# Extrinsic Ice Nucleation in Plants: What Are The Factors Involved and Can They Be Manipulated

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## 1. INTRODUCTION

In the early 1980's a considerable amount of research focused on the role of extrinsic ice nucleation and its' role in inducing plants to freeze at warm sub-zero temperatures. The working hypothesis was that by controlling extrinsic nucleation events, plants could supercool well below 0 °C and thus avoid freezing (Lindow, 1995). It was felt that such a strategy could provide a significant level of frost protection to frost sensitive plants or plant parts. While the majority of reports dealt with the role of ice-nucleating-active (INA) bacteria (e.g. *Pseudomonas syringae*), related research focused on the role of other extrinsic nucleating agents and whether or not plants could actually supercool to temperatures several degrees below 0 °C due to the presence of intrinsic nucleating agents which induced the plants to freeze at warm temperatures (Ashworth and Kieft, 1995). The identification of a wide range of both extrinsic and intrinsic ice nucleating agents made the practical application of blocking extrinsic ice nucleation complex. Since that time, research emphasis has switched to identifying genes that impart cold tolerance and the transcriptional activators that regulate cold hardiness genes (Thomashow, 1998; Jaglo, et al., 2001). The hypothesis here is that by the overexpression of these types of genes, a non-acclimated or freeze-sensitive plant could be made freezing tolerant. While great progress has been made in understanding the genetic basis of cold hardiness, manipulation of this trait by molecular biology has also demonstrated itself to be complicated due to the "additional" effects of the overexpression of several cold hardiness genes on the physiology and development of the target plant. Therefore, blocking extrinsic ice nucleation, although complicated, may still be a valuable approach to providing protection to frost sensitive plants.

In order for ice to form on or within a plant, ice nucleation must first occur. Although the melting point of ice is 0 °C, the freezing temperature of water is not as defined (Ashworth, 1992). In fact, although it is not commonly recognized, pure water has a low probability of freezing at temperatures warmer than -40 °C (Franks, 1985). This is because a small ice crystal embryo is necessary in order for ice to form and grow to any substantial size. The probability of forming such an ice crystal embryo in pure water, as well as the half-life of such a crystal, is low until temperatures approaching -40 °C. This temperature is referred to as the homogeneous ice nucleation point.

In nature, it is rare for water to exist in a pure state but it rather exists as an ionic or colloidal solution. In such solutions heterogeneous ice nucleation is initiated on the surface of objects or on suspended particles (Ashworth, 1992). Heterogeneous ice nucleators are very effective in inducing ice

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formation and are very abundant. As a consequence, freezing occurs in nature at much warmer temperatures than the homogeneous nucleation temperature.

The role of heterogeneous ice nucleators in inducing ice formation in plants is important because if methods can be developed for regulating ice nucleation, significant advances could be made in limiting frost injury to both freezing-sensitive and cold adapted plants. A major question concerns the relative importance of extrinsic ice nucleation agents, such as ice-nucleation-active (INA) bacteria (e.g. *Pseudomonas syringae*), and intrinsic nucleation agents synthesized by plants (Ashworth and Kieft, 1995). While all plants can supercool (i.e., have tissues below 0 °C without freezing) to some degree (Ashworth and Kieft, 1995; Burke, et al., 1976; Lindow, 1995; Lindow, et al., 1978), the extent of supercooling varies between plant species and is influenced by the presence of ice nucleating agents which may be of plant (Ashworth, 1992; Fuller, et al., 1994; Gross, et al., 1988) or bacterial (Lindow, 1978; Hirano, et al., 1985; Lindow, 1983) origin.

The abundance of ice nuclei on plants can be estimated by freezing of droplets of plant macerates or small portions of plant tissue (Ashworth and Kieft, 1995; Lindow, 1983) but these procedures are destructive and do not provide information on where ice formation was initiated. Ice formation in intact plants can be readily detected by measuring, with thermocouples, the heat that is released upon the freezing of the water in the plant (Ashworth, 1992; Cary and Mayland, 1970; Proebsting, et al., 1982; Quamme, et al., 1972). Nevertheless, even when arrays of temperature measuring devices are attached to plants, the actual site of ice initiation and the temperature at the site where ice nucleation occurred can only be inferred (Ashworth, et al., 1985). This is a significant technical limitation and more details of the freezing process are required in order to accurately predict freezing patterns and determine under what conditions the reduction or interference of extrinsic ice nuclei would provide significant frost protection. Recently, the ability to use infrared video thermography to directly observe ice nucleation (i.e,, initial ice formation) and propagation in plants has been demonstrated (Carter, et al., 2001, 1999; Ceccardi, et al., 1995; Fuller and Wisniewski, 1998; LeGrice, et al., 1993; Wisniewski, et al., 1997; Wisniewski, 1998; Wisniewski and Fuller, 1999; Workmaster et al., 1999). The temperature and spatial resolution of the device used in these studies has enabled the researchers to clearly define the initial site of ice nucleation as well as monitor the ice front as it spread into the surrounding tissues. Using infrared thermography it is possible to determine the role of extrinsic and intrinsic ice nucleating agents in the freezing process, rates of ice propagation, the effect of plant structure on the freezing process, and how the specific pattern of freezing relates to visual patterns of injury. It is also possible to clearly evaluate if the reduction of ice nuclei or inhibiting their activity is a feasible approach to frost protection. The present report will provide an overview of these various studies and detail the factors that apparently play a significant role in determining when a plant will freeze and how ice will propagate through a plant.

## 2. ROLE OF MOISTURE AND EXTRINSIC ICE NUCLEATING AGENTS

One of the critical factors in determining when a plant will freeze is the presence or absence of surface moisture (Ashworth, 1992). Dry plants will always supercool to a lower temperature than wet plants. Secondly, if ice nucleating agents, such as INA bacteria, are present, they will induce plants to freeze at a warmer temperature than just the moisture alone (Wisniewski, et al., 1997, Fuller and Wisniewski, 1998). The presence of nucleators on the surface without moisture is not effective because nucleators are only active in aqueous solutions.

In order for the presence of external ice (frozen moisture on a leaf surface) to induce ice formation in a plant, the ice must physically grow through a break in the surface of the cuticle (eg. crack or broken hair cells) or through a stomatal opening (Wisniewski and Fuller, 1999). A thick cuticle, such as found on evergreen leaves (eg. azalea, cranberry) serves as an effective barrier to external nucleation (Wisniewski and Fuller, 1999; Workmaster, et al., 1999). Water can freeze on the upper surface of these plants and the plant will continue to supercool. When external ice does induce the plant to freeze it is through the growth of ice through a stomatal opening on the abaxial surface. In many herbaceous plants, the cuticle is not an effective barrier, or there are sufficient avenues of ingress that allow ice to readily propagate from either the upper or lower surface. Providing a barrier of silicone grease sufficiently prevents external ice from inducing herbaceous plants to freeze (Wisniewski and Fuller, 1999).

## 3. HYDROPHOBIC BARRIERS APPLIED TO HERBACEOUS PLANTS CAN BLOCK ICE NUCLEATION

Our previous research indicated that by somehow blocking the activity of extrinsic nucleating agents one may allow plants to supercool to a lower temperature and thereby provide some frost protection. In a subsequent study we used infrared thermography to examine freezing in young tomato (*Lycopersicon esculentum*) plants and determine if a hydrophobic barrier on the plant surface could prevent the action of extrinsic nucleating agents such as Ice-Nucleating-Active (INA or $Ice^+$) bacteria (*Pseudomonas syringae,* strain Cit7) from initiating freezing within a plant. To provide a barrier to the action of extrinsic ice-nucleating agents, M-96-018, a hydrophobic kaolin particle film (Engelhard, Islin, NJ, USA) was applied to the plant surface before applying an extrinsic nucleating agent (Wisniewski, et al., In Press).

Tomato plants were grown in a greenhouse in individual pots and used when they were 4-6 weeks old. Freezing tests were conducted in a programmable freezing chamber, a radiative frost chamber, and outdoors. Freezing was visualized and recorded on videotape using an infrared radiometer. Freezing of the plants was extrinsically induced by the application of droplets (5 $\mu$l) of water containing Cit7. To provide a barrier to the action of extrinsic ice nucleating agents, an emulsion of hydrophobic kaolin (Engelhard, Inc.) was applied to the plant surface prior to application of an extrinsic nucleating agent. Results indicate that dry, young tomato plants can supercool to as low as -6 °C whereas plants having a single droplet of Cit7 would freeze at -1.5 to -2.5 °C. Application of the hydrophobic barrier blocked the effect of Cit7 and allowed whole plants to also supercool to -6 °C, despite the presence of frozen droplets on the surface of leaves and stems (Fig. 1).

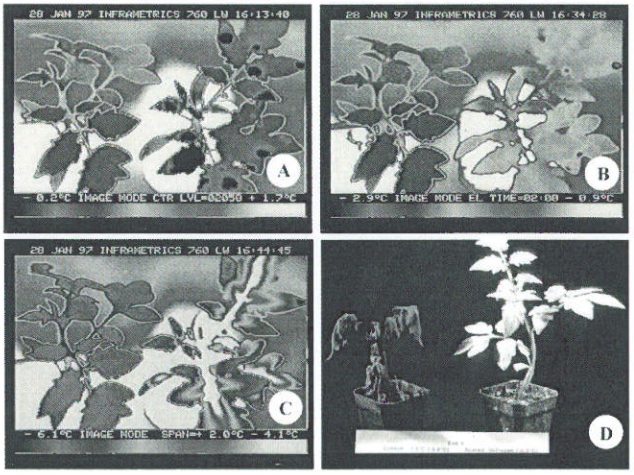


**Fig. 1.** Infrared thermography (**A - C**) and a photograph (**D**) of M96-018 coated and uncoated tomato plants subjected to freezing temperatures. Each plant was sprayed with water containing $Ice^+$ bacteria using a hand-operated aerosol sprayer. The coated plant is on the left in **A-C** and on the right in **D**. Large drops of water can be seen as black areas on the uncoated plant in **A**. The black areas are a result of these areas being colder than the set temperature range of the camera (-0.2 to 1.7 °C). The lower temperature of these areas is due to evaporative cooling. In **B**, the uncoated plant is in the process of freezing, as evidenced by the exothermic reaction of ice formation, while the coated plant is unfrozen at approximately -2.5 °C. In **C**, the uncoated plant has almost completely frozen, except along the stem, petiole, and mid-vein. The coated plant in **C** is still unfrozen at approximately -6.0 °C. In **D**, uncoated (left) and coated (right) plants can be seen after exposure to -6.0 °C, removal from the chamber, and thawing. The uncoated plant is completely killed while the coated plant is uninjured.

When whole plants were sprayed with water and Cit7 using an aerosol sprayer and exposed to -3 °C, plants coated with the hydrophobic particle film exhibited a significant increase in survivability over untreated plants (Fig. 2). Similar results were obtained using a radiative frost chamber (Fig. 3). Experiments conducted under natural frost conditions also resulted in less injury in the coated plants, although due to the small sample size the difference in injury between the coated and uncoated plants was not significant. The hydrophobic kaolin particle film was better at preventing plants from freezing due to extrinsic ice nucleation than normal kaolin alone or anti-transpirants with putative frost protection

properties.

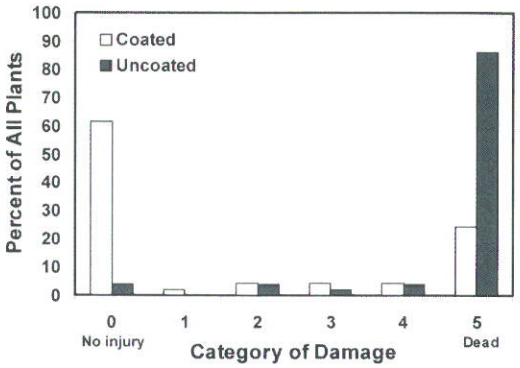


**Fig. 2**. Percent of M96-018-coated and uncoated tomato plants in different injury classes after exposure to -3.0 °C. Each plant was sprayed with water containing Ice$^+$ bacteria prior to being placed in the environmental chamber. The damage levels, combined for all classes, was analyzed by a t-test for coated vs. uncoated. The probability of a difference based on the t-test is $P > 0.0001$. $n = 51$ for uncoated plants and $n = 49$ for coated plants.

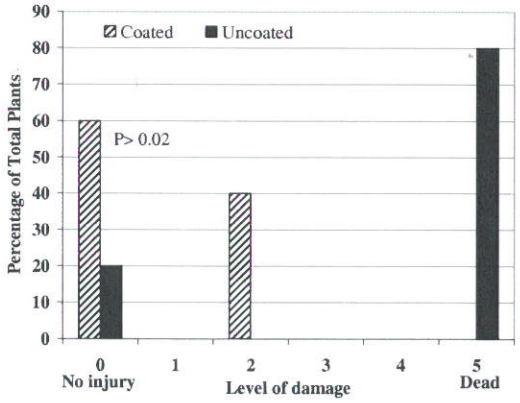


**Fig. 3.** Percent of M96-018-coated and uncoated tomato plants in different injury classes after exposure to -1.6 °C for one hour in a radiative frost chamber. All plants were sprayed with water containing Ice$^+$ bacteria prior to being placed in the frost chamber. A t-test indicated that there was a significant difference in the level of injury between coated and uncoated plants ($P > 0.02$; $n = 6$).

The material used in the study consists of a proprietary formulation of kaolin particles that have been coated to impart hydrophobicity. It is our hypothesis that if moisture can be prevented from forming on the plant surface and activating epiphytic ice nucleators, or itself freezing and acting as source of nucleation activity, then there would be a higher probability of the plant expressing its intrinsic ability to supercool. The results of the study supported this hypothesis on both individual leaves and whole plants of tomato. It appeared that the particle film, due to its hydrophobicity, prevented moisture from being deposited on the plant surface (in many cases water would simply roll off) and lowered the amount of contact that any individual droplet had with the plant surface, effectively raising the droplet above the leaf surface (Fig. 4). Additionally, it is assumed that the moisture barrier prevented the wetting of epiphytic ice nucleators present on the leaf surface and hence their activation. Collectively, this reduced the number of potential extrinsic nucleation events, and diminished the ability of ice crystals that did form from propagating into the internal portion of the leaf and inducing the leaf to freeze. Access of ice crystals to the internal portion of the leaf is believed to occur through stomates, cracks in the cuticle, broken epidermal hairs, etc. (Wisniewski and Fuller, 1999).

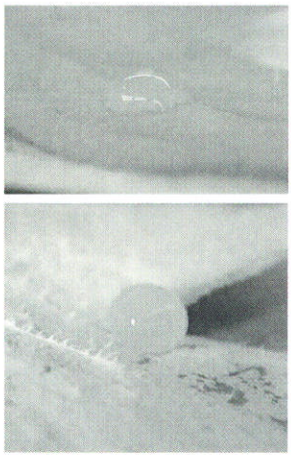

**Fig. 4.** Photographs of droplets of water containing Ice$^+$ bacteria, strain Cit7 of *Pseudomonas syringae*, on the surface of an uncoated tomato leaflet (upper panel) and a tomato leaflet coated with the M96-018 hydrophobic-kaolin-particle-film (lower panel). Note how the droplet on the coated leaflet is more spherical and has less contact with the leaf surface than the droplet on the uncoated leaflet.

Relevant to that study, is the work on ice nucleation in tomato plants reported by Anderson and Ashworth (1985). Using thermocouples and monitoring populations of ice-nucleation-active bacteria *(Pseudomonas syringae)*, they observed both a size and time dependency on the extent of supercooling. As plant mass increased, the extent of supercooling decreased. Additionally, at any particular sub-zero temperature the percentage of samples that would freeze increased with time. They also noted that a prime determinant of the extent of supercooling was the activity of extrinsic nucleating agents (INA bacteria, moisture). The results reported by Wisniewski, et al. (In Press) differ only in the extent to which whole plants have the potential to supercool. We have definitely observed that plant parts (leaves and leaflets) supercool to a greater extent than whole plants but have commonly seen supercooling of whole plants to temperatures of at least -6.0 °C using cooling rates of -2.0 to -2.5 °C · $h^{-1}$. Their inability to see supercooling below -2.0 °C in whole plants may have been due to the nucleation activity of the thermocouples themselves. Our inability to prevent some plants from freezing at -3.0 °C, however, may reflect both the size and time dependency aspects of intrinsic nucleation rather than our failure at blocking extrinsic nucleation.

The ability of the hydrophobic particle film to protect plants from frost damage has also been demonstrated for potatoes, grapevines, and citrus plants under both convective and radiative frost conditions where plants were exposed to -3 °C for two hours (Fuller, et al., submitted). In these studies, the presence of the hydrophobic particle film consistently led to less damage than that observed in uncoated plants. The particle film delayed ice crystal growth from a frozen droplet present on leaf surfaces for an average of one hour, and in some cases for the whole duration of the frost test. This time delay is significant in that it is representative of the duration of transient radiation frosts under field conditions (Fuller and Le Grice, 1998). Large scale studies under field conditions will be needed, however, to determine if the hydrophobic particle film (or a similar type of compound) can be used to provide frost protection under the complex freezing conditions that are present during natural frost episodes.

## 4. FREEZING OF WOODY STEMS AND BARRIERS TO ICE PROPAGATION

As previously documented and reviewed by Ashworth and Kieft (1995), the presence of effective, intrinsic nucleators, appears to be common in woody plants. These nucleators appear to be as effective as external ice nucleators, such INA bacteria, and induce stems to freeze at warm, subzero temperatures. Barriers appear to exist, however, that prevent ice propagation into lateral appendages such as buds, or newly extended primary tissues (flowers, inflorescences, etc.) (Carter et al., 2001; Wisniewski, 1997; Workmaster, 1999). These barriers are most effective if the initial freezing event occurs at a relatively warm temperature. Barriers have been observed in the propagation of ice into the strigs of Ribes and grapevines, the pedicel of cranberry fruits, and flowers of peach indicating that the ability of buds, flowers, and inflorescences to supercool in the presence of frozen stem material may be an active mechanism of

freeze avoidance.

## 5. INFLUENCE OF COLD ACCLIMATION AND ANTIFREEZE PROTEINS ON ICE NUCLEATION

When plants are cold acclimated, they develop a greater ability to supercool. This has been demonstrated in canola and barley, and rye plants (unpublished data). In one experiment, cellular extracts of canola (*Brassica napus)* were placed on long, rectangular strips of filter paper, and the rate of freezing from one end of the strip to the other was determined using infrared thermography. Uniform times and temperatures of ice nucleation were controlled by placing a drop of ice-nucleating-active bacteria (*Pseudomonas syringae,* strain Cit7) at the bottom of each strip. Results indicated that both the sugars and proteins present in acclimated plants may play a role in regulating the rate of ice crystal growth (Fig. 5). Cellular extracts from acclimated plants delayed ice crystal growth more than extracts from non-acclimated plants. Additionally, extracts from a hardy cultivar (Express) had a greater effect than extracts from a less hardy cultivar (Quest). An effect of unidentified proteins could be seen over and above that of sugars by boiling the samples and also comparing the response of cellular extracts to pure sugar solutions with similar osmolality.

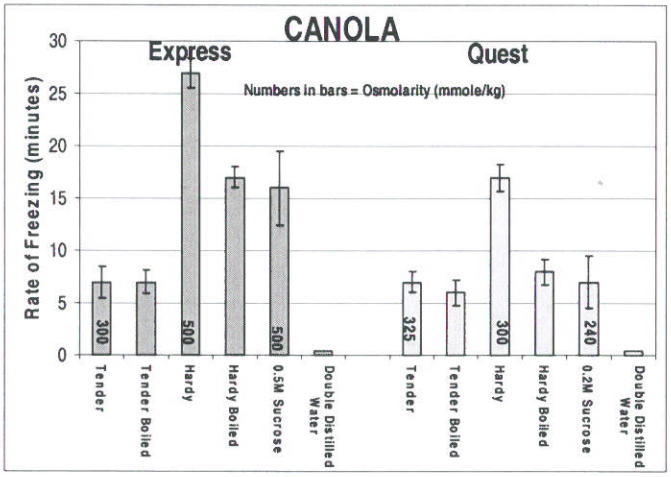


**Fig. 5.** Effect of cellular extracts from acclimated and non-acclimated plants of two cultivars (Express and Quest) of canola on the rate of freezing of strips of filter paper. The effect of the fresh cellular extracts was compared to solutions of sucrose with similar osmolality, boiled samples of the extracts, and deionized water.

Antifreeze proteins (AFPs), also known as hysteresis proteins (THPs), inhibit ice crystal growth by a non-colligative mechanism, lowering the freezing point of water below the melting point, thereby producing a so-called thermal hysteresis (DeVries, 1971). First described in fish, they have also been widely reported in insects (Duman, et al., 2001), but insect AFPs are structurally different than those of fishes and have higher activities. Antifreeze protein activity has also been identified in many plants (Griffith, et al., 1992; Hon, et al, 1995; Duman, 1994) but the levels of thermal hysterisis activity are comparatively low (generally 0.2-0.5 °C), as compared to fishes (genrally 0.7-1.5 °C), and insects (generally 3-6 °C). Transgenic Arabidopsis plants expressing an insect antifreeze gene derived from *Dendroides canadensis* exhibited an enhanced ability to supercool (Fig. 6). The enhanced ability to supercool was only present in the transformed lines in which the construct also coded for a signal peptide that allowed for extracellular secretion of the protein (340 lines). Transformants (270 lines) that expressed the protein but did not have the coding for the signal peptide did not supercool to a greater extent than the wild-type plants.

When acclimated canola plants were allowed to supercool to low temperatures (-12 to -15 °C) and then frozen, they exhibited no injury despite the rapid rate of ice formation and propagation. This indicates that the acclimated plants have the ability to rapidly lose water in order to prevent intracellular ice

formation (unpublished data). Distinct differences in the freezing response of acclimated and non-acclimated rye plants have also been observed that may be attributed to the presence or absence of antifreeze proteins (see Chapter by Griffith, et al., this volume).

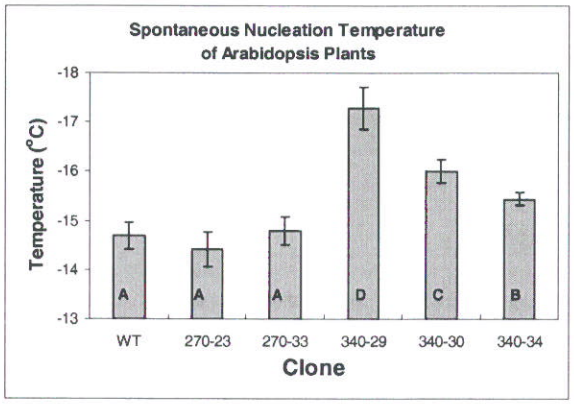


**Fig. 6.** Spontaneous nucleation temperature of wild-type (WT) and transgenic Arabidopsis plants expressing a gene coding for an insect antifreeze protein derived form *dendroides Canadensis*. Lines 270-23 and 270-33 are transformants that express the protein intracellularly while 340-29, 340-30, and 340-34 have a signal peptide that allows for extracellular secretion of the protein.

## 5. SUMMARY

The factors that determine when and to what extent a plant will freeze are complex. In herbaceous plants, it appears that extrinsic nucleating agents play a key role in initiating ice formation. Ice-nucleating-active bacteria and moisture are two major extrinsic agents, however, their influence on the freezing process can be moderated by the presence of natural or applied hydrophobic barriers. Evidence suggests that ice crystals formed on the surface of plants must physically grow into the interior of the plant in order to initiate freezing of the plant. This can occur through stomates, or cracks in the cuticle. Thick cuticles, as found on many evergreen plants, and the application of synthetic, hydrophobic materials appear to serve as effective barriers and can inhibit the effect of extrinsic ice nucleating agents by preventing moisture from collecting on the surface of the plant and/or inhibiting the growth of ice crystals into the interior of the plant. In this regard, the application of hydrophobic barriers may provide a new approach to frost protection.

The situation in woody plants is different than in herbaceous plants. In general, woody plants appear to possess native, intrinsic nucleating agents that are just as active as many extrinsic ice nucleating agents. The exact identification of the intrinsic nucleating agents in woody plants, however, is unkown. Despite the presence of internal nucleating agents that are active at warm temperatures, barriers exist in woody plants that inhibit growth of ice from older stems into primary, lateral appendages. This is important because many of tissues in woody plants that are frost sensitive are flowers and primary, elongating, shoot tissues that arise from buds attached to older stems. The barriers that prevent ice propagation into the lateral appendages are most effective when ice forms in the main stem tissues at warm temperatures (-1.0 to -2.5 °C). If significant supercooling occurs in the main stem prior to ice formation, the rate of ice crystal growth is so rapid that it easily penetrates any existing barrier between the main stem and lateral appendages. While the identification of the barrier is undefined, vascular continuity (as well as the size and amount of the vascular elements) are believed to play a key role.

Cold acclimation appears to influence the freezing response of plants. In general, acclimated plants will supercool to a greater extent than non-acclimated plants and also have the ability to lose water very rapidly, without being injured, when ice formation does occur. This can be true even when acclimated plants are supercooled to -10 to -15 °C. Evidence suggests that native antifreeze proteins can affect the freezing process in plants and also that plants transformed to express insect antifreeze proteins will supercool to a greater extent than wild-type plants.

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