BOTANICAL BRIEFING

Plant Freezing and Damage

ROGER S. PEARCE*

Department of Biological and Nutritional Sciences, The University of Newcastle upon Tyne, Newcastle upon Tyne, NE1 7RU, UK

Imaging methods are giving new insights into plant freezing and the consequent damage that affects survival and distribution of both wild and crop plants. Ice can enter plants through stomata and hydathodes. Intrinsic nucleation of freezing can also occur. The initial growth of ice through the plant can be as rapid as 40 mm s^{-1}, although barriers can limit this growth. Only a small fraction of plant water is changed to ice in this first freezing event. Nevertheless, this first rapid growth of ice is of key importance because it can initiate further, potentially lethal, freezing at any site that it reaches. Some organs and tissues avoid freezing by supercooling. However, supercooled parts of buds can dehydrate progressively, indicating that avoidance of freezing-induced dehydration by deep supercooling is only partial. Extracellular ice forms in freezing-intolerant as well as freezing-tolerant species and causes cellular dehydration. The single most important cause of freezing-damage is when this dehydration exceeds what cells can tolerate. In freezing-adapted species, lethal freezing-induced dehydration causes damage to cell membranes. In specific cases, other factors may also cause damage, examples being cell death when limits to deep supercooling are exceeded, and death of shoots when freezing-induced embolisms in xylem vessels persist. Extracellular masses of ice can damage the structure of organs but this may be tolerated, as in extra-organ freezing of buds. Experiments to genetically engineer expression of fish antifreeze proteins have not improved freezing tolerance of sensitive species. A better strategy may be to confer tolerance of cellular dehydration.　　　　© 2001 Annals of Botany Company

Key words: Freezing, dehydration, infrared video thermography, low temperature scanning electron microscopy, NMR micro-imaging.

INTRODUCTION

Freezing is a major environmental stress, inflicting economic damage on crops and limiting the distribution of both wild and crop species. Therefore, understanding freezing and how it damages plants is of extensive practical importance. Improvements in imaging methods are providing a precise description of the initiation and spread of ice and of the nature of damage. This Botanical Briefing emphasizes the advances in current understanding that imaging methods provide, against a background of the broad concepts of the subject. I have not, with few exceptions, referred directly to the older literature, which can be consulted in Burke *et al.* (1976), Levitt (1980) and Sakai and Larcher (1987). Acclimation, which is not the primary focus of this Briefing, has recently been extensively reviewed by Thomashow (1999), Xin and Browse (2000) and Pearce (1999).

A number of methods can provide important information about freezing and damage in plants. Differential thermal analysis (DTA) and nuclear magnetic resonance (NMR) spectroscopy are important for analysing plant freezing (Burke *et al.*, 1976). DTA compares the output from thermocouples attached to a sample and reference. The reference is a dry inert material held in the same environment as the sample. Freezing is identified when the sample temperature rises above the reference temperature.

This indicates a release of heat (an exotherm) occurring when water in the specimen forms ice. However, DTA cannot detect the location at which ice is nucleated or the path of the initial spread of freezing. Infrared video thermography (IRVT) also detects exotherms, but in addition gives a real-time image of the temperature of the plant surface, thus revealing the location at which ice first forms and showing the route and rate of initial growth of ice through the plant (Wisniewski *et al.*, 1997).

NMR spectroscopy quantifies liquid water. However, NMR cannot show where ice and liquid water are located. In contrast, NMR micro-imaging (magnetic resonance imaging, MRI) has sufficient resolution to identify supercooled water in small organs or in tissues of the dimension of xylem (Ishikawa *et al.*, 1997).

Several longer-established imaging methods continue to be important. Low-temperature scanning electron microscopy (LTSEM) shows the location of ice relative to cells. The identity of ice is confirmed by subliming it away in the microscope, and by the appearance of fracture faces before and during sublimation (Pearce, 1988). Freeze-substitution EM shows ice only indirectly, by the cavities left after dissolution during the solvent-substitution step. In freeze-fracture EM, a fracture plane passes through cell membranes revealing details of damage.

These methods differ greatly in spatial resolution: freeze-fracture and freeze-substitution EM > LTSEM > light microscopic methods > MRI > IRVT > the unaided

* Fax +44 (0) 191 222 8684, e-mail R.S.Pearce@ncl.ac.uk

eye > DTA. Collectively, these methods can deliver information across a range of scales, linking information at the whole plant and organ level to phenomena at the tissue, cellular and sub-cellular level. The methods also differ in their ability to resolve events in time. Thus, while IRVT gives real-time images, it takes 30 min to obtain one MRI image; microscopic images are obtained from single time points.

HOW DO PLANTS FREEZE?

Plants and plant parts freeze when they cannot avoid nucleation (see below) and cannot prevent the growth of ice. Freezing-point depression, caused by the presence of solutes (1–2 °C: Levitt, 1980; Franks, 1985) and by supercooling, is often too slight to prevent freezing in moist temperate and colder climates. Consequently, plants of contrasting types nucleate at mild temperatures. In the field, peach freezes between −0·6 and −2·6 °C and other over-wintering temperate woody species freeze between −1·2 and −2.1 °C (Ashworth and Davis, 1986): grasses can freeze between −1·5 and −2·5 °C (Pearce and Fuller, 2001). Laboratory tests often give lower nucleation temperatures than those reported under natural conditions and so can be unreliable indicators of behaviour in the field (Ashworth *et al.*, 1985; Flinn and Ashworth, 1994).

Nucleation

Water molecules come together to form a stable ice nucleus, either spontaneously (homogeneous nucleation), or when catalysed so to do by another substance (heterogeneous nucleation). Homogeneous nucleation is unlikely at temperatures just below 0 °C but, in contrast, in a moist climate, heterogenous nucleation is difficult to avoid.

The amount of pure water needed to generate a single stable ice nucleus by homogeneous nucleation at modest sub-zero (Celsius) temperatures is extremely large; $2·3 \times 10^{312}$ kg at −10 °C (Franks, 1985). The amount falls rapidly with a drop in temperature, but remains large even at −30 °C ($3·8 \times 10^{11}$ kg). The amount falls further as the temperature is reduced until the homogeneous nucleation temperature of −38·5 °C is reached, at which point homogeneous freezing is likely even in a very small volume of water (Franks, 1985). This sets a lower limit to freeze-avoidance for plants and their organs and tissues. Even for single cells, if they have not frozen or been freeze-dehydrated at a higher temperature, then they will freeze internally near −40 °C (the homogeneous nucleation point being depressed by solutes). However, cells with highly viscous contents, such as any cells dehydrated by growth of extracellular ice, are likely exceptions. These may form a glass (vitrify) rather than freeze (Franks, 1985). Vitrification occurs in cells of deeply frozen popular (Hirsh *et al.*, 1985), and this may explain why some tree species can survive temperatures down to liquid nitrogen and liquid helium temperatures (Sakai and Larcher, 1987).

Substances in nature than can act as heterogeneous nucleators include: (*a*) ice nucleation-active (INA) bacteria; (*b*) other biological molecules and structures; and (*c*) organic and inorganic debris. Nucleators may be on the plant surface (extrinsic) or, in some cases, within the plant (intrinsic; see below). To function, a potential heterogeneous nucleator must be in contact with water. Consequently, if the plant surface is dry, extrinsic nucleators will be ineffective. However, during radiation frosts in many climates, moisture will tend to condense onto plant surfaces so giving an opportunity for any heterogeneous nucleators present on the plant surface to function. Snow and sleet can also initiate freezing in plants.

Nucleators operating in nature are partly understood. INA bacteria commonly found on plants comprise strains of several species (such as *Pseudomonas syringae* and *Erwinia herbicola*), which produce a protein able to nucleate freezing at temperatures as high as −2 °C. However, the presence of INA bacteria is not a universal explanation of nucleation of ice in plants. Sizes of INA bacterial populations vary greatly between plant species, sites, climates and seasons, and only a small percentage of cells in a population are effective nucleators (Lindow, 1990). Lindow argued that a very small population of INA bacteria could nucleate freezing throughout citrus trees since once nucleated freezing would spread rapidly. However, this argument would not carry to herbaceous plants, where each leaf (in grasses) or main shoot (in a dicot) would freeze separately because of the higher temperature of the crown compared to the leaves (Fuller and Wisniewski, 1998; Pearce and Fuller, 2001).

It is difficult to study nucleators produced by plants: attempts to isolate the causal agent often result in a reduction or loss of activity. However, plants themselves do contain non-bacterial nucleating agents and some of these agents function at high freezing temperatures e.g. in *Prunus* at about −2 °C (Gross *et al.*, 1988). Some Afro-alpine species and some *Opuntia* contain polysaccharide nucleators operating above −4 °C (Griffith and Antikienen, 1996).

In laboratory tests of freezing-tolerance, the absence of surface moisture can result in artifactual supercooling. Consequently, researchers carrying out freezing tests often ensure nucleation by applying a fine spray of water to the plants (Fuller and Wisniewski, 1998). IRVT shows that even a single droplet of water on a leaf of herbaceous species can freeze first, before the plant, and then becomes the focus from which ice grows within the plant (Wisniewski *et al.*, 1997; Wisniewski and Fuller, 1999).

Leaves with dry surfaces can be used in tests to identify the presence of internal (intrinsic) nucleators. These may operate at temperatures as high as −2 °C, or at much lower temperatures, depending on species and plant part tested (Kaku, 1973; Griffith and Antikienen, 1996). Where intrinsic nucleation can occur, its biological significance would need to be verified since it could be pre-empted by a surface nucleation event. However, even when surface water is present, surface nucleation is not inevitable: nucleation of holly leaves can occur either from frozen surface droplets or internally in the petiole or midrib, and ice then spreads from there (Fig. 1). In some evergreen woody plants, intrinsic nucleators in the stem may operate before the leaves freeze, freezing then spreading to the leaf
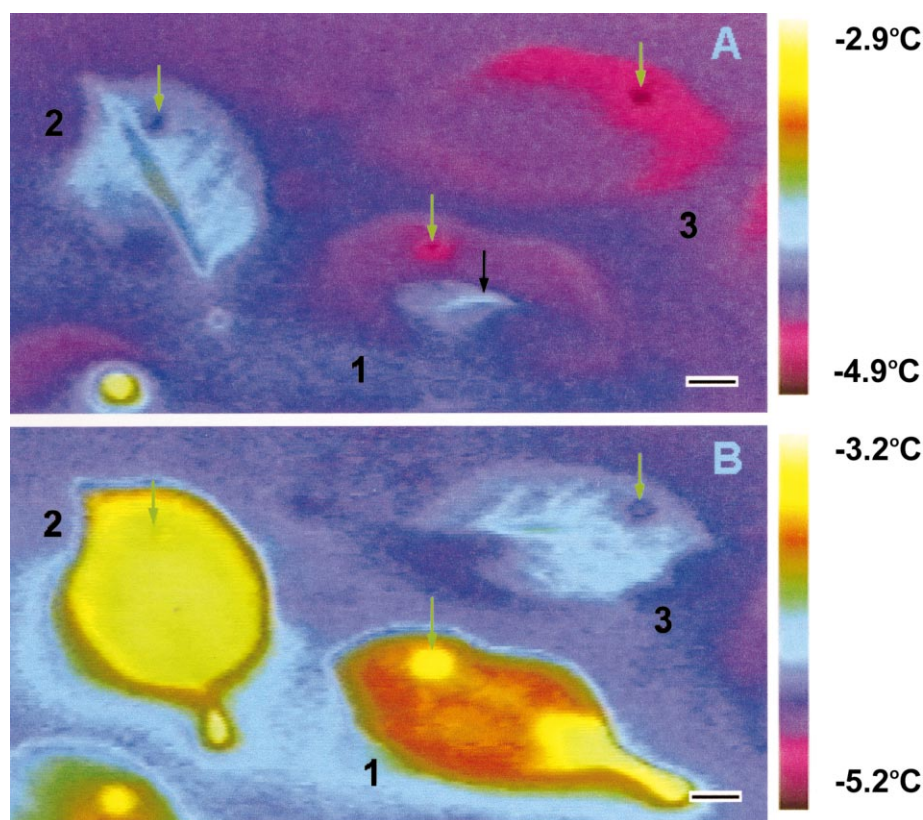
FIG. 1. Infrared video thermography of holly leaves during freezing in a cooling controlled environment chamber (Atherton, Pearce and Fuller, unpubl. res.). Methods were as described in Pearce and Fuller (2001). Freezing is exothermic and thus can be detected by detecting warming. An infrared imaging camera was used to monitor exothermic events in the freezing leaves. The image displays temperature as false-colours, running from 'cold' colours (pink and blue) to 'warm' colours (yellow and red). The scale to the right of each image shows the temperatures that correspond to the false colours. The temperature range detected was adjusted during cooling to maintain a 2 °C window to include the current range of temperatures of the specimens within the field of view. Image A was captured first and image B was captured 156 s later. The pale-blue/white area in leaves 1 and 2 in A and in leaf 3 in B indicate the area of the initial spread of a low-intensity exotherm up to that moment in each leaf, following nucleation of ice respectively 0·9, 4·9 and 2·9 s earlier. Green arrows indicate droplets of water placed on the leaf surfaces before cooling. Nucleation of leaves was internal in leaf 1 (black arrow indicates where nucleation occurred) and in leaves 2 and 3 it occurred from the site of the frozen droplet on the surface. The warmer colours in leaves 1 and 2 in B indicate where larger amounts of water had subsequently frozen during a second freezing event in each leaf, when water is drawn from the cells and freezes extracellularly. Bars = 1 cm.

(e.g. rhododendron: Wisniewski *et al.*, 1997; Wisniewski and Fuller 1999).

Leaves of some species, including Mediterranean sclerophyllous woody plants such as olive, some plants of tropical mountains, sub-tropical and warm temperate palms, and bamboo remain supercooled for long periods, as a freeze-avoidance strategy (Sakai and Larcher, 1987). Recently, IRVT showed that supercooling of *Eucalyptus pauciflora* leaves could be altered by environmental change: the leaves supercooled to $-4·7 \pm 0·5$ °C when the plants were grown in an atmosphere with current CO_2 concentration, but to $-3·5 \pm 0·4$ °C when grown in a CO_2-enriched atmosphere (Lutze *et al.*, 1998).

It has been argued that freezing in trees would occur first in the xylem, based on the total volume of liquid in vessels and on vessel dimensions (Sakai and Larcher, 1987). As mentioned above, a very large volume of water would be required for homogeneous nucleation at the high temperatures at which plants freeze. The volume required (more than 10^{312} kg at temperatures above -10 °C) would be far more than the xylem elements of a tree typical of temperate

regions could contain. Thus when freezing occurs at these temperatures, whether inside or outside the xylem vessels, it is nucleated heterogeneously and the location of surface or intrinsic (internal) heterogeneous nucleators would be the main factor determining the location of nucleation, rather than the total volume of liquid. On the other hand, xylem parenchyma deep supercools in some trees, down to -40 °C, at which temperature it then freezes homogeneously. However, this is not the first freezing event in the tree, other parts having already frozen at a much higher temperature (Burke *et al.*, 1976).

Antifreeze factors

Plants also produce factors that can inhibit ice formation or its growth such as cereal cell wall polysaccharides (Olien and Smith, 1981) and antifreeze proteins (AFPs) (Griffith and Antikienen, 1996). AFPs are defined by their ability to modify freezing *in vitro*, and the name does not necessarily indicate their function *in vivo*. AFPs produced by cereals have strong sequence similarity to the pathogenesis-related

proteins, class I endochitinase, β-1,3-endoglucanase and thaumatin. The full activity of AFPs probably depends on the formation of oligomers containing more than one type of AFP (Yu and Griffith, 1999). The cereal leaf AFPs were found in apoplasmic extracts and the extracellular location of the glucanase-like AFP was confirmed by immuno-localization, indicating their role relates to ice outside the cell (Pihakaski-Maunsbasch *et al.*, 1996).

It is unlikely that the role of AFPs is to prevent plants from freezing. Cereal leaves and leaves of many other species do not use a freeze-avoidance strategy (discussed below). Plant AFPs would not effectively prevent freezing since they only lower the temperature at which ice is stable by 0·3 °C (Griffith and Antikienen, 1996). Suggested roles include controlling the sites of ice formation, the rate at which ice grows, or inhibiting recrystallization (Griffith and Antikienen, 1996).

GROWTH OF ICE INTO AND THROUGH THE PLANT

Wisniewski and Fuller (1999) compared nucleation by droplets placed on the adaxial or abaxial surface of bean and rhododendron leaves and found that propagation of ice through the adaxial surface was delayed. They concluded that stomata provide an important route by which ice, nucleated on a leaf surface, can enter the plant. This would be true when stomata are open in the day. On the other hand, at night, which in many climates is often when freezing begins, stomata would be closed and this might reduce the role of this route for entry of ice. In experiments using IRVT at night in the field, ice entered leaves of a grass, *Holcus lanatus*, through hydathodes (Pearce and Fuller, 2001). It was also found that ice forming on the leaf surface below the tip did not directly nucleate the leaves, indicating that closed stomata may not allow entry of ice.

The use of IRVT shows initial rapid spread through the leaves of a low-intensity thermal signal, indicating rapid growth of ice. This is followed throughout the leaf by a more prolonged high-intensity signal indicating substantial further freezing. This pattern occurs whether freezing is initiated by nucleation within the leaf or on the leaf surface (Fig. 1). The rapid initial growth of ice comprises freezing of only a very small part of the total leaf water, about 0·4 % in barley (Pearce and Fuller, 2001). In the second, more intense freezing event, water is drawn from cells and freezes extracellularly (see below). This second freezing event may only occur at sites that the initial spread of freezing has reached (Pearce and Fuller, 2001).

Barriers often slow or prevent ice growing from stems into flowers or fruits. In cranberry, IRVT showed that ice could not penetrate into the fruit from the stem or the fruit surface but could enter via the calyx (Workmaster *et al.*, 1999). On the other hand, barriers between organs do not occur in all species: freezing readily spreads from one apple flower to another (Wisniewski *et al.*, 1997). Barriers that slow the spread of freezing also occur at nodes and in the crowns of cereals and bean (Single and Marcellos, 1981; Zámećník *et al.*, 1994; Wisniewski *et al.*, 1997; Pearce and Fuller, 2001).

The initial growth of ice is very rapid, between 4 and 40 mm s^{-1} (Single and Marcellos, 1981; Zámećník *et al.*, 1994; Wisniewski *et al.*, 1997; Pearce and Fuller, 2001). The fastest rates are higher than those reported for the growth of ice without the involvement of a plant, possibly because the amount of water freezing at the growing ice front in plants can be very small and thus the heat of fusion can be rapidly dissipated (Hobbs, 1974; Pearce and Fuller, 2001). Freezing spreads at 10 mm s^{-1} in mulberry twigs even when the bark is peeled off leaving only the wood, indicating an association with the xylem (Kitaura, 1967). There is no doubt that the xylem vessel contents do freeze at some stage during freezing of the plant (see below). However, views differ as to whether or not the initial spread of freezing is in the xylem vessels themselves (Single and Marcellos, 1981; Sakai and Larcher, 1987) or may be extracellular (Pearce and Fuller, 2001). In peach stems, ice is nucleated in the cortex and grows from there to the xylem (Wisniewski *et al.*, 1997).

FINAL LOCATION OF ICE

LTSEM shows that ice is usually extracellular in frozen leaves. In acclimated rye and wheat, ice occupies gas spaces and the cells are collapsed and dehydrated (Pearce, 1988; Pearce and Ashworth, 1992). It is less widely realized that extracellular freezing, and consequent cellular dehydration, also occurs in non-acclimated leaves (in barley: Pearce, 1988) and in leaves of species with no capacity for acclimation (Fig. 2).

Ice is also extracellular in the cortex of woody shoots (Burke *et al.*, 1976; Ashworth *et al.*, 1988). In contrast, xylem parenchyma may or may not freeze, depending on the species. For example, in apple the xylem parenchyma deep supercools (Ashworth *et al.*, 1988), only freezing at the homogeneous nucleation temperature (Burke *et al.*, 1976). Freezing is then intracellular, killing the cells. However, xylem parenchyma cells of other woody species do not deep supercool. Their exact state was only revealed recently, by freeze-substitution microscopy of red osier dogwood (Ashworth, 1996). The xylem parenchyma cell walls were not collapsed, as they would have been if they had been dehydrated by extracellular ice. In contrast, the cytoplasmic contents were collapsed but were otherwise undamaged. Probably, ice had formed between the cell wall and the protoplast, thus dehydrating and collapsing the protoplast. As far as is known for plants in nature, this occurs only in xylem parenchyma of species able to survive below −40 °C.

In different species, bud parts either freeze-dehydrate or supercool. Generally, ice is present in bud scales and in the subtending immature stem. In conifers and *Cornus officinalis* this causes dehydration of the interior bud organs and is known as extra-organ freezing (Sakai and Larcher, 1987). However, in *Forsythia* and peach the interior bud organs are supercooled, mostly to between −10 and −30 °C (Ashworth *et al.*, 1989; Ashworth, 1990). Supercooling is lost in spring when xylem vessels develop into the bud and thus give a route for ice to propagate, killing the bud (Ashworth *et al.*, 1989).
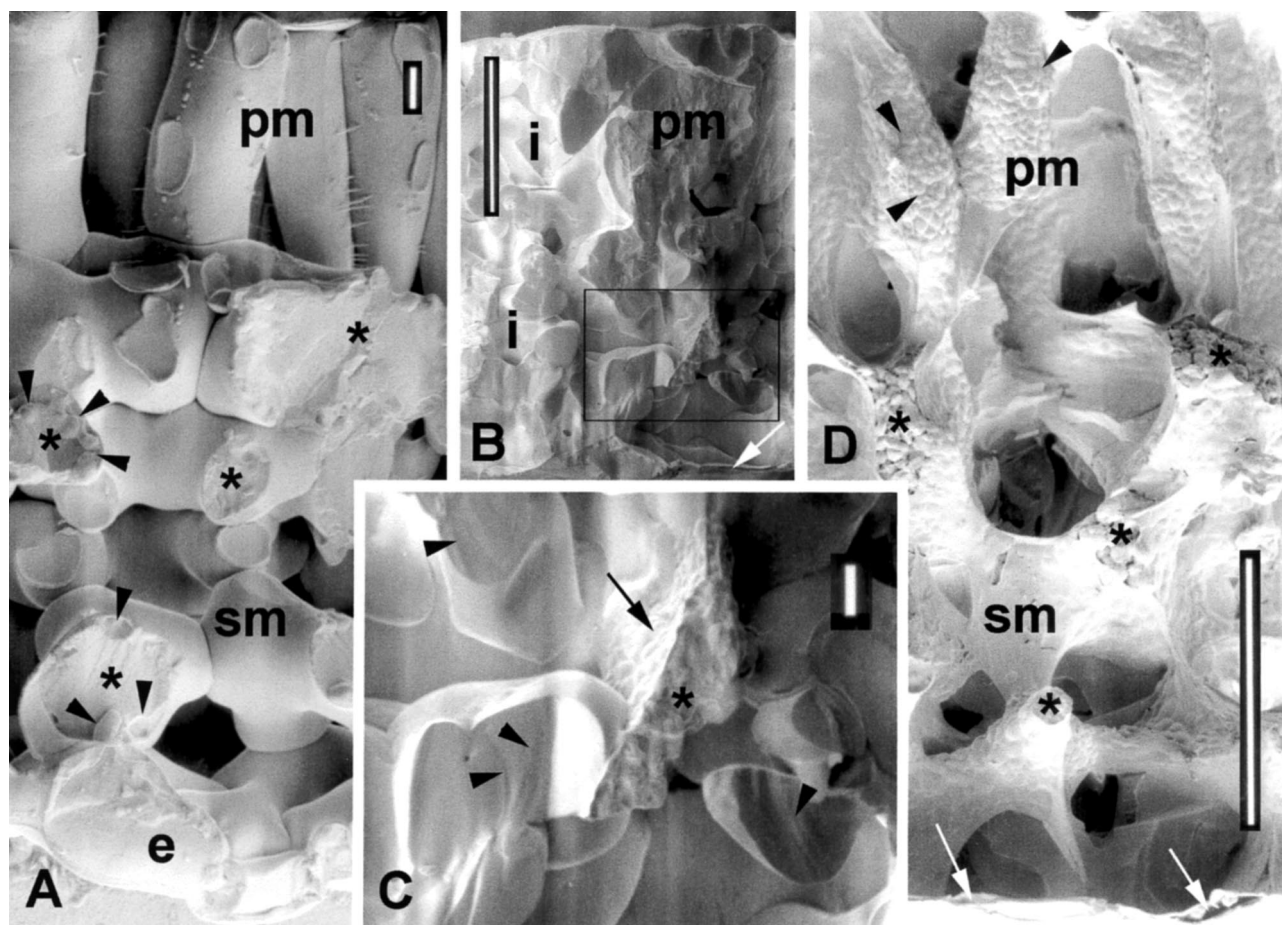
FIG. 2. Low temperature scanning electron microscopy of control (A) and freezing-stressed tobacco leaves (B–D) (Ashworth and Pearce, unpubl. res.). The micrographs show transverse fractures through the leaves. Young potted plants were grown in a warm greenhouse and were tested at the two-to-four leaf stage. The plants were sprayed with water and cooled at 2 °C h^{-1} to −20 °C then freeze-fixed in melting freon 12. DTA showed the leaves froze between −2·0 °C and −3·0 °C. Details of the microscopical methods are as in Pearce and Ashworth (1992). A, Control sample showing turgid cells and absence of extracellular ice in all tissues (e, epidermis; pm, palisade mesophyll; sm, spongy mesophyll). Note that organelle profiles were visible where the fracture plane had cut through the cells (stars indicate cross-fractured cells in the spongy mesophyll; arrow heads indicate organelles in two example cells). The epidermal cells (e) were also cross-fractured. B and C, Sample frozen to −20 °C showing extensive extracellular ice (i). C is an enlargement of the area boxed in B. In this example ice ramified extensively through the gas spaces but did not fully occlude them. The white arrow (B) indicates the collapsed epidermis. At low magnification (B) the ice appeared superficially similar to turgid cells. However, when enlarged (C) the cross-fracture of these structures showed them to contain no organelle profiles and instead the fractured surface had steps (arrow heads) typical of fractured ice. The cells were mostly hidden by the ice. However, in the area enlarged in C a collapsed cell (star) was easily identified by the organelles it contained: the arrow indicates the impress on the cell wall of organelles in a cell. D, Sample frozen to −20 °C. Ice was removed from the specimen by sublimation in the microscope, thus revealing the collapsed, dehydrated cells. The mesophyll cells (pm, palisade mesophyll; sm, spongy mesophyll) and epidermis (white arrows) were collapsed. The outer surface of cell walls showed an impress of the organelles within the cells (examples indicated by arrowheads). Stars indicate where the fracture plane has cut through the cells, again revealing organelles. All gold-coated. 2 kV. Bars = 10 μm (A and C) or 100 μm (B and D).

MRI shows that the terminal buds and flower primordia of a maple supercool, but reveals progressive slight dehydration as the temperature is lowered, thus indicating dehydration-avoidance is only partial (Ishikawa *et al.*, 1997). Lateral buds are much more fully freeze-dehydrated. Extra-organ freezing and slow dehydration of florets also occurs in *Rhododendron japonicum* buds, again indicating some supercooling and only partial avoidance of dehydration (Price *et al.*, 1997).

Recrystallization—the growth of larger ice crystals at the expense of smaller ice crystals (Franks, 1985)—could alter the final location of most ice and lead to the formation of large ice masses. Recrystallization is favoured by prolonged exposure to moderate and high freezing temperatures, as readily happens in nature. Plant AFPs are potent inhibitors of recrystallization (Griffith and Antikienen, 1996; Sidebottom *et al.*, 2000), thus it would be interesting to investigate whether the sites where ice masses form correlate negatively with sites where AFPs are present.

DAMAGE

Freezing-induced cellular dehydration is the most widespread cause of damage. However, damage in specific organs and species may occur for other reasons. For example, cells that deep supercool will die if their capacity

for supercooling is exceeded (above). Other factors include: large ice masses affecting tissue or organ structure; embolisms in xylem vessels; frost-burn caused by exposure to wind or sun; disease, which may enter through lesions or exacerbate damage; ice-encasement, which causes hypoxic stress.

Although ice can be evenly distributed throughout some organs, such as many leaves, it can also form ice masses at predictable sites (as discussed above). Ice masses can separate cell layers and create cavities. An example is separation of epidermis from underlying tissues (Levitt, 1980). Despite such separation, microscopic inspection shows cytoplasmic streaming in the epidermal cells, which thus are not killed. When the structure of organs is disrupted in this way, it need not jeopardise plant survival directly. Thus, ice can form in dispensable organs such as bud scales (Ashworth, 1990), and ice-masses formed in the stem immediately below buds (discussed above) create cavities but both the ice and cavities are tolerated.

Frost cracks in trees are a good example of structural damage as an indirect result of freezing. This involves sudden radial splitting of a tree trunk from its centre through to the bark, said to make a sound like a gunshot. The crack is not created directly by local growth of an ice mass; instead, it is the result of tension in the wood. Several factors may contribute to this, including freezing out of water from the cell walls into the lumen of dead wood cells, and faster cooling of outer wood than inner wood (Kubler, 1983; Sakai and Larcher, 1987). Frost cracks do not directly kill the tree; the cracks can persist for years, opening again each winter, and can cause economic loss in forestry (Sano and Fukazawa, 1996).

As well as growing extracellularly, ice also forms in xylem vessels. Formation of ice forces gases out of solution. LTSEM reveals very small bubbles in xylem vessels (Utsumi *et al.*, 1999). During thawing these bubbles are either reabsorbed or expand, forming embolisms. A large percentage of the vessels can be affected (Utsumi *et al.*, 1998). On the other hand, vessels may refill with water and resume functioning (Canny, 1997; Utsumi *et al.*, 1998). Direct water-stress can also cause embolisms and this could be expected to impede refilling. Thus in dry climates, frost-induced embolisms could be damaging. Consistent with this, shoots of *Rhus laurina*, a chaparral species, appear to be killed by frost-induced embolisms and, though capable of regenerating from the base, this may be an important factor affecting the distribution of this species (Langan *et al.*, 1997).

Freezing-induced cellular dehydration and damage

The water potential of ice is lower than that of liquid water. Consequently, extracellular ice crystals grow by drawing water from cells until the water potential of ice and cell are equal, thus dehydrating the cell contents. The water potential of ice falls as temperature falls, hence cellular dehydration becomes progressively greater as temperature falls (Gusta *et al.*, 1975), down to a limit set by vitrification. In some species, cell walls partially resist the collapse in cellular volume, creating a divergence from equilibrium and

reducing the extent of dehydration; however, substantial cellular dehydration still occurs (Zhu and Beck, 1991).

Membrane structure is damaged when freezing-induced dehydration exceeds the dehydration-tolerance of a cell (Steponkus, 1984; Pearce and Willison, 1985; Pearce, 1985; Steponkus *et al.*, 1993). The physiological consequence—loss of compartmentation—is detectable as leakage of electrolytes and other solutes even before thawing (Stout *et al.*, 1980; Zhang and Willison, 1992*a*). In cereals, the plasma membrane is the membrane most vulnerable to this kind of damage; other membranes are also affected but often at a lower temperature. However , in other species and organs, damage to the tonoplast limits survival (Stout *et al.*, 1980; Zhang and Willison, 1992*b*; Murai and Yoshida, 1998).

Cereal cell membranes have been intensively studied in relation to freezing damage. The mechanism of damage involves phase separation in the membrane. Pearce and Willison (1985) thought that membrane failure occurred by the same mechanism in acclimated and non-acclimated plants, whereas Steponkus *et al.* (1993) argue that important details are different in non-acclimated plants. However, there is agreement that the stress leads to a phase change in a fraction of the membrane lipids, from a bilayer to a non-bilayer structure and that this is a key step in destabilization.

Thus, protection of cell membranes against freeze-dehydration-induced damage is a major factor in freezing tolerance. This is probably achieved both by changes in membrane lipid composition (Steponkus *et al.*, 1988) and by accumulation of substances in the surrounding cytosol. Solutes accumulate during acclimation, including sugars, proline and betaines (reviewed by Xin and Browse, 2000). In addition, highly hydrophilic, boiling-stable proteins accumulate, particularly group 2 LEA (late-embryogenesis-abundant) proteins, also called dehydrins (Close, 1996). It is proposed that solutes and dehydrins stabilize membranes either by direct interaction with membrane surfaces or, indirectly, by their strong interaction with the surrounding water (Crowe *et al.*, 1992; Close, 1996).

Are cells of freezing-tolerant and freezing-sensitive species freeze-killed by similar or markedly different mechanisms? Leaves of sensitive as well as tolerant species freeze extracellularly and thus they are both killed by dehydration (above). Although it is clear that cell membranes are a target for damage in the tolerant species, the subcellular target in the sensitive species is unknown. The most obvious possibility to test is that the membrane lesions found in non-acclimated tolerant species also explain damage in sensitive species.

PROSPECTS FOR MODIFYING FREEZING AND TOLERANCE

One approach to improving freezing-tolerance would be to modify factors regulating acclimation (Thomashow, 1999). This is a powerful approach when applied to freezing-adapted species. The *Arabidopsis thaliana* transcription factor, CBF1, has a central role in acclimation, binding to *cis*-acting regulatory elements of cold-expressed genes

(Stockinger *et al.*, 1997). Constitutive expression of CBF1 in *A. thaliana* confers constitutive freezing-tolerance (Jaglo-Ottosen *et al.*, 1998). However, this approach will not necessarily work with species having no innate capacity to acclimate, since they may be impaired in the functional as well as the regulatory components of acclimation.

In susceptible species, a small improvement in freezing-tolerance may be economically important and achievable by manipulating a single aspect of structure or physiology. Thus, an alternative approach for species that cannot acclimate is to try to increase freeze-avoidance, even if only by one or a few degrees Celcius. Infiltration of a fish antifreeze protein (AFP) into leaves lowered the freezing temperature by 1·8 °C (Cutler *et al.*, 1989). On the other hand, expression of fish AFPs in transgenic plants has not resulted in improvement in survival attributable to anti-freeze effects (Wallis *et al.*, 1997; Kenward *et al.*, 1999). In general, the role of intrinsic plant AFPs is, as discussed above, probably to control rather than prevent freezing. Where plants do avoid freezing by supercooling substan-tially, the mechanisms involved are not fully understood but, at least in the case of buds and xylem parenchyma of woody species, they include structural features (Wisniewski and Fuller, 1999) that could prove difficult to manipulate with precision by genetic engineering.

Susceptible species can freeze extracellularly (Fig. 2), and hence it is possible to aim to improve their tolerance of the consequent dehydration. Small but important improve-ments might be achieved by aiming to alter just one area of structure or biochemistry that might be needed for tolerance. A limitation in choosing this area is that the subcellular target for damage in susceptible species is, at present, only speculative (discussed above). Also, tests of the effect of changes in single areas of function have only been made in non-acclimated plants of species that do, nevertheless, have the capability to acclimate to freezing, so the results are only an indication of what may be possible with susceptible species. As an example, since membrane stability is a key factor in tolerance this is a possible target for improvement (other possibilities are outlined in Pearce, 1999). The lipid composition of rye plasma membrane was experimentally manipulated and *A. thaliana* was trans-formed to express constitutively a chloroplast protein thought to interact with membranes. These changes conferred 1–2 °C improvement in tolerance (Steponkus *et al.*, 1988; Artus *et al.*, 1996).

ACKNOWLEDGEMENTS

LITERATURE CITED

Artus NN, Uemura M, Steponkus PL, Gilmour SJ, Lin CT, Thomashow MF. 1996. Constitutive expression of the cold-regulated *Arabidopsis thaliana cor15a* gene affects both chloroplast and protoplast freezing tolerance. *Proceedings of the National Academy of Sciences, USA* **93**: 13404–13409.

Ashworth EN. 1990. The formation and distribution of ice within forsythia flower buds. *Plant Physiology* **92**: 718–725.

Ashworth EN. 1996. Responses of bark and wood cells to freezing. *Advances in Low Temperature Biology* **3**: 65–106.

Ashworth EN, Davis GA. 1986. Ice formation in woody plants under field conditions. *HortScience* **21**: 1233–1234.

Ashworth EN, Davis GA, Anderson JA. 1985. Factors affecting ice nucleation in plant tissues. *Plant Physiology* **79**: 1033–1037.

Ashworth EN, Davis GA, Wisniewski ME. 1989. The formation and distribution of ice within dormant and deacclimated peach flower buds. *Plant, Cell and Environment* **12**: 521–528.

Ashworth EN, Echlin P, Pearce RS, Hayes TL. 1988. Ice formation and tissue response in apple twigs. *Plant, Cell and Environment* **11**: 703–710.

Burke MJ, Gusta LV, Quamme HA, Weiser CJ, Li PH. 1976. Freezing and injury in plants. *Annual Review of Plant Physiology* **27**: 507–528.

Canny MJ. 1997. Vessel contents during transpiration—embolisms and refilling. *American Journal of Botany* **84**: 1223–1230.

Close TJ. 1996. Dehydrins: Emergence of a biochemical role of a family of plant dehydration proteins. *Plant Physiology* **97**: 289–290.

Crowe JH, Hoekstra FA, Crowe LM. 1992. Anhydrobiosis. *Annual Review of Physiology* **54**: 570–599.

Cutler AJ, Saleem M, Kendall E, Gusta LV, Georges F, Fletcher GL. 1989. Winter flounder antifreeze proteins improve the cold hardiness of plant tissues. *Journal of Plant Physiology* **135**: 351–354.

Flinn CL, Ashworth EN. 1994. Blueberry flower-bud hardiness is not estimated by differential thermal analysis. *Journal of the American Society for Horticultural Science* **119**: 295–298.

Franks F. 1985. *Biophysics and biochemistry at low temperatures.* Cambridge: Cambridge University Press.

Fuller MP, Wisniewski M. 1998. The use of infrared thermal imaging in the study of ice nucleation and freezing of plants. *Journal of Thermal Biology* **23**: 81–89.

Griffith M, Antikienen M. 1996. Extracellular ice formation in freezing-tolerant plants. *Advances in Low-Temperature Biology* **3**: 107–139.

Gross DC, Proebsting EL Jr, Macrindle-Zimmerman H. 1988. Development, distribution, and characteristics of intrinsic, non-bacterial ice nuclei in *Prunus* wood. *Plant Physiology* **88**: 915–922.

Gusta LV, Burke MJ, Kapoor A. 1975. Determination of unfrozen water in winter cereals at subfreezing temperatures. *Plant Physiology* **56**: 707–709.

Hirsh AG, Williams RJ, Meryman HT. 1985. A novel method of natural cryoprotection—intracellular glass-formation in deeply frozen *Populus*. *Plant Physiology* **79**: 41–56.

Hobbs PV. 1974. *Ice physics.* Oxford: Clarendon Press.

Ishikawa M, Price WS, Ide H, Arata Y. 1997. Visualization of freezing behaviors in leaf and flower buds of full-moon maple by nuclear magnetic resonance microscopy. *Plant Physiology* **115**: 1515–1524.

Jaglo-Ottosen KR, Gilmour SJ, Zarka DG, Schabenberger O, Thomashow MF. 1998. *Arabidopsis CBF1* overexpression induces *COR* genes and enhances freezing tolerance. *Science* **280**: 104–106.

Kaku S. 1973. High ice nucleting ability in plant leaves. *Plant and Cell Physiology* **14**: 1035–1038.

Kenward KD, Brandle J, McPherson J, Davies PL. 1999. Type II fish antifreeze protein accumulation in transgenic tobacco does not confer frost resistance. *Transgenic Research* **8**: 105–117.

Kitaura K. 1967. Freezing and injury of mulberry trees by late spring frost. *Bulletin of the Sericulture Experimental Station (Tokyo)* **22**: 202–323.

Kubler H. 1983. Mechanism of frost crack formation in trees—a review and synthesis. *Forest Science* **29**: 559–568.

Langan SJ, Ewers FW, Davis SD. 1997. Xylem dysfunction caused by water stress and freezing in two species of co-occurring chaparral shrubs. *Plant, Cell and Environment* **20**: 425–437.

Levitt J. 1980. *Responses of plants to environmental stresses*, Vol. 1. New York: Academic Press.

Lindow SE. 1990. Use of genetically altered bacteria to achieve plant frost control. In: Nakas JP, Hagedorn C, eds. *Biotechnology of plant-microbe interactions*. New York: McGraw-Hill, 85–110.

Lutze L, Roden JS, Holly CJ, Wolfe J, Egerton JJG, Ball MC. 1998. Elevated atmospheric [CO_2] promotes frost damage in evergreen tree seedlings. *Plant, Cell and Environment* **21**: 631–635.

Murai M, Yoshida S. 1998. Vacuolar membrane lesions induced by a freeze-thaw cycle in protoplasts isolated from deacclimated tubers of Jerusalem artichoke (*Helianthus tuberosus* L.). *Plant and Cell Physiology* **39**: 87–96.

Olien CR, Smith MN. 1981. Protective systems that have evolved in plants. In: Olien CR, Smith MN, eds. *Analysis and improvement of plant cold hardiness*. Boca Raton: CRC Press, 61–87.

Pearce RS. 1985. A freeze-fracture study of membranes of rapidly drought-stressed leaf bases of wheat. *Journal of Experimental Botany* **36**: 1209–1221.

Pearce RS. 1988. Extracellular ice and cell shape in frost-stressed cereal leaves: A low temperature scanning electron microscopy study. *Planta* **175**: 313–324.

Pearce RS. 1999. Molecular analysis of acclimation to cold. *Plant Growth Regulation* **29**: 47–76.

Pearce RS, Ashworth EN. 1992. Cell shape and localization of ice in leaves of overwintering wheat during frost stress in the field. *Planta* **188**: 324–331.

Pearce RS, Fuller MP. 2001. Freezing of barley (*Hordeum*) studied by infrared video thermography. *Plant Physiology* **125**: 227–240.

Pearce RS, Willison JHM. 1985. A freeze-etch study of the effect of extracellular freezing on the cellular membranes of wheat. *Planta* **163**: 304–316.

Pihakashi-Maunsbach K, Griffith M, Antikienen M, Maunsbach AB. 1996. Immunogold localization of glucanase-like antifreeze protein in cold acclimated winter rye. *Protoplasma* **191**: 115–125.

Price WS, Ide H, Arata Y, Ishikawa M. 1997. Visualisation of freezing behaviours in flower bud tissues of cold-hardy *Rhododendron japonicum* by nuclear magnetic resonance micro-imaging. *Australian Journal of Plant Physiology* **24**: 599–605.

Sakai A, Larcher W. 1987. *Frost survival of plants*. Berlin: Springer.

Sano S, Fukazawa K. 1996. Timing of the occurrence of frost cracks in winter. *Trees* **11**: 47–53.

Sidebottom C, Buckley S, Pudney P, Twiggs S, Jarman C, Holt C, Telford J, McArthur A, Worrall D, Hubbard R, Lillford P. 2000. Heat-stable antifreeze protein from grass. *Nature* **406**: 256.

Single WV, Marcellos H. 1981. Ice formation and freezing injury in actively growing cereals. In: Olien CR, Smith MN, eds. *Analysis and improvement of plant cold hardiness*. Boca Raton: CRC Press, 17–33.

Steponkus PL. 1984. Role of the plasma membrane in freezing injury and cold acclimation. *Annual Review of Plant Physiology and Plant Molecular Biology* **35**: 543–584.

Steponkus PL, Uemura M, Webb MS. 1993. A contrast of the cryostability of the plasma membrane of winter rye and spring oats—two species that widely differ in their freezing tolerance and plasma membrane lipid composition. *Advances in Low Temperature Biology* **2**: 211–312.

Steponkus PL, Uemura M, Balsamo RA, Arvinte T, Lynch DV. 1988. Transformation of the cryobehaviour of rye protoplasts by modification of the plasma membrane lipid composition. *Proceedings of the National Academy of Sciences, USA* **85**: 9026–9030.

Stockinger EJ, Gilmour EJ, Thomashow MF. 1997. *Arabidopsis thaliana* CBF1 encodes an AP2 domain-containing transcription activation that binds to the C-repeat/DRE, a *cis*-acting DNA regulatory element that stimulates transcription in response to low temperature and water deficit. *Proceedings of the National Academy of Sciences, USA* **94**: 1035–1040.

Stout DG, Majak W, Reaney M. 1980. *In vivo* detection of membrane injury in freezing temperatures. *Plant Physiology* **66**: 74–77.

Thomashow MF. 1999. Plant cold acclimation: Freezing tolerance genes and regulatory mechanisms. *Annual Review of Plant Physiology and Plant Molecular Biology* **50**: 571–599.

Utsumi Y, Sano Y, Funada R, Fujikawa S, Ohtani J. 1998. Visualization of cavitated vessels in winter and refilled vessels in spring in diffuse-porous trees by cryo-scanning electron microscopy. *Plant Physiology* **117**: 1463–1471.

Utsumi Y, Sano Y, Funada R, Fujikawa S, Ohtani J. 1999. The progression of cavitation in earlywood vessels of *Fraxinus mandshurica* var *japonica* during freezing and thawing. *Plant Physiology* **121**: 897–904.

Wallis JG, Wang HY, Guerra DJ. 1997. Expression of a synthetic antifreeze protein in potato reduces electrolyte releasing at freezing temperatures. *Plant Molecular Biology* **35**: 323–330.

Wisniewski M, Fuller M. 1999. Ice nucleation and deep supercooling in plants: new insights using infrared thermography. In: Margesin R, Schinner F, eds. *Cold-adapted organisms—Ecology, physiology, enzymology and molecular biology*. Berlin: Springer, 105–118.

Wisniewski M, Lindow SE, Ashworth EN. 1997. Observation of ice nucleation and propagation in plants using infrared video thermography. *Plant Physiology* **113**: 327–334.

Workmaster BAA, Palta JP, Wisniewski M. 1999. Ice nucleation and propagation in craneberry uprights and fruit using infrared video thermography. *Journal of the American Society for Horticultural Science* **124**: 619–625.

Xin Z, Browse J. 2000. Cold comfort farm: the acclimation of plants to freezing temperatures. *Plant, Cell and Environment* **23**: 893–902.

Yu X-M, Griffith M. 1999. Antifreeze proteins in winter rye leaves form oligomeric complexes. *Plant Physiology* **119**: 1361–1369.

Záměćník J, Bieblováá J, Grospietsch M. 1994. Safety zone as a barrier to root-shoot ice propagation. *Plant and Soil* **167**: 149–155.

Zhang MIN, Willison JHM. 1992a. Electrical impedance analysis in plant tissues: *in vivo* detection of freezing injury. *Canadian Journal of Botany* **70**: 2254–2258.

Zhang MIN, Willison JHM. 1992b. Electrical impedance analysis in plant tissues: The effect of freeze-thaw injury on the electrical properties of potato tuber and carrot root tissues. *Canadian Journal of Plant Science* **72**: 545–553.

Zhu J-J, Beck E. 1991. Water relations of *Pachysandra* leaves during freezing and thawing. *Plant Physiology* **97**: 1146–1153.