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Expression of a Bacterial Ice Nucleation Gene in Plants[1]

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ABSTRACT

We have introduced an ice nucleation gene (*inaZ*) from *Pseudomonas syringae* pv. *syringae* into *Nicotiana tabacum*, a freezing-sensitive species, and *Solanum commersonii*, a freezing-tolerant species. Transformants of both species showed increased ice nucleation activity over untransformed controls. The concentration of ice nuclei detected at −10.5°C in 15 different primary transformants of *S. commersonii* varied by over 1000-fold, and the most active transformant contained over 100 ice nuclei/mg of tissue. The temperature of the warmest freezing event in plant samples of small mass was increased from approximately −12°C in the untransformed controls to −4°C in *inaZ*-expressing transformants. The threshold nucleation temperature of samples from transformed plants did not increase appreciably with the mass of the sample. The most abundant protein detected in transgenic plants using immunological probes specific to the *inaZ* protein exhibited a higher mobility on sodium dodecyl sulfate polyacrylamide gels than the *inaZ* protein from bacterial sources. However, some protein with a similar mobility to the *inaZ* protein could be detected. Although the warmest ice nucleation temperature detected in transgenic plants is lower than that conferred by this gene in *P. syringae* (−2°C), our results demonstrate that the ice nucleation gene of *P. syringae* can be expressed in plant cells to produce functional ice nuclei.

Considerable evidence indicates that some hardy plants can attain their hardiness potential only if ice formation occurs at warm subzero temperatures before extensive supercooling occurs. Both hardy and nonhardy plant species can supercool extensively because most plants have no internal ice-nucleating agents active at temperatures higher than −5°C (16). Nucleation at high subfreezing temperatures facilitates slow dehydration of cells in equilibrium with extracellular ice. Siminovitch and Scarth (30) reported that lethal intracellular freezing occurs in hardy cells that are allowed to supercool and that initiation of ice formation at temperatures close to 0°C favored cell survival. Olein (24) considered supercooling to be injurious to hardy plants because it promoted nonequilibrium freezing. Winter wheat crown tissue that froze after supercooling to −3°C resulted in death at higher temperatures than when freezing was initiated at temperatures just below 0°C (10). Rajashekar et al. (28) found that *Solanum acuale* leaves from nonhardened and hardened plants frozen at −1°C could survive cooling to −5.5 and −8.5°C, respectively. However, the leaves were killed at the temperature of initial ice formation if freezing was initiated at or below −2 or −2.6°C in nonhardened and hardened plants, respectively. Similar behavior was observed in the survival of supercooled and nucleated *Prunus* flower buds (2, 3, 9, 27). In all of these studies, prevention of supercooling by initiation of ice formation at temperatures close to 0°C allowed hardy plants to survive to lower temperatures.

Ice formation at relatively warm temperatures leads to controlled propagation of ice through the apoplast (16). Water is drawn from the cells by a gradient of water potential, leading to an increase in solute concentration and a depressed freezing point within the cells. Alternatively, supercooling followed by spontaneous ice nucleation at colder temperatures cause tissues to freeze too rapidly for cell dehydration to occur. In this situation, ice penetrates cells, ruptures membranes, and causes cell death (21).

Ice nucleation active bacteria such as *Pseudomonas syringae*, *P. fluorescens*, *P. viridiflava*, *Erwinia herbicola*, *Erwinia ananas*, and *Xanthomonas campestris* pv. *vesicatoria* can catalyze ice formation at temperatures as warm as −2°C (18). These bacterial species were shown to be the predominant ice-nucleating agent on the surface of plants and the cause of frost injury in many plant species (18). The molecular basis for ice nucleation activity of *P. syringae* has been extensively investigated (5, 20, 33). Genes (*ice*, *ina*) encoding ice nucleation proteins in different strains of *P. syringae* have been cloned and structurally characterized (8, 25).

We have hypothesized that: (a) the efficient expression of ice nuclei in plant cells would reduce the degree of supercooling, and (b) the presence of extracellular ice nuclei in plant cells would increase the ability of freeze-tolerant plants to tolerate the freezing process. Because most plant cells do not contain internal ice nuclei, it would be desirable to introduce the genetic determinants of ice nucleation proteins into plant cells to enable at least a few cells in a tissue to contain ice nuclei active at high subzero temperatures. As part of our efforts to produce heterogeneous ice nuclei extracellularly in plant cells, we report here the stable introduction and functional expression of the *inaZ* gene into *Nicotiana*

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tabacum, a freezing-sensitive plant, and *Solanum commersonii*, a freezing-tolerant plant.

MATERIALS AND METHODS

Construction of pHIZ1, pHIZ5, pJIZ3, and pJIZ7

To facilitate the constructions described below, a 3.7-kb *Eco*RI fragment carrying the *inaZ* gene from *Pseudomonas syringae* without its promoter from plasmid pMWS10 (33) was first cloned in the vector pUC119 (22) digested with *Eco*RI. A derivative designated pUZ119 was selected that had the *inaZ* fragment in the appropriate orientation to be re-excised by digestion with *Bam*HI. The *Bam*HI fragment from pUZ119 containing *inaZ* was then cloned into the *Bam*HI site of pGSJ280 (4) to produce pJIZ3 and pJIZ7. The first plasmid has the *inaZ* gene oriented in the direction of the CaMV 35S promoter in the vector (sense orientation, Fig. 1) and the second in the reverse orientation. A similar cloning procedure using the vector pGSH160 (4) produced pHIZ1 (*inaZ* in the sense orientation relative to the pT2'promoter) and pHIZ5 (*inaZ* in the reverse orientation). The cloning procedures were according to Sambrook et al. (29). In both pJIZ3 and pHIZ1, the *inaZ* region is placed in the translational frame of the first ATG codon downstream from the 35S or pT2' promoter in the vectors.

Plant Transformation

pHIZ1, pHIZ5, pJIZ3, and pJIZ7 were transferred into *Agrobacterium tumefaciens* strain pGV2260 by triparental matings (6). Transformations of leaf tissues from *Nicotiana tabacum* cv Petit Havana SR1 and *Solanum commersonii* (PI458317) were made using the leaf disc method of Deblare et al. (4), except that the regeneration medium for *S. commersonii* was Murashige-Skoog medium (23) supplemented with 5 mg/L IAA and 5 mg/L zeatin (13). Regeneration media contained 100 μg/mL kanamycin to select for transformed plants. To determine whether single or multiple insertions had occurred in the *Nicotiana*$_1$ primary transformants, plants were selfed and the seeds (T_1 generation) were germinated on kanamycin-containing Murashige$_1$-Skoog medium. The ratio of kan^r to kan^s seedlings in the T_1 population was 3:1 for all transformants tested, indicating that they had been transformed at a single locus.

Northern Blot Analysis

Total RNA was isolated by the guanidine isothiocyanate/cesium chloride centrifugation method of Lee et al. (15). RNA (20 μg) was electrophoresed in 1.3% formaldehyde agarose gels (29), blotted onto nylon membrane (Zeta-Probe, Bio-Rad), and hybridized with ^{32}P-labeled probe using the protocol described by the manufacturer (Bio-Rad). A 3.7-kb *Bam*HI fragment from pUZ119 containing *inaZ* was used as a probe after random labeling as described (7). Membranes were exposed to x-ray film (Kodak, XAR-5) with an intensifier screen for 36 h at −70°C.

Western Blot Analysis

Proteins were extracted from plants by grinding leaves in liquid nitrogen, followed by the addition of 2 to 3 volumes of ice-cold extraction buffer consisting of 50 mM Tris pH 8.0, 2% SDS, 1 mM PMSF, and 5% 2-mercaptoethanol. Samples were then homogenized with a Polytron at setting 5 for about 1 min and centrifuged at 10,000*g* for 20 min at 4°C. The supernatant was then clarified by pouring through four layers of cheesecloth, and protein was precipitated with 5 volumes of acetone at −20°C overnight. The pellet was dissolved in Laemmli sample buffer (14), boiled for 10 min, and run on a 7.5% SDS-PAGE gel.

For blotting, SDS-PAGE gels were soaked for 5 min in transfer buffer (192 mM glycine, 25 mM Tris base, and 5% methanol) containing 0.1% SDS. Proteins were then transferred to nitrocellulose using a Bio-Rad Trans-Blot cell containing transfer buffer (see above). Gels were electroblotted for 2 h at 100 V with a water-circulating cooling coil following the method of Deininger et al. (5). Blots were probed with an antibody (DNAP code No. anti-Inab-R1-B4) raised against InaW. InaW is an ice nucleation protein from *P. fluorescens* (5) and has a high degree of homology to *InaZ*. Blots were then developed using the Vectastain ABC horseradish peroxidase kit (Vector Laboratories).

Ice Nucleation Assays

Three different quantitative assays were used to determine the ice nucleation activity of plant samples. Unless otherwise noted, all tissues were acclimated at 4°C for at least 48 h prior to the assay to maximize ice nucleation activity (unpublished data). The numbers of ice nuclei per unit mass of tissue active at a given temperature were determined by a droplet-freezing assay (19) as follows: Leaf tissue was ground thoroughly in 0.1 M sodium phosphate buffer, pH 6.7, or suspensions of protoplasts were isolated according to the method of Potrykus and Shillito and suspended in K3M medium (26). Forty 10-μL drops of a given dilution of tissue macerate or protoplast suspension were then placed on paraffin-coated aluminum foil. When ice nucleation activity was assessed at a single temperature, the foil was floated on a bath containing 70% ethanol that was maintained at −10.5°C. The number of frozen drops was recorded after about 3 min. When the concentration of ice nuclei active over a range of temperatures was desired, the foil was placed on a temperature-controlled aluminum block that was slowly cooled by circulating cold 70% ethanol through it. Thermal equilibrium of the foil and the block was ensured by using petroleum jelly as a heat conductor between these two surfaces. The cumulative number of drops that froze as the temperature was dropped from 0 to −15°C was recorded. The number of ice nuclei per unit volume of suspension was calculated according to Vali (32). The number of ice nuclei per unit mass of plant tissue was calculated by normalizing the number of ice nuclei per volume of suspension by the mass of tissue contained per unit volume.

The threshold nucleation temperature of intact leaves was measured using a tube nucleation assay (11). Culture tubes (25 mL) containing 10 mL of distilled water were precooled

to −9°C, and those that froze due to ice nuclei present in them or in the water were discarded. Individual *N. tabacum* leaves were rolled into individual ice nucleus-free tubes. The tubes were then submerged in a refrigerated bath circulating 70% ethanol. The temperature of the bath was dropped abruptly in 0.5°C decrements and the tubes were allowed to equilibrate for 20 min at each temperature. Frozen tubes were then counted and removed from the bath. Data were plotted on a Rankit scale (12) as the cumulative percent of tubes frozen as a function of assay temperature.

The threshold ice nucleation temperature of leaf disks was estimated by differential thermal analysis. Thermocouples were attached to small samples of leaf tissue (about 0.1 g fresh weight for *S. commersonii* or 0.5 g for *N. tabacum*) harvested from plants grown under sterile conditions. Samples were then wrapped in aluminum foil, placed in a freezer, and cooled at a rate of 5°C/h. Temperatures were monitored by routing thermocouple signals through an analog multiplexer board (EXP-16, MetraByte, Stoughton, MA) and into an analog to digital convertor (Das 8, MetraByte) to generate a logic level signal readable by a personal computer. The signal was then processed to convert microvolts to degrees Celsius. The temperature at which the first ice nucleation event occurred was determined as the coldest temperature to which the sample could be cooled before nucleation of the water, an exothermic process, caused transient warming of the sample.

RESULTS

The *inaZ* gene from *P. syringae* pv. *syringae* was successfully transferred into both *N. tabacum* cv SR1 and *S. commersonii* by *Agrobacterium*-mediated transformation. The *inaZ* gene was cloned into the *Bam*HI site of pGSJ280 (4) positioned downstream of the 35S promoter and in frame with the vector's ATG start codon to produce the plant transformation vector pJIZ3 (Fig. 1). A similar construct, pJIZ7, contained *inaZ* in the reverse orientation. Two other constructs, pJIZ1 and pJIZ5, were made by inserting *inaZ* in the sense and antisense orientation, respectively, into the transformation vector pGSH160 downstream from the pT2′ promoter. In *N.*$_1$ *tabacum*, a ratio of 3:1 kan^r to kan^s seedlings from the T_1 generation of all transformants was obtained, demonstrating that a single linkage group carried the gene for kanamycin resistance and, therefore, that *inaZ* was probably located at a single insertion site. Southern blot analysis of DNA isolated from these plants also revealed a single copy of the *inaZ* gene in all transformants studied (data not shown). Tobacco plants homozygous for *inaZ* were generated by selfing the transformants. Homozygous members of the T_2 generation were identified by their ability to produce only kan^r progeny. Only these plants were extensively investigated.

Northern blot analysis revealed that transgenic plants contained an RNA band of about 3.6 kb that hybridized to the *inaZ* DNA probe (Fig. 2). The size of the transcript corresponds closely to the size expected of the *inaZ* gene (Fig. 1). The *inaZ* mRNA was more abundant in the homozygous tobacco than *S. commersonii* (Fig. 2). Besides the 3.6-kb RNA species that was prominent in transformants of both species,

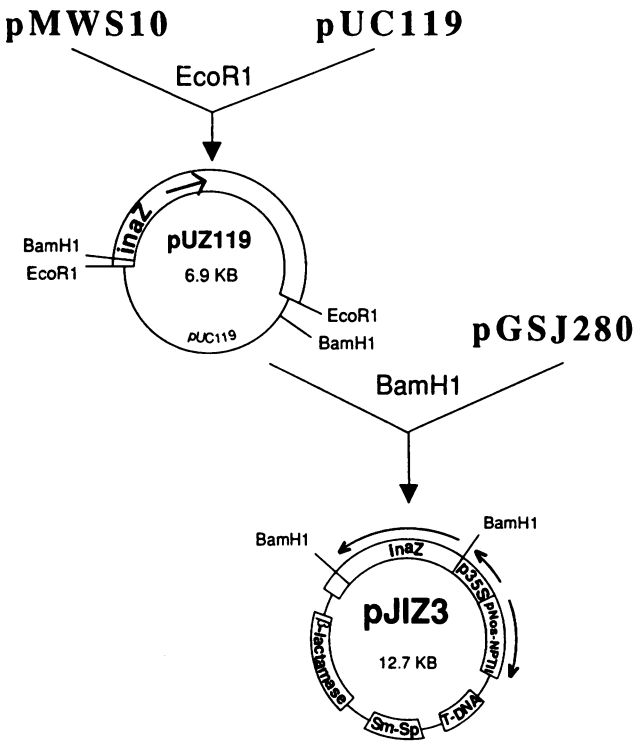

Figure 1. Construction of plasmid pJIZ3. The 3.7-kb *Bam*HI fragment of pUZ119 (see text) carrying the *inaZ* gene was cloned into the *Bam*HI site of pGSJ280 (18). In the pJIZ3 plasmid, the *inaZ* region is in frame with the vector ATG start codon.

there also was a considerable amount of smaller sized RNAs that hybridized to the *inaZ* probe.

InaZ protein was detectable by western blot analysis in transformed plants expressing ice nucleation activity (Fig. 3). Although a small amount of protein recognized by anti-*inaW* antibodies exhibited the same mobility on SDS-PAGE gels as the *inaZ* protein recovered from bacterial cells, the most prominent *inaZ* species isolated from *N. tabacum* migrated more rapidly than did the *inaZ* protein from bacterial sources (Fig. 3). More numerous but less abundant protein species with high mobility in SDS-PAGE gels were seen in preparations from both *N. tabacum* and *S. commersonii* transformants as well as from bacterial cells. These low mol wt proteins have been hypothesized to be due to degradation of the *inaZ* protein at regularly spaced sites in the repeated sequence of ice nucleation proteins (5).

Both *N. tabacum* and *S. commersonii* plants transformed with both pHIZ1 and pJIZ3 exhibited significantly higher ice nucleation activity than untransformed plants. The highest ice nucleation activity was observed with pJIZ3 transformants. Plants transformed with either pJIZ5 or pJIZ7, in which *inaZ* was in the reverse orientation relative to the relevant vector promoter, exhibited little ice nucleation activity and were indistinguishable from the untransformed controls with regard to this phenotype. Expression of the *inaZ* gene in transgenic plants increased the concentration of ice nuclei relative to that in control plants primarily at temperatures below −7°C (Fig. 4, A and B). Higher ice nucleation activity

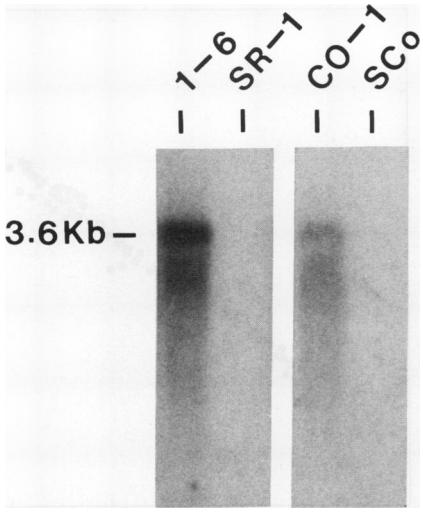

Figure 2. Northern blot analysis of RNA extracted from transgenic *N. tabacum* (1-6) and *S. commersonii* (CO-1) carrying *inaZ* and from untransformed control plants of each species (SR-1 and SCo, respectively).

was also detected in preparations of *N. tabacum* protoplasts isolated from transgenic plants than in those from nontransformed control plants (Fig. 5). Although no nucleation activity was detected in protoplasts from untransformed control tobacco plants at temperatures above −9°C, approximately 10% of the cells from plants transformed with pJIZ3 froze at this temperature. Approximately 1 transformed cell in 10^5 froze at temperatures as high as −4.5°C. Although no ice nucleation activity was detected in macerates of untransformed *S. commersonii* above −15°C, this species contained substantial numbers of ice nuclei active at colder temperatures (Fig. 4B).

Protoplasts isolated from transformed *N. tabacum* initiated ice formation at temperatures as high as −4.2°C, whereas untransformed controls showed no nucleation at temperatures above −9°C (Fig. 5). Differential thermal analysis of larger (approximately 50 mg) samples of transgenic leaf tissues also revealed threshold ice nucleation temperatures of about −4°C (Table I). Because the threshold nucleation temperature should increase with the mass of the tissue being tested (3, 18), we examined the warmest temperature at which nucleation occurred within entire leaves using a tube nucleation assay. The presence of the *inaZ* gene raised the highest threshold ice nucleation temperature in the plants whether this was measured in samples of small or large size. As for samples of smaller mass, the threshold ice nucleation temperature of whole transformed *N. tabacum* leaves was about −4°C (Fig. 6). In contrast, sterile leaf tissue from untransformed plants with masses as large as 40 g never exhibited ice nucleation events at this temperature using this assay.

Large differences were found in the amount of ice nuclei produced in different primary plant transformants. The 15 different primary transformants of *S. commersonii* tested exhibited a wide distribution of ice nucleation activity (data not shown). A similar range in ice nucleation activity was also observed in over 40 primary transformants of *N. tabacum* (data not shown). However, the most active transformants in each species had similar ice nucleation activity levels. The ice nucleation activity in transformed plants was influenced by gene dosage. For example, nucleation activity in diploid plants homozygous for *inaZ* was approximately 4 times that in heterozygous plants (Fig. 7).

DISCUSSION

The results of the various types of freezing assays clearly show that the *inaZ* gene was functionally expressed in two different higher plant species. The levels of ice nucleation activity measured at cold temperatures (i.e. below −9°C) was comparable to that of naturally occurring *P. syringae* pv. *syringae* on a per cell basis (17). However, there was a paucity of warm temperature ice nuclei (i.e. ice nuclei active above −5°C) in the transgenic plants compared to ice-nucleating bacteria (18). For example, in both plants (Fig. 5) and *Escherichia coli* (data not shown) carrying pJIZ3, about 10% of the cells contained ice nuclei active at −10°C. In contrast, less

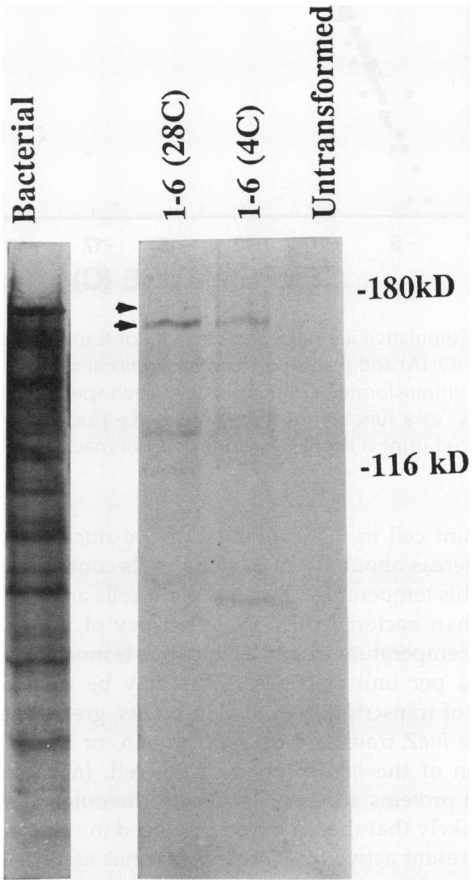

Figure 3. Western blot analysis of proteins extracted from untransformed *N. tabacum* plants and plants carrying *inaZ* (center and right lanes, respectively) and from *E. coli* pICE1.2 (16) (left lane). The top arrow indicates the position of the large dominant immunologically staining protein species in both *E. coli* and in transgenic *N. tabacum*, and the lower arrow indicates the predominant immunologically staining species in *N. tabacum* (1-6).

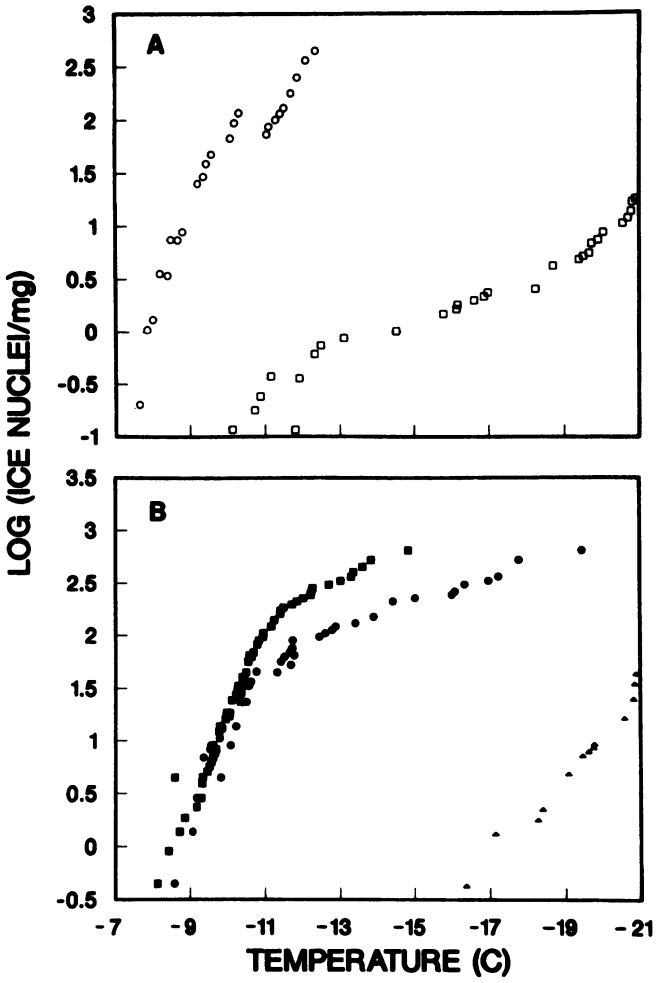

Figure 4. Cumulative ice nucleation activity of transgenic *N. tabacum* plants (O) (A) and plants of *S. commersonii* carrying *inaZ* (●, ■) (B), and of untransformed control plants of each species (□ and ▲), respectively, as a function of temperature. Ice nucleation activity was estimated using a droplet freezing assay of macerated tissue.

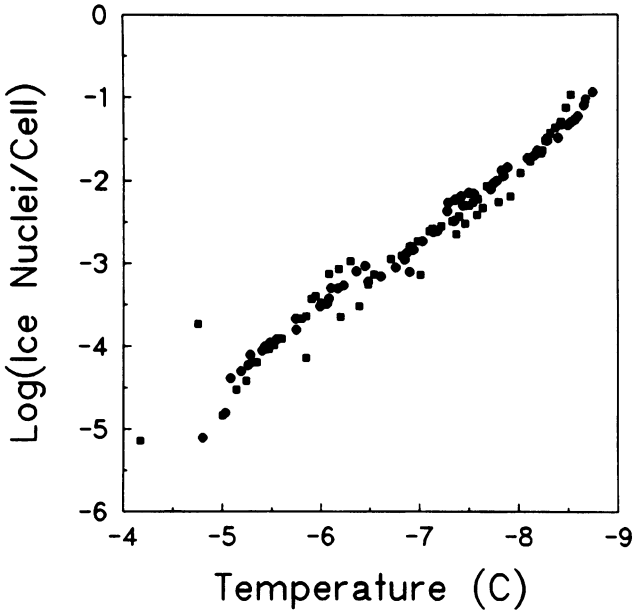

Figure 5. Cumulative ice nucleation activity of protoplasts isolated from two different transgenic *N. tabacum* plants that were homozygous for *inaZ*. Ice nucleation activity was determined by a droplet-freezing assay. No freezing events occurred in untransformed control plants at these temperatures; therefore, no control points were plotted.

than 1 plant cell in 10^5 contained an ice nucleus active at −5°C, whereas about 1% of bacterial cells contain ice nuclei active at this temperature. Because plant cells are much more massive than bacterial cells, the efficiency of expression of even cold temperature ice nuclei in plants is much lower than in bacteria per unit cell mass. This may be due to lower efficiency of transcription of *inaZ* in plants, greater degradation of the *inaZ* transcript or InaZ protein, or inappropriate localization of the *inaZ* protein in the cell. In bacteria, ice nucleation proteins are associated with the outer membrane (20). It is likely that the ice nuclei produced in our transgenic plants represent active *inaZ* protein that has associated with a membrane. However, it is not yet known what fraction of the *inaZ* protein is associated with the different membrane in the plant cell or whether different membranous associations are equal in the efficiency with which they allow ice nucleus assembly and function.

A broad range of ice nucleation activities were observed among a collection of primary transformants of *N. tabacum* and *S. commersonii* (data not shown). Variations in the expression efficiency of heterologous genes, presumably via differences in transcription rates, strongly depends on the location of insertion into the host genome (1). The frequency of occurrence of plants having relatively high ice nucleation activity (greater than about 100 ice nuclei/mg tissue) probably approaches zero asymptotically, when more primary trans-

Table I. *The Warmest Freezing Temperature, Determined by Differential Thermal Analysis, of Tobacco Plants Transformed with inaZ*

←, →, Relative orientations of the CaMV 35S promoter and the *inaZ* frame.

Construct	Plant	Warmest Freezing Temperature
		°C
$P_{35S} \rightarrow \rightarrow$ *inaZ* →[a] (pJIZ3)	1-6A:12[b]	−3.8 ± 0.9
	1-6A:56	−4.3 ± 0.7
	1-6A:24	−4.1 ± 0.9
	1-6A:61	−4.0 ± 0.5
	1-6A:38	−4.4 ± 0.7
$P_{35S} \rightarrow \leftarrow$ *inaZ*[c] (pJIZ7)	1-1:3	−11.6 ± 1.5
	1-2:10	−8.2 ± 0.8
	1-2:9	−11.9 ± 0.9
	SR1[d]	−10.0 ± 1.0

[a] Plants transformed with *inaZ* in sense orientation. [b] Each number is referred to an individual transformant. [c] Plants transformed with *inaZ* in antisense orientation. [d] Untransformed control plant.

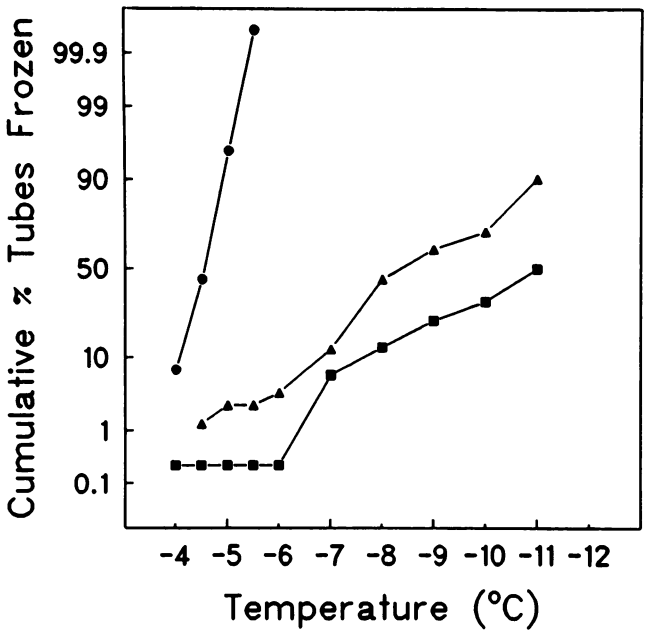

Figure 6. Cumulative percentage of detached leaves of transgenic *N. tabacum* (1-6) carrying *inaZ* (●) and from untransformed control plants (▲) and water-filled test tubes alone (■), which froze as a function of assay temperature. Data are plotted on a Rankitt scale.

formants are examined. Because we already tested substantial numbers of *inaZ* transformants, the likelihood of identifying plants with substantially higher ice nucleation activity than those characterized here by further screening of different primary transformants is low.

Ice nucleation activity was strongly influenced by the number of copies of the *inaZ* gene in transgenic plants. In bacteria, the relationship between *inaZ* protein concentration and ice nucleation activity can be expressed as:

$$\log_{10}(\text{ice nuclei/cell}) = 2(\log_{10}[\textit{InaZ} \text{ protein concentration}]) - 5$$

(17, 31). Based on this relationship, a doubling of protein concentration leads to an increase of about 4-fold in the ice nucleation activity. If we assume that the amount of InaZ protein in plants homozygous for *inaZ* is twice that of heterozygous plants having only one copy of *inaZ*, we should expect about 4 times more ice nuclei to be present. Ice nucleation activity in plants homozygous for *inaZ* was, in fact, approximately 4 times that in heterozygous plants (Fig. 7). This result suggests that our transgenic plants are capable of producing more ice nucleation sites than that observed here because the cells apparently are able to accommodate and assemble concentrations of *InaZ* proteins higher than that produced to date. Efforts to increase the transcription of *inaZ* or enhance translation of these transcripts should, therefore, lead to enhanced ice nucleation activity.The *inaZ* gene product was readily detected by western blot analysis of total cellular protein preparations from transformed tobacco plants (Fig. 3). A "ladder" of degradation products can be seen both in the plant and the bacterial lanes. This ladder has been speculated to represent successive removal of 2.6-kD peptide fragments from the highly repetitive ice nucleation proteins (5). Although a band comigrating with the bacterial *inaZ* can be distinguished in some blots, the most prominent high mol wt *inaZ* protein species extracted from the transgenic plant appear to be slightly reduced in size (by 10–20 kD) as compared to *inaZ* isolated from *P. syringae*. This suggests greater protein degradation in the plant than in the bacterial system. As a control, bacterial proteins were coextracted from untransformed or transformed plant tissues to ensure that the milieu of compounds released upon extraction of *inaZ* from plants did not contribute to degradation. The high mol wt *inaZ* protein of bacterial origin was always quantitatively recovered (data not shown), indicating that any degradation or modification of the *inaZ* protein in transgenic plants was not an artifact of extraction. It is possible that extraction of the *inaZ* protein from plant membranes is more difficult than from bacterial membranes. The low number of high mol wt *inaZ* protein from plant extracts may be a result of incomplete extraction of this protein when it is incorporated into plant membranes. Alternatively, posttranslational modification of the protein, such as the addition of lipids, could result in abnormally low mobility in PAGE. This possibility was not investigated in our study.

The demonstration that the *inaZ* gene from *P. syringae* can be functionally expressed in two different higher plant species opens the possibility of engineering a novel form of frost protection in plants capable of surviving warm-temperature extracellular ice formation. We have been able to increase the temperature of the warmest temperature freezing event in small plant samples from approximately −12 to −4°C and in intact leaves from about −7 to −4°C. The warmest temper-

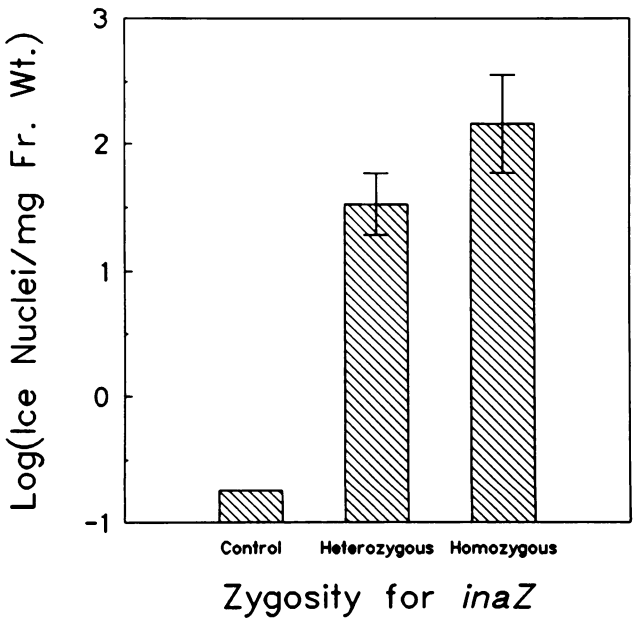

Figure 7. Ice nucleation activity of *N. tabacum* SR1 plants having zero, one, or two copies of *inaZ* (untransformed control, and heterozygous or homozygous for *inaZ*, respectively). Ice nucleation activity of macerated leaf was estimated using a droplet-freezing assay at −9.5°C.

ature of ice nucleation determines whether slow controlled extracellular freezing or uncontrolled lethal freezing will occur in plants. To maximize frost protection by escaping supercooling, the warmest temperature nucleation event must take place above −4°C (28). The *inaZ* protein is capable of nucleating ice in bacteria at this temperature. The warmest nucleation temperature in plant tissues can potentially be increased by increasing *inaZ* copy number, level of transcription, or transcript stability, by targeting the *inaZ* protein to cellular locations where it might be more stable, or by modifying the *inaZ* protein to a form(s) that might be more stable in plants. We are currently exploring some of these possibilities.

ACKNOWLEDGMENTS

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