (–150 °C). Freeze-fixed specimens were stored in liquid dinitrogen before inspection in the low-temperature scanning-electron microscope. All manipulations in the walk-in freezer were conducted using equipment equilibrated to freezer temperature. All specimens remained frozen during manipulation. Control leaf pieces were cut at room temperature from plants not exposed to freezing stress and were immediately freeze-fixed in the same way as described for the freeze-stressed specimens.

Low-temperature SEM

Specimen stubs were loaded into the specimen holder and attached to the transfer rod of a Hexland cold stage (Hexland, East Challow, Oxfordshire, UK) while submerged in liquid dinitrogen. Shrouded specimens were then rapidly (< 1 s) transferred to the cryochamber of the microscope. Specimens were fractured inside the cryochamber using a cooled scalpel blade and subsequently transferred to the microscope cryostage. Three to ten specimens (average 6) of each species were examined for each treatment. The temperatures of the cryochamber and cryostage were maintained below –150 °C. Specimens were examined using a JEOL JSM-840 scanning-electron microscope (JEOL, Tokyo, Japan). Often, specimens were first examined without coating. It was then possible to sublime ice by

raising the set temperature of the stage to between –90 °C and –50 °C, and examining the specimen again after re-cooling the stage to –150 °C. Either initially or following sublimation of ice, the specimens were sputter-coated with gold-palladium within the cryochamber.

Results

Dicotyledonous species

The cells of control leaves were turgid-like in appearance and there was no evidence of extracellular ice (Fig. 1a–d). In contrast, the extracellular spaces of leaves of freezing-stressed plants contained abundant ice and normal cell shapes were absent (Fig. 2a–f). Fracture steps were visible in the fractured ice surfaces (Fig. 2a–f). The fracture faces of leaves of freezing-stressed bean and tomato were fully occluded with ice (Fig. 2a, b). The appearance of freezing-stressed leaves of tobacco and cucumber was similar to that in bean and tomato except that in parts of the leaves the ice did not completely fill the extracellular spaces (Fig. 2c–f).

Usually, the mesophyll cells had collapsed profiles embedded in the ice. These profiles could be confirmed as cells by the organelles contained within (Fig. 2a, b). Where, in tobacco and cucumber leaves, ice did not totally fill the extracellular spaces (Fig. 2c), it was possible to see a much larger part of the cells than was possible in the bean and tomato samples (Fig. 2d–f). The appearance confirmed that the cells had lost turgor. Ice and cells could again be distinguished. As in cells embedded in ice, where the collapsed cells and ice particles were fractured the cells revealed organelles while the ice did not (compare d and e in Fig. 2). In addition, the surfaces of collapsed cells often showed the imprint of organelles lying within the cells (Fig. 2d, f), whereas ice surfaces were smooth (Fig. 2e).

Ice was confirmed as such by subliming it from specimens while in the microscope. During sublimation, the ice surfaces showed stippling and ridges (Fig. 3a–d). The appearance of the cells after partial or complete sublimation of the ice confirmed that the cells were collapsed, often appearing flattened (Fig. 3a–e). Again, these profiles were confirmed as cells by the organelles they contained (Fig. 3a, c–e). Also, again, cell wall surfaces often showed the imprint of organelles within the cells (Fig. 3d, e). The insert in Fig. 3e shows the close contact which existed between the cell wall and cell contents in the freezing-stressed samples.

Maize

Cells of the control maize leaves were turgid-like in appearance and there was no evidence of extracellular ice (Fig. 4a). Like the other species tested, the mesophyll of the freezing-stressed maize leaf samples was filled with ice (Fig. 4b), and this could be removed by sublimation to reveal that the mesophyll cells were collapsed (Fig. 4c). However, in complete contrast to the other species, in maize both the epidermal cells and the bundle-sheath cells retained their original shape and volume

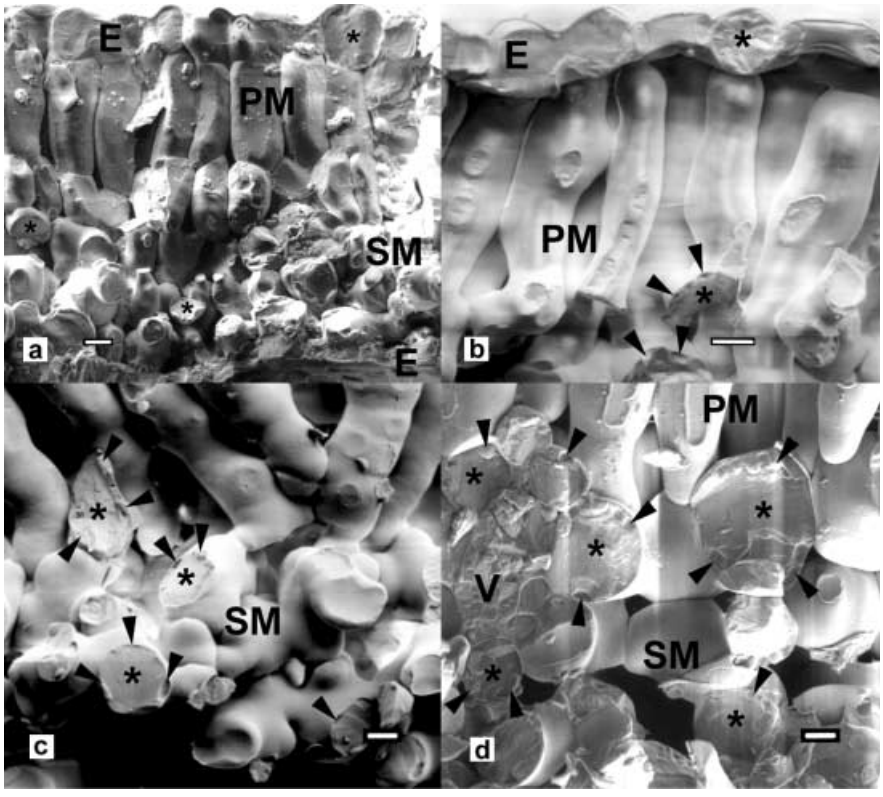

Fig. 1 Transverse fractures through control leaves of bean (*Phaseolus vulgaris*; **a**), tomato (*Lycopersicon esculentum*; **b**), cucumber (*Cucumis sativus*; **c**) and tobacco (*Nicotiana tabacum*; **d**). Note that extracellular ice was absent and cells were turgid-like in appearance. *Stars* indicate cell profiles revealed when the fracture plane passed through cells; *arrowheads* indicate organelles in the profiles. *E* Epidermis, *PM* palisade mesophyll, *SM* spongy mesophyll, *V* vascular tissue. All coated. 5 kV (**a**) or 2 kV (**b–d**). Bars = 10 μm

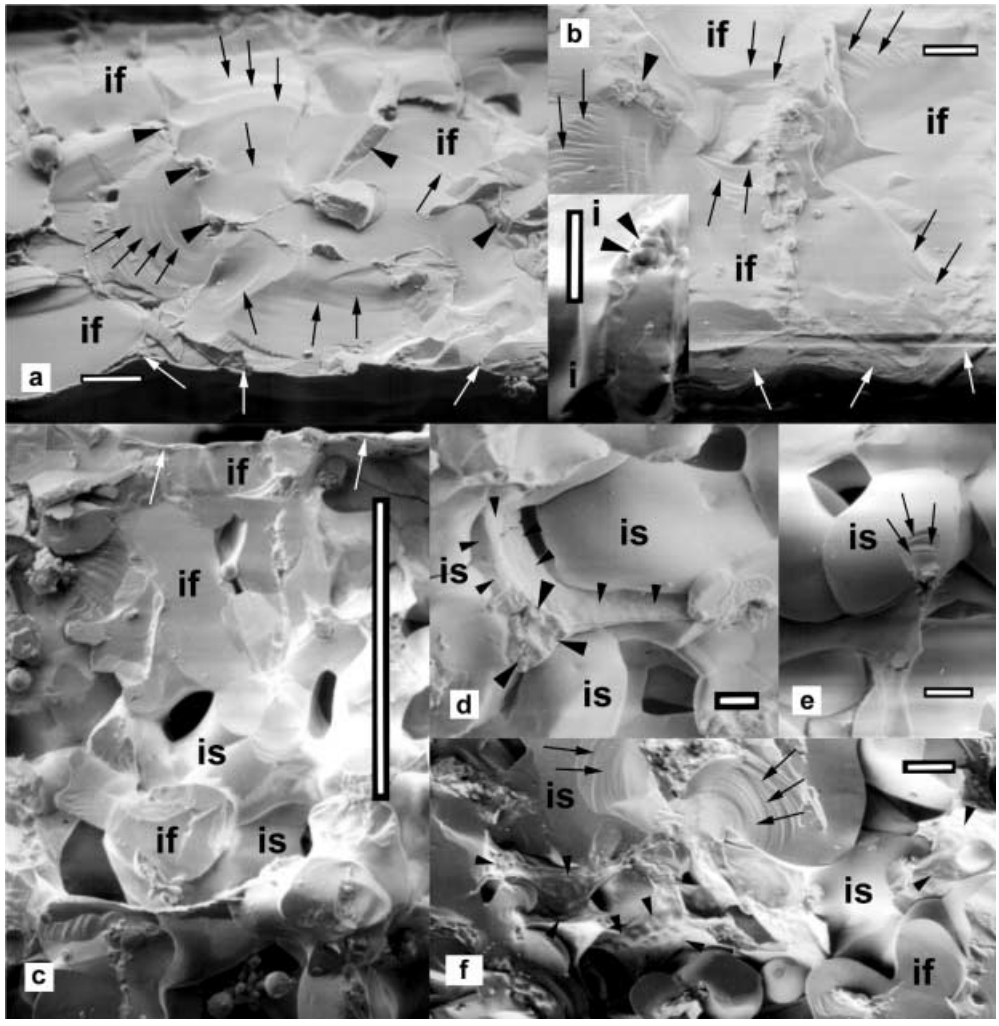

Fig. 2 Transverse fractures through leaves of freezing-stressed bean (**a**), tomato (**b**), tobacco (**c–e**) and cucumber (**f**). **a–c** The fracture faces were fully occupied (**a, b**) or extensively occupied (**c**) by ice. Some parts of the ice had smooth fracture profiles and other parts had fracture steps (*arrows* in **a, b**), sometimes giving a conchoidal appearance (**a**). Note the flattened fracture profiles of mesophyll cells (*arrowheads*) and epidermal cells (*white arrows*). Organelles were visible in the cell profiles embedded in the ice (*arrowheads* in the *insert* in **b**). **d–f** Where extracellular spaces were only partly occluded by ice, the ice was visible as smooth outer surfaces of ice particles (*is*) as well as fracture surfaces (*if*). Fractures through ice showed fracture steps (*arrows* in **e, f**). The outer surfaces of cell walls of some cells were visible and carried imprints of the organelles contained in the cells (*small arrowheads* in **d, f**). Fractures through these cells showed organelles (*large arrowheads* in **d**). Note the different appearance of fractures through a cell (**d**) and an ice particle (**e**). *i* Ice, *if* fracture through ice, *is* surface of ice. Not coated (**a**) or coated (**b–f**, *insert* in **b**). 5 kV (**a**) or 2 kV (**b–f**, *insert* in **b**). Bars = 10 μm (**a, b**; *insert* in **b**; **d–f**), 100 μm (**c**)

during freezing-stress (Fig. 4b, c), the exception, within the epidermis, being the subsidiary and guard cells, which did collapse (compare d and e in Fig. 4).

Discussion

In freezing-tolerant plants, extracellular ice grows by withdrawing water from the cells, the extent of withdrawal increasing with fall in temperature (Mazur 1969; Gusta et al. 1975). The limit to the plants freezing-tolerance is thus set by the amount of cellular dehydration that it can survive. Our results show that when bean, tomato, tobacco and cucumber leaves are exposed to a freezing stress, freezing and dehydration occur in the same way as in the freezing-tolerant species: ice formed extracellularly, withdrawing water from the cells, and the cells became dehydrated. Thus in these species, injury from freezing is not due to intracellular formation of ice and instead results from freezing-induced dehydration. The results indicate two further conclusions: (i) it is often not the manner of freezing that differs between susceptible and tolerant species but the capacity to tolerate the dehydration that occurs, and (ii) often no special mechanism may be needed to avoid intracellular freezing. However, maize was exceptional, freezing extracellularly in only some tissues.

It may be argued that the cells could have been killed not by the dehydration, which eventually led to the abundant extracellular ice evident at –14 °C and –20 °C, but by intracellular ice crystals formed when freezing first began, with dehydration occurring only later. We think this is unlikely. Any intracellular ice crystal formed during the slow cooling treatment used would be expected to grow at least as rapidly as any extracellular ice formed at the same time and probably more rapidly due to its immediate contact with intracellular water. Thus intracellular freezing would have led to a quite different appearance from that seen in the dicotyledonous species. This expectation is illustrated by the different freezing behaviours of different tissues in the maize leaf.

Identification of extracellular ice and collapsed cells

The freeze-fixation method results in conversion of all water in the specimens from liquid to ice or a glass. The

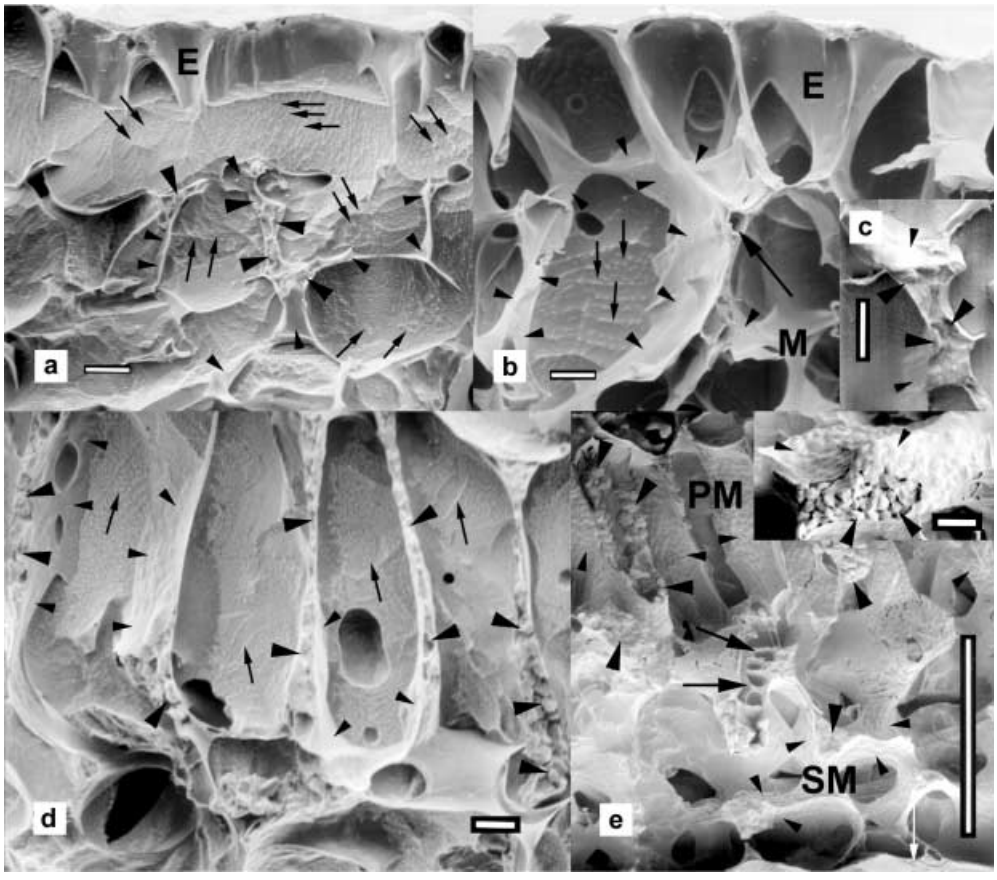

Fig. 3 Transverse fractures through leaves of freezing-stressed bean (**a, b**), tomato (**c**) and tobacco (**d, e**). Ice was partly (**a–d**) or wholly removed (**e**) by sublimation. **a–d** Ice had been removed to various extents by the sublimation, leaving the cells projecting slightly (**a**) or considerably (**b**) above the receding ice surface. The remaining ice was evident as ridged and stippled surfaces (*small arrows* indicate examples). The mesophyll (*arrowheads*) and epidermal cells (E) had flattened profiles. *Small arrowheads* indicate cell surfaces. Some cell surfaces carried the imprint of organelles contained within the cells (**d**). *Large arrowheads* indicate organelles in the fractured cell profiles (**a, c, d**). Note the minor vein in **b**: the xylem element (*large arrow*) was surrounded by collapsed cells. **e** Almost complete removal of ice by sublimation gave a more extensive view of the cells. Mesophyll (*PM*; *SM*) and epidermal cells (*white arrow*) were collapsed. *Small arrowheads* indicate outer surfaces of mesophyll cells. These showed an imprint of organelles contained within the cells. *Large arrowheads* indicate organelles where the fracture cut through cells. Note the xylem elements (*large arrows*) surrounded by collapsed cells. *Insert* Detail of a typical mesophyll cell at greater enlargement. *E* Epidermis, *PM* palisade mesophyll, *SM* spongy mesophyll. Not coated (**a, b**) or coated (**c–e** and *insert* in **e**). 5 kV (**a, b**) or 2 kV (**c–e** and *insert* in **e**). Bars = 10 μm (**a–d**; *insert* in **e**), 100 μm (**e**)

controls, freeze-fixed from room temperature, showed a normal appearance, with cells appearing turgid and absence of material filling the gas spaces. This indicated that the freeze-fixation method did not cause an artifactual redistribution of water in the specimens and provides good preservation of leaf architecture. Hence the finding of abundant ice filling the gas spaces in the freezing-stressed specimens was an accurate reflection of the distribution of ice in the freezing-stressed specimens before freeze-fixation.

A key factor in identifying ice in LTSEM specimens is that no other abundant substance in biological materials can sublime at temperatures above –100 °C. Thus the material filling the extracellular spaces could be sublimed away, confirming it as ice. In addition, the occurrence of this extracellular material was accompanied by volumetric collapse of the cells, indicating that the material had been drawn from the cells, again a point explicable only if the sublimable extracellular material is ice.

The fracture faces often presented a solid mass of ice in which collapsed cell profiles were embedded. Ice and cells were distinguished by the following features. The cell profiles showed organelles and a cell wall, whereas fractures through ice showed neither. The ice profiles were often larger than the fractures through control cells. During sublimation, the ice surface showed stippling and ridges typical of etching of ice (Davey and Branton 1970; Staehelin and Bertaud 1971). Sublimation removed the ice; the absence of residual cytoplasmic material when sublimation of the purported extracellular ice was completed, and the appearance of the collapsed cells remaining after sublimation, confirmed that the ice had been extracellular.

Sometimes ice did not fill the fracture face. In these cases the ice particles often had the shape of branched and fusing tubes and therefore might, in a superficial

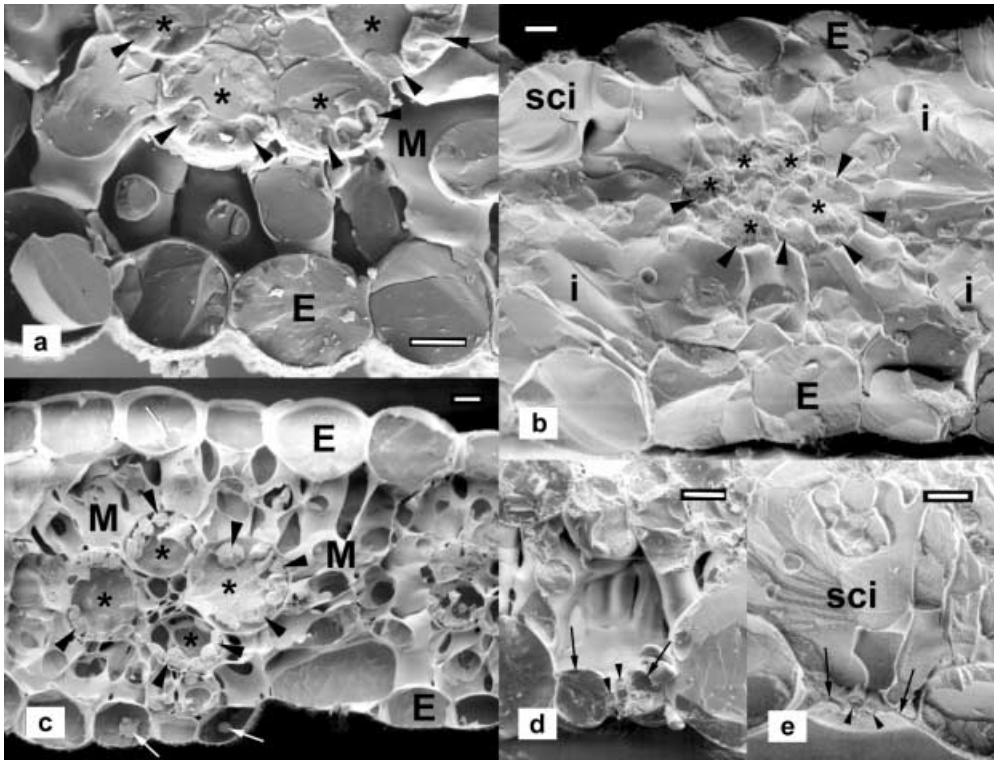

Fig. 4a–e Transverse fractures through leaves of maize (*Zea mays*).**a** Control leaf showing absence of extracellular ice. Cells in all tissues were turgid-like in appearance. Organelles (*arrowheads*) can be seen in the bundle-sheath cell profiles (*stars*). **b, c** Leaves from plants exposed to freezing stress. The mesophyll was filled with ice (*i* in **b**) and the mesophyll cells were collapsed, but the cells in the epidermis and bundle sheath remained turgid-like in appearance. In **c** ice was partly removed by sublimation. Organelles (*arrowheads*) can be seen in the bundle-sheath cells (*stars* in **b, c**). *White arrows* (**c**) indicate residual cytoplasm in epidermal cells after sublimation of surrounding ice. **d, e** Substomatal cavities in control (**d**) and freezing-stressed leaves (**e**). The subsidiary cells (*arrows*) were turgid-like in appearance in the control and collapsed in the freezing-stressed sample. The guard cells (*arrowheads*) were also smaller in the stressed sample. Note that the extracellular ice formed in the mesophyll in stressed samples, penetrated into the substomatal cavity (*sci* in **e** and also top left region of **b**). *E* Epidermis, *M* mesophyll. Not coated (**e**) or coated (**a–d**). 5 kV (**a**), 3 kV (**b, d**) or 2 kV (**c, f**). Bars = 10 μm

examination, be confused with cells. However, several features distinguished them. Where the cells and ice particles were fractured the cells revealed organelles while the ice did not. The cell surfaces often showed the imprint of organelles lying within the cell, whereas ice surfaces were smooth.

Explanations for the different freezing patterns

In leaves of freezing-tolerant plants the volumetric collapse of cells during extracellular freezing is due to withdrawal of water from the cells by the growth of extracellular ice, and is not due to direct ice-pressure (Gusta et al. 1975; Burke et al. 1976; Pearce 1988). In the experiments here, ice often filled the fracture profile and the cells were embedded, visible as flattened profiles, in the ice. This could give the impression that the ice had compressed the cells. However, because in some parts of the tobacco and cucumber leaves ice did not fill all the fracture profiles, yet the cells were always collapsed, it is clear that in freezing-sensitive plants, too, the collapse of cells was due to freezing-induced dehydration, not ice pressure.

In freezing-tolerant species both the protoplast and the enclosing cell wall were collapsed by the freezing-induced dehydration (Pearce 1988). This was also the case for the freeze-dehydrated cells of the freezing-sensitive species. The imprint of the enclosed organelles on the cell wall surfaces indicated that the cell walls and cell contents remained in close contact. This was confirmed by the appearance of fractures through cells, where the enclosing cell wall and the cell contents were in contact and had the same profile.

Like the freezing-sensitive dicotyledonous species tested here, previous LTSEM studies of freezing of leaves of freezing-tolerant cereals have shown that all cells, whether mesophyll, epidermal or vascular, were freeze-dehydrated (Pearce 1988; Pearce and Ashworth 1992). Thus maize is the first case where different tissues within the leaf behaved differently: the mesophyll was freeze-dehydrated whereas the epidermis (except the subsidiary and guard cells) and bundle sheath were not.

In principle, the observation that the epidermal and bundle-sheath cells remained turgid-like in appearance during freezing stress in maize could be explained by either intracellular freezing early during cooling, or by supercooling. In the former case, intracellular freezing

of the epidermis and bundle sheath would need to have preceded extracellular freezing of the mesophyll, otherwise they would be freeze-dehydrated. In contrast, supercooling would have been followed by freezing at some lower temperature. Substantial supercooling would only occur if freezing were not nucleated in the cells and the cells avoided dehydration.

There are, in principle, two ways in which dehydration could be avoided in the epidermal and bundle-sheath cells. The tissues could be hydraulically isolated from the mesophyll, so providing no pathway for transfer of water to the mesophyll, or, alternatively, the individual cell walls or the tissue structure might be sufficiently rigid in the epidermis or bundle sheath to prevent cell-wall collapse, thus providing a force that would oppose the movement of water to the mesophyll. The latter occurs in leaves of a number of species, reducing the movement of water from cells to growing ice particles (Anderson et al. 1983; Hansen and Beck 1988; Zhu and Beck 1991). However, while this reduces movement of water out of the cells, it does not prevent it, and the cells still experience substantial dehydration. The epidermis and bundle sheath of maize are not known to be more rigid than in rye, wheat or barley, yet in these three species extracellular freezing, and in the case of barley also drought, cause cell collapse throughout the epidermis and bundle sheaths (Pearce and Beckett 1987; Pearce 1988; Pearce and Ashworth 1992). Thus, though rigidity, speculatively, might be capable of causing or contributing to supercooling, there is at present no evidence that this is the explanation in maize.

The shape and cell wall composition of maize leaf epidermal cells is known to differ between leaves formed at different stages during plant development (Moose and Sisco 1994): in young plants they are uniform but in mature plants the cell shapes and cell wall compositions differ between different files of cells, including suberisation of some. However, we used leaves from young plants in our experiments. The epidermis of young maize leaves stains pink with toluidine blue O (Moose and Sisco 1994). This indicates a probable absence of lignin from the cell walls but is otherwise uninformative (O'Brien et al. 1964).

Hydraulic isolation is, on the other hand, a possible explanation for the freezing behaviour of the bundle sheath. The cell walls of the maize bundle-sheath cells are suberised, and intermediates of C4 metabolism pass between the bundle sheath and mesophyll through abundant plasmodesmata (Evert et al. 1977). Thus direct movement of water from the lumen of the bundle-sheath cells to the extracellular ice in the mesophyll would be prevented. Water could move to the extracellular ice in the mesophyll via the plasmodesmata and mesophyll cells but the restricted route and longer pathway would reduce the flux. Because cold affects membrane structure, speculatively there could be an additional impairment of plasmodesmatal water transport by cold. If these explanations are correct, then other C4 species may behave similarly to maize.

Although the epidermis and bundle sheath of maize behaved differently, the mesophyll of maize and all the leaf tissues of the dicotyledonous species tested, froze extracellularly, dehydrating the cells. However, maize itself is not typical of monocotyledonous species. Thus, with some exceptions, leaves of herbaceous species, whether innately freezing-sensitive or capable of acclimating, freeze extracellularly and are killed by the freezing-induced dehydration.

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