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MEASUREMENT AND CONTROL

A 50

Measurement and Control

With contributions by
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Genetically Engineered Microorganisms to Rescue Plants from Frost Injury

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Ice nucleation active bacteria belonging to genera *Pseudomonas*, *Xanthomonas* and *Erwinia* contribute to frost damage to plants by initiating the formation of ice in plants that would otherwise supercool and avoid the damaging ice formation. The biological control of frost injury can be achieved by the application of non-ice nucleation active bacteria to the plant surfaces before they become colonized by Ice$^+$ species. *ice* genes have been cloned from *Pseudomonas* and isogenic Ice$^-$ derivatives constructed via genetic manipulations. These genetically engineered microorganisms (GEMs) have been released into the environment to control the frost damage. The incidence of frost injury to the plants has, thereby, been reduced by 50–85% during natural frosts. These GEMs do not survive in soil and show no aerial dispersal in the environment.

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1 Introduction

The use and release of genetically engineered microorganisms (GEMs) into the environment has become a tenable proposition in the last decade. Such organisms include both those produced by recombinant techniques as well as those derived by intensive selection of native species [1]. A variety of uses of GEMs has been identified in crop production and protection, degradation or sequestration of environmental pollutants, extraction of metals from ores, industrial bioconversions and the production of enzymes [2]. Recently, GEMs have been successfully exploited to control frost injury to plants [3]. The ability of some species of Gram negative bacteria and lichens to nucleate the crystallization of water at low temperature demonstrates a unique type of manipulation of the environment by microorganisms. Such microorganisms are major ice nucleating agents found on the leaves and flowers of many plants and initiate much of damage done to crops by frost besides having a role in the rain cycle and in freeze texturing food [4–7]. Much attention has recently been paid to the role of ice nucleating activity in the process of plant damage by *Pseudomonas syringae* since ice formation is a prerequisite for many diseases induced by this bacterium.

The various parameters of Ina (ice nucleation active) bacteria such as their physiology, biochemical and genetical characteristics as well as their ecology have been studied in detail in recent years [8–10]. The molecular basis of ice nucleation has also been worked out. The ice nucleating activity in different microorganisms is conferred by a single structural gene. GEMs have been constructed by manipulating this gene and isogenic mutants (Ice^- or Ina^-) are currently being used to control frost injury to plants [11]. In the present article, the bacterial induction of ice nucleation, the extent of frost damage to plants and its control by GEMs under field conditions are discussed.

2 Frost Injury in Plants

Frost damage is one of the major cause of crop loss in temperate and subtropical regions and low temperature has been reported as being the most limiting factor to natural plant distribution [11a]. Frost sensitive plants are distinguished from frost tolerant plants by their relative inability to tolerate ice formation within their tissues. Tolerant plants can do so thereby avoiding the frost damage [12–15] which occurs to sensitive plants between -2 to $-5\,^{\circ}C$ under natural conditions [16–18]. At these temperatures, ice formed from super-cooled water propagates throughout the tissue system (inter- and intra-cellularly) in such plants and results in frost damage. In the absence of site capable of ice nucleation, the water in the plants can supercool and freezing will not occur until the temperature becomes low enough to allow plant components to catalyze crystallization of supercooled water.

The frost damage is mostly prevalent in temperate regions and to some extent in sub-tropical areas during winter and spring seasons. The severe damage occurs to foliage and flowers in field and horticultural crops. Such damage at the time of budding and flowering in pear, peach, almond and other crops not only reduces yield but also predisposes the development of other diseases such as dieback, canker etc. [8, 9, 19, 20]. Even shoots and stems of trees like pine and eucalyptus and crop plants such as papaya, mango, wheat, potato, tomato etc. become susceptible to such injury. Most of the frost sensitive plants have no mechanism for frost tolerance or for recovery from damage, thus they must be protected from ice formation to avoid frost injury [12, 14, 15].

3 Bacterial Induction of Ice Formation

Although ice melts at 0 °C, water does not necessarily freeze at this temperature. Instead, it can be supercooled to much lower temperatures (as low as − 40 °C) before freezing occurs [21, 22]. This is because the initiation of ice crystallization depends on the presence of ice nuclei – the particles of critical size and specific shape that allows formation of an ice lattice around them. In pure water, ice nuclei can be created only by chance orientation of water molecules. However, in the presence of some contaminants in water, freezing occurs at higher temperature. For instance, dust particles can raise the freezing temperature to − 10 °C and mineral particles such as silver iodide can cause ice nucleation at − 8 °C [23]. However, the members of bacterial genera such as *Pseudomonas*, *Erwinia*, *Xanthomonas* and a few lichen of the genera *Rhizoplaca*, *Xanthoparmelia*, *Xanthuria* are most active ice nucleating agents [24–29].

Most phyllosphere colonizing organisms show ice nucleation activity at temperatures warmer than − 15 °C [3, 30, 31]. However, seven bacterial species namely *Pseudomonas syringae* [24, 32]; *Pseudomonas fluorescens* [3, 26, 38]; *Pseudomonas viridiflava* [3, 38]; *Erwinia herbicola* [33]; *Erwinia ananas* [35, 122]; *Erwinia uredovora* [35] and *Xanthomonas campestris* pv. *translucens* [34] are active in ice nucleation in vitro as well as on the plant surface at temperatures warmer than − 1.5 °C or when the temperature falls below zero as may happen in early spring or autumn (Table 1). The plant may supercool but the capacity to supercool is reduced in the presence of Ina bacteria in the phyllosphere which increases the frost injury to the plant. There is direct correlation between frost injury and population of epiphytic Ina bacteria. The Ina bacteria occur in several habitats including water, plant surfaces and in the environment above the plant [24, 36, 37, 84]. Many strains of these colonizers are either epiphytic saprophytes or conditional plant pathogens. The most ubiquitous Ina bacterium *P. syringae*, a pseudomonad pathogenie to liliac, is a common harmless commensal epiphyte for most other plants from widespread geographic locations [6, 24, 38, 39]. Of the 44 pathovars (host range variants) of *P. syringae* known so far, about 50% are active ice nucleators [38–40].

Table 1. List of micro-organisms involved in ice nucleation at temperatures warmer than − 1.5 °C and the corresponding host plants

Sr. No.	Micro-organisms	Host plants	Reference No.
A. Bacteria			
1.	*Pseudomonas syringae*		
	pv. *syringae*	Tomato, beans, corn	38, 51, 112, 117
	pv. *pisi*	Pea	38, 51
	pv. *coronafaciens*	Oats	38
	pv. *lachrymans*	Cucumber	38
	Other epiphytic strains	Cereals, corn, soyabean, beans, potato, tomato, hairy vetch and some other vegetables, clover lilae, strawberries, citrus, coniferous trees, olive, pear, peach, cherry, almond, apricot and other deciduous fruit trees.	5, 18, 19, 32, 33, 86, 110, 111, 112, 118
2.	*P. fluorescens* (epiphytic)	Pine and some forest plants, some cereals, vegetables and fruit crops etc.	3, 38, 61, 76, 116
3.	*P. viridiflava* (epiphytic)	– do –	3, 38, 121, 122
4.	*Erwinia herbicola* (epiphytic)	Corn, cereals, sugarcane, clover, timothy, perennial ryegrass and other grasses, vegetables, fruits and flowers of some deciduous plants etc.	17, 89, 104, 113, 114
5.	*E. ananas* (epiphytic)	Vegetable crops	122
6.	*E. uredovora* (epiphytic)	Strawberry	35
7.	*Xanthomonas campestris* pv. *translucens*	Rice, wheat, corn, oat, various vegetable crops etc.	25, 104
B. Lichens			
1.	*Rhizoplaca*	–	28
2.	*Xanthoparmelia*	–	29
3.	*Xanthuria*	–	29

The ice nucleation frequency (the ratio of the number of ice nuclei to the number of bacterial cells capable of ice nucleation) on plant varies from 10^{-8} to 10^{-1} with an average frequency of 10^{-3} [41–45]. One ice nucleus is sufficient to cause ice formation and thereby frost damage to the entire leaf [43]. Thus, the disease basically abiotic in nature becomes biotic at temperatures warmer than − 1.5 °C when the Ina bacterial population exceeds a threshold number of about 10^3 g^{-1} fresh weight of plant tissue or 10^{12} cfu (colony forming unit) cm^{-2} of leaf surface [44, 46]. Detection of Ina bacteria under laboratory conditions has been reviewed earlier [47]. The Ina bacterial colonies are detected by replica freezing of leaf material on laboratory media at − 5 °C, followed by a fine mistspray of ice-nucleation-free water on the surface. The Ina^+ colonies appear as discrete frosty white areas of ice [48–49]. Measurement of the supercooling point of leaves by the tube nucleation assay is also predictive of plant frost sensitivity under field conditions [43, 48].

The rate of nucleation at a particular nucleating site increases drastically over a narrow range of temperatures [21]. It is, therefore, essential to consider a point in this range to know the threshold temperature at and below which the sites become active in nucleation. Each particle that can nucleate ice crystallization has its own characteristic threshold temperature. The ice nucleation sites formed by bacteria vary in their threshold temperature from $-2.5\,^{\circ}C$ to $-14\,^{\circ}C$. Only an extremely small fraction of cells in the culture show ice nucleation activity at various temperatures above $-5\,^{\circ}C$. At temperatures below $-10\,^{\circ}C$, essentially all cells exhibit nucleation activity. A plot of the number of cells capable of nucleation versus temperature gives a cumulative nucleus spectrum [21]. On the basis of cumulative nucleus spectra three types of nucleating activity have been differentiated viz. cells active at $-5\,^{\circ}C$ or warmer designated as type I; $-5\,^{\circ}C$ to $-8\,^{\circ}C$ as type II; and $-8\,^{\circ}C$ or below as type III activity [33, 50]. Subsequently, this classification was modified on the basis of nucleus spectrum, sensitivity of pH, water miscible solvent and has been designated as class A, B and C type nucleating activity [51]. The class A structure is formed on only a small fraction of cells in the culture, nucleate water at a temperature of $-4.4\,^{\circ}C$ and is an effective nucleator of D_2O. A second class of structure called class B is formed on a large proportion of cells, nucleate water between $-4.8\,^{\circ}C$ to $-5.7\,^{\circ}C$ and is a relatively poor nucleator of supercooled D_2O. The class C structure is formed on almost all the cells and nucleates at $-7.6\,^{\circ}C$ or cooler. These three classes are also differentiated by their sensitivity to low concentration of dioxane and dimethyl sulfoxide. The addition of these solvents lowers nucleation activity by 1000-fold or more in class A while in classes B and C, it is lowered by 20–40% and 70–90%, respectively.

4 Genetics of Ice Nucleation

A single gene is responsible for the Ina^+ phenotype in each of the bacterial species. This gene has been designed as *ice* E, *ice* C (*ina* Z) and *ina* W in *E. herbicola*, *P. syringae* and *P. fluorescens*, respectively [52–54]. The Ina proteins from different species show a high degree of homology indicating that these genes have descended from a common ancestral gene.

The involvement of the *ina* gene product in nucleating activity has been shown by deletion, mutagenesis or cloning studies [55–56]. Transfer and expression of this gene into other Gram negative hosts has confirmed that each of the known genes is sufficient for the Ina^+ phenotype. Each *ina* gene contains three orders of internal repetition. The first order repeat is eight codons in length. The two first order repeats make up a second order repeat of 16 codons. A triplet of second order repeat make up the third order repeat of 48 codons [8, 53]. Translation of the consensus of 48 codons repeat gives the following peptide: AGYGST-TAG-SSLIAGYGSTQTAG-S-LT-AGYGSTQTAQ-S-LT.

In most species this repetition is perfect throughout the length of gene while in others, it becomes imperfect. However, deviation from the perfect repetition is similar in each gene suggesting that the pattern of imperfection is functional.

The *ina* genes of different bacterial species encode for a single protein of 150–180 kDa [57]. This protein is found in low quantities, and forms about 0.01% of the total membrane protein [58]. However, this small amount of protein is sufficient to confer Ice^+ phenotype against all heterogenic backgrounds tested [53, 55]. The Ina proteins have been identified in Ina^+ transformants of *E. coli* using antibodies directed against Ina W protein [59] and a synthetic peptide homologous to part of the predicted amino acids sequence responsible for the nucleation activity [60].

5 Structural Basis for Ice Nucleation in Bacteria

The bacterial ice nucleation phenotype is very sensitive to heat (74 °C), pH, proteases, 2-mercaptoethanol, urea, SDS and heavy metals [26, 61, 62] indicating that a protein is required for ice nucleation activity. Ice nucleation activity of *P. syringae* and *E. herbicola* is also partially sensitive to membrane perturbant compounds such as borate or lectins; and the membrane structure changes associated with lysis by a virulent phage [63]. For a bacterium to nucleate ice formation in external super-cooled water, one would expect that the nucleating structure must be freely exposed and should be exterior to the cell. The bacterial ice nucleating site may be localized in the outer membrane of the source organism [37]. Subsequently, it was found that it may be a membrane-bound protein aggregate [4]. Ice nucleation activity has been enriched by fractionation of bacterial membranes in subcellular particles [4, 37]. Isolated outer membranes of *P. syringae*, *E. herbicola* and *E. coli* HB 101 containing a cloned *ice* gene show ice nucleation activity [55]. Further, membrane vesicles shed off from strains of Ina bacteria also show ice nucleation activity [50]. However, it is not true in *P. syringae* since the product of the *ina* Z gene is localized both in the outer and inner cell membrane.

Bacterial Ina proteins require lipids for their activity as the delipidation of the membrane abolished ice nucleation activity, which was then reconstituted by addition of specific lipids [63, 64]. But, lipopolysaccharides (LPS) that occur exclusively in the outer membrane are not required for ice nucleation activity [58]. The removal of LPS up to 78% did not reduce the ice nucleation activity significantly. The hydrophobic environment supplied by phospholipids greatly favours both the assembly and proper orientation of the membrane protein complex. From the different classes of phospholipids, phosphatidyl inositol (PI) which constitutes 0.1 to 1.0% of total phospholipids in Ice^+ bacterial strains is essential for the Ina protein of membranes. The Ice^- *E. coli* strains contained traces of PI that amount to 2–30% of the level found in the Ice^+ transformed

E. coli. The relative ice nucleation activity at $-4\,^{\circ}C$ was proportional to PI content. It was, therefore, concluded from these studies that PI plays an important role in ice nucleation at warm temperatures and is a likely component of ice nucleating sites [65]. However, overproduction of Ina proteins in *E. coli* results in the accumulation of a large number of nucleating sites in the inner membrane. It appears that assembly of nucleating sites requires only common lipids and may take place on the inner membrane prior to transport to the outer membrane. No detectable signal peptide for the translocation of Ina proteins is present. However, the mechanism of translocation of the protein is still not fully understood. It is probable that Ina proteins utilize signal peptides with unusual sequences. The three orders of periodicity in the Ina proteins probably reflect the hierarchy of the three motif of structural repetition. It is possible to construct a model matching the hexagonal symmetry of ice by virtue of the 2×3 organisation of the repeat sequences.

Cell nucleating activity is proportional to the size of the nucleating structure [53, 63]. The target size of the ice nucleation structure is roughly proportional to the ability of cells to nucleate at the warmest temperature [58]. A non-linear but positive relationship between the concentration of the *ina* gene product and the ice nucleation activity of a bacterial culture has been reported [66]. Larger aggregates of interacting ice nucleation proteins are required at warmer temperatures. However, there is no evidence of chemical heterogeneity between structures active at different temperatures. The cross-sectional area of a nucleation site (i.e. the size of target it represents) can be inferred from the dosage of γ-radiation required to inactivate it. Assuming that a single hit is sufficient for inactivation of an Ina site, it has been concluded that nucleation sites, with a $-12\,^{\circ}C$ threshold possess an average molecular size of approximately 150 kDa and a nucleation at $-3\,^{\circ}C$ required a structure with an apparent size of 700 kDa [64]. Nucleating sites with a higher threshold required smaller radiation doses for inactivation which results in the estimation of a much larger molecular size (19 000 kDa) for nucleation sites with a threshold above $-3\,^{\circ}C$. However, the large size estimated for nucleation sites with a $-3\,^{\circ}C$ threshold from theoretical predictions demonstrate that some bacterial ice nucleating sites are considerably larger than a single molecule of Ina protein. A plausible model is that of co-operative homo-aggregates of Ina proteins, embedded in and stabilized by a lipid membrane [8]. This model is supported by the fact that nuclei which are active at warmer temperatures are assembled more slowly [67].

6 Construction of Ice$^-$ GEMs

Genomic libraries of *P. syringae* and *E. herbicola* have been constructed. This greatly facilitated the manipulation of genes responsible for the Ice$^+$ phenotype. Expression of cloned *ice* genes have been observed in the heterogenous Gram

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