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Factors involved in ice nucleation and propagation in plants: an overview based on new insights gained from the use of infrared thermography

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SUMMARY

The use of infrared thermography has revealed new details about the freezing process in every plant species in which it has been used. Thus far, it has indicated several new possibilities for enhancing frost protection. Development of thicker cuticles, or providing hydrophobic barriers, may provide a method of blocking extrinsic ice propagation. Selecting for the presence of barriers to ice propagation in woody plants, that allow expanded flowers and inflorescences to supercool despite the presence of ice in woody stems, may also provide a method for enhancing cold hardiness during spring frosts. Finally, evidence has been obtained supporting the role of antifreeze proteins in enhancing supercooling in plants. In this report we summarize previously published data and present the results of current ongoing research.

Key words: antifreeze proteins, frost protection, ice nucleation, ice propagation, infrared thermography, plants.

YFIRLIT

Þættir sem hafa áhrif á myndun og dreifingu ískristalla í plöntum: yfirlit byggt á nýrri þekkingu sem aflað hefur verið með innrauðri hitamyndatöku

Notkun innrauðra hitaljósmynda hefur opnað mönnum sýn á smáatriði í frostferli í öllum þeim plöntutegundum sem prófaðar hafa verið. Þessi þekking bendir til möguleika á að auka varnir gegn frystingu. Þróun þykkari yfirhúðar eða myndun vatnsfælinna hindrana geta stöðvað ísmyndun utan frá. Val á hindrunum fyrir dreifingu á ís í trjákenndum plöntum sem þola undirkælingu blómskipana, þrátt fyrir ís í stofninum, geta leitt til aukins þols gegn vorfrostum. Loks hafa komið fram vísbendingar um að frostþolsprótein auki undirkælingu plantna. Í greininni er gefið yfirlit yfir fyrri niðurstöður og greint frá rannsóknum sem unnið er að.

INTRODUCTION

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 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 extent (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 (Lindow *et al.*, 1978; Fuller *et al.*, 1994; Andrews *et al.*, 1984; Gross *et al.*, 1988) or bacterial (Gross *et al.*, 1984; 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, however, 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 condi-

tions the reduction or interference of extrinsic ice nuclei would provide significant frost protection.

Recently the ability to use infrared video thermography (Figure 1) to directly observe ice nucleation (i.e., initial ice formation) and propagation in plants has been demonstrated (Carter *et al.*, 1999; Ceccardi *et al.*, 1995; Fuller and Wisniewski, 1998; LeGrice *et al.*, 1993; Wisniewski *et al.*, 1997; Wisniewski, 1988; Wisniewski and Fuller, 1999; Workmaster *et al.*, 1999). The use of this technology to study the freezing process is based on the fact that ice formation is an exothermic event and the release of the heat of fusion as water changes phase from a liquid to a solid can be monitored and visualized. 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.

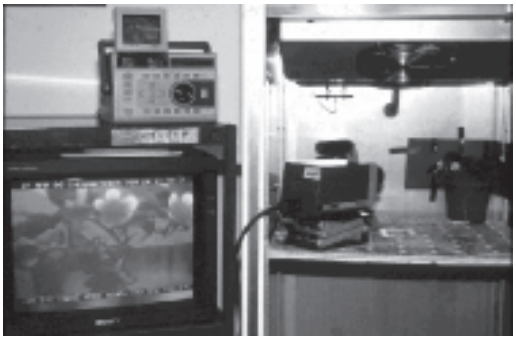


Figure 1. Use of infrared thermography to study freezing in plants. Infrared camera (Inframetrics Model 760 Infrared Radiometer) is set up inside an environmental chamber along with a potted strawberry plant. Control unit sitting on top of a video monitor is used to adjust the camera parameters while the experiment is in progress. The entire experiment is videotaped for future evaluation.

1. mynd. Notkun innrauðrar hitaljósmyndunar til rannsókna á frostferli í plöntum. Innrauð ljósmyndavél (Inframetrics Model 760 Infrared Radiometer) er komið fyrir inni í frystiklefanum, ásamt jarðarberjaplöntu í potti. Stýribúnaður ofan á myndbandsskjá er notaður til að stilla breytur myndavélarinnar á meðan á tilrauninni stendur. Öll tilraunin er tekin upp á myndband og árangur metinn síðar.

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. 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.

ROLE OF THE CUTICLE AND STOMATES IN EXTERNAL INDUCTION OF FREEZING

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 (e.g. cracks or broken hair cells) or through a stomatal opening (Figure 2). A thick cuticle, such as found on evergreen leaves (e.g. azalea, cranberry), serves as an effective barrier to external nucleation (Wisniewski and Fuller, 1999; Workmaster *et al.*, 1999). Wa-

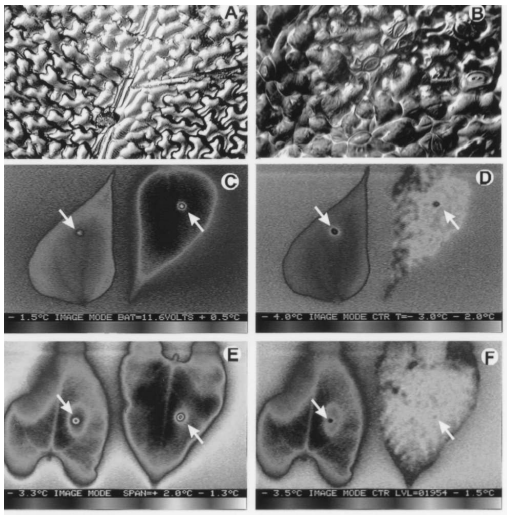


Figure 2. Top panel: Upper (top left) and lower (top right) surface of a bean (*Phaseolus vulgaris*) leaf showing the presence of stomates on the abaxial surface. Middle panel: Infrared images of bean leaf with drops of water containing ice-nucleation-active (INA) bacteria (*Pseudomonas syringae*, strain Cit7). On the left, drops have frozen but leaves have not, while on the right the leaf with the frozen droplet on the abaxial surface has frozen but the leaf with the frozen droplet on the adaxial surface remains unfrozen. Bottom panel: Infrared images of abaxial surface of bean leaves with drops of water containing INA bacteria on the surface. The leaf on the left has a layer of silicone grease under the droplet of INA bacteria. On the left, droplets have frozen but leaves have not while on the right, the leaf with the silicone grease subtending the droplet has not frozen while the leaf with only the droplet of INA bacteria has frozen. This indicates that ice must physically grow through a stomate or crack in the cuticle in order to initiate freezing of the leaf. (Reprinted from Wisniewski and Fuller, 1999, with permission).

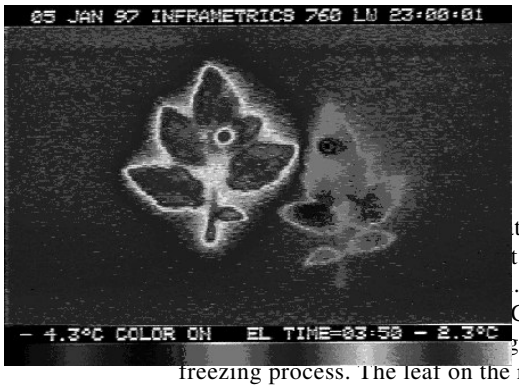
*2. mynd. Efsta röð: Efra borð (til vinstri) og neðra borð (til hægri) laufblaðs baunaplöntu (*Phaseolus vulgaris*) sýnir loftauga á ásfrálægu yfirborði. Miðröð: Innrauð mynd af baunablaði með vatnsdropa sem inniheldur ískristallamyndandi (INA) bakteríu (*Pseudomonas syringae*, stofn Cit7). Vinstra megin hefur dropinn frosið, en laufblaðið ekki, og hægra megin hefur laufblaðið á ásfrálæga yfirborðinu frosið, en laufblaðið á ásaðlæga yfirborðinu er ófrosið. Neðsta röð: Innrauð mynd af ásaðlæga yfirborðinu með dropa með ískristallamyndandi bakteríu á yfirborðinu. Laufblaðið til vinstri er með lag af silikonfeiti undir dropanum. Til vinstri hafa droparnir frosið, en laufblaðið ekki, og hægra megin hefur dropinn á silikoninu ekki frosið, en dropinn sem bara hefur ískristallamyndandi bakteríu er frosinn. Þetta sýnir að ísinn hlýtur að vaxa gegnum loftaugun eða sprungur í yfirhúðinni til að koma af stað frystingu í laufblaðinu (endurprentað eftir Wisniewski og Fuller, 1999, með leyfi).*

ter 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 by the growth of ice through a stomatal opening on the abaxial surface. In 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. To test the hypothesis that a hydrophobic barrier can prevent plants from freezing, an emulsion of hydrophobic kaolin (Englehard, Inc.) was applied to the surface of leaves (Figure 3) or whole 4–6 week old tomato plants prior to application of an extrinsic nucleating agent (Cit7 strain of Ina$^+$ *Pseudomonas syringae*). Results indicated 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 the plants to supercool to –6°C, despite the presence of frozen droplets on leaf surfaces.

THE INITIAL FREEZING PROCESS AND SUBSEQUENT ICE PROPAGATION

Ice nucleation, when it occurs at warm temperatures in herbaceous plants, is a two step process. In the first step, only water that is present along the surface of cells is induced to freeze. This can be seen as a small exothermic response after which the plant tissue quickly

cools back down to ambient temperatures. In the second stage, which can often be distinctly separated from the first step, the extracellular ice induces a much more substantial freezing event where bulk water in the xylem conducting elements freezes, water is drawn out of cells and freezes extracellularly. This is seen as a substantial exothermic event that persists for an extended period of time. This second freezing event is easily propagated throughout the rest of the plant. Ice does not, however, move down through a stem into a below-ground portion of a plant and then back up into another above-ground portion of a plant. For example, ice does not propagate down a potato stem into a tuber and then back up another stem (Fuller and Wisniewski, 1998). If ice formation occurs at a warm temperature, appendages attached to the organ that initially froze may escape freezing for a period of time, indicating that some barriers to ice propagation do exist. The two steps of the freezing process become superimposed on each other if freezing is initiated after a significant amount of supercooling has occurred.



to (*Lycopersicon*
t has been coated
. The leaf on the
C and is warmer
given off by the
freezing process. The leaf on the right is unfrozen at –3.8°C despite the presence of a frozen droplet of INA bacteria (round dot) on its surface. This indicates that hydrophobic films may represent a method of frost protection.

*3. mynd. Innrauð mynd af tómatlaufblaði (*Lycopersicon esculentum*). Blaðið til hægri hefur verið þakið með vatnsfælinni húð. Laufblaðið til vinstri hefur verið frosið til –2,3°C og er heitara en umhverfið vegna hitagjafar við frystinguna. Blaðið til hægri er ófrosið við –3,8°C, þrátt fyrir frosinn dropa með ískristallamyndandi bakteríu (kringlóttur punktur) á yfirborðinu. Þetta sýnir að vatnsfælin himna getur verndað gegn frosti.*

FREEZING OF WOODY STEMS AND BARRIERS TO ICE PROPAGATION

As previously documented, 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 the 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.*, 1999; Workmaster, 1999). These barriers are most effective if the initial freezing event occurs at a relatively warm temperature. These 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.

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. When cellular extracts of canola were placed on filter discs, similar responses to those of intact plants were made, indicating that sugars and proteins present in acclimated plants may play a role in enhancing supercooling. Transgenic Arabidopsis plants expressing an insect antifreeze protein also exhibited an enhanced ability to supercool (Tao

et al., 2000). Interestingly, 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 acclimated plants have the ability to rapidly lose water in order to prevent intracellular ice formation. Distinct differences between acclimated and non-acclimated rye plants have also been observed that may be attributed to the presence or absence of antifreeze proteins.

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