In vitro freezing in microtitre plates applied to tobacco plants transformed with the inaZ gene of Pseudomonas syringae

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Received 11 December 2001; Accepted 7 June 2002

Abstract
High throughput assays have been developed to measure the ice nucleation activity of transgenic tobacco, Nicotiana tabacum L. cv. Petit Havana SR1 plants expressing the ice nucleation gene, inaZ, from Pseudomonas syringae at a young seedling stage, as well as in leaf tissue. Both assays are carried out in 96-well microtitre plates. The first assay involves direct seeding in vitro, one seed per microtitre plate well containing Murashige-Skoog agar. When seedlings reach the two-leaf stage, they are exposed to freezing temperatures by floating the plates on a circulating alcohol bath set at temperatures colder than –9 °C. The second assay involves placing small leaf discs individually in microtitre plate wells containing sterile distilled water. The assays complement each other, give highly reproducible results, are technically simple and enable the detection of freezing events in large numbers of plants. The utility and limitations of these assays are discussed.

Key words: Cold, freezing assay, ice nucleation, microtitre plates, Nicotiana tabacum.

Introduction
Plant freezing tests may be performed in the field, in controlled temperature chambers, or in vitro. Depending on the experimental material being used, freezing may be applied to whole plants, plant organs or tissues (e.g. leaves, flowers, shoot tips, leaf discs, calluses etc) (Lindow, 1982; Rajashekar et al., 1983) or to homogenized plant material (Ashworth and Kieft, 1995; Rajashekar et al., 1983). Environmental conditions in the field vary and are not predictable, which is why it is often impossible to reproduce a freezing assay under identical field conditions (Ashworth and Kieft, 1995). Freezing plants in the field is ultimately necessary, after there has been a first evaluation of the plant material in the laboratory or in a controlled temperature chamber. In the latter cases the rate at which the temperature drops may be controlled, allowing experiments to be repeated under almost identical conditions. Freezing assays differ not only in the way freezing is applied but also in the recording of plant freezing events, or the rating of freezing damage.

Various viability tests have been used over the years that measure the damage of plants subjected to low temperatures. A period of 5 weeks may be long enough for freezing-resistant plants to recover in certain cases (Steponkus, 1978). More sophisticated viability tests have been proposed, among them the electrolyte leakage (EL) assay (Dexter et al., 1932), the TTC (triphenyl tetrazolium chloride) assay (Steponkus and Lanphear, 1967), and the amino acid leakage assay based on ninhydrin-reacting substances (Wiest et al., 1976). Plant freezing events may be recorded using the Differential Thermal Analysis (DTA) (Quamme et al., 1972), acoustic emissions (Weiser and Wallner, 1988), in situ counting of electric conductivity (Prive and Zhang, 1996) or thermal imaging (Wisniewski et al., 1997).

The molecular basis of bacterial ice nucleation activity (INA) has been extensively investigated (Warren, 1995). Single, highly conserved genes have been cloned from several gram negative epiphytic or plant pathogenic bacteria. They encode large outer membrane proteins

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with many consensus repeats. Apart from bacteria, other organisms have been shown to possess ice nucleation properties (Ashworth and Kieft, 1995; Costanzo and Lee, 1995; Duman et al., 1995).

In an earlier study *Nicotiana tabacum* and *Solanum commersonii* plants were transformed with the ice nucleation gene, inaZ from *P. syringae*. These plants exhibited reduced supercooling capacity, which caused them to freeze at warmer subzero temperatures compared to the controls (Baertlein et al., 1992). In that study the ice nucleation activity of plant samples was determined by a droplet freezing assay of macerated leaf tissue or suspension protoplasts and by using a tube nucleation assay on intact leaves. Though suitable for small numbers of plant samples, these assays are not suitable for the detection of ice nucleation activity in large populations.

As part of a project aimed at determining the factors that influence the freezing behaviour of plants, high-throughput screening procedures were developed to distinguish susceptible (ice nucleation-active, INA+) from control (INA-) plants among the progeny of INA+ parental lines, by using seedlings grown directly in microtitre plates and, as a complementary test, leaf discs in microtitre plates filled with water.

**Materials and methods**

**Plant material: kanamycin test**

The ice nucleation gene inaZ of *P. syringae* had been introduced in *Nicotiana tabacum* cv. Petit Havana SRI plants at a single locus (Baertlein et al., 1992). T1 plants homozygous for the T-DNA insertion (Baertlein et al., 1992) were self-fertilized and their progeny (T2 seeds) was subjected to a kanamycin test by sowing on Petri dishes containing MS salts (Murashige and Skoog, 1962) solidified with 0.7% agar and supplemented with 500 μg ml⁻¹ kanamycin (Sigma K4378). The seeds were germinated at 25 °C for 16 h in continuous light (7 W m⁻²) and 8 h in the dark at 20 °C. Kanamycin-resistant (Km⁺) seedlings were counted after 10–15 d.

**In vitro freezing assays**

Non-contaminated seeds from plants homozygous and heterozygous for the inaZ gene (INA⁺, Km⁺) and non-transformed (INA⁻, Km⁻) plants were sown directly in 96-well microtitre plates (1 seed well⁻¹) (Falcon 3075) half-filled with MS agar medium (150 μl well⁻¹). From each plant 24 seeds were placed randomly in the microtitre plate wells. In addition, 24 wells were left without seeds to record the freezing of the MS medium. The plates were sealed with Parafilm™ and placed in the growth chamber set as described above. After 10–15 d the plates were removed and placed on the surface of a circulating alcohol bath (Lauda RM20) that was maintained at −9 °C. The bath temperature was lowered by one-degree steps every 30 min to −21 °C. The freezing of the seedlings was recorded, based on the change in opacity of the agar medium in the well, at every 1 °C temperature change.

For leaf freezing assays 24 leaf discs, 4 mm diameter, were cut from the lowest, well-developed leaf from each plant (leaves bearing signs of mechanical injury or possible infection were rejected). Leaves were rinsed in sterile distilled water prior to use. The leaf discs were individually placed at random in microtitre plate wells containing 150 μl sterile distilled water. The plates were sealed with Parafilm™ and freezing assay proceeded as described above.

**Results and discussion**

T1 plants were initially checked for zygosity by selfing. Homozygous and heterozygous lines were identified from the segregation of the Km⁻ trait among their progeny. Heterozygous seeds were obtained after crossing the homozygous T1 plants with non-transformed plants. Ice nucleation activity always co-segregated with Km⁻.

The freezing assay involving seedlings was carried out when the seedlings were at the two-leaf stage (≤0.5 cm tall) after direct seeding on microtitre plate wells filled with MS agar. An equal number of plate wells containing MS agar served as controls in order to monitor the nucleation activity of the medium throughout the temperature range employed in the assay. Wells containing only MS agar froze at much colder temperatures than those containing either INA⁺ and INA⁻ seedlings, indicating that freezing of the MS agar did not mask the seedling-freezing events (Fig. 1).

The freezing behaviour of non-transformed seedlings was also recorded and compared with that of homozygous and heterozygous transformed seedlings. Seedlings that were homozygous for the inaZ gene (lines 1–6) exhibited a distribution of freezing temperatures with a midpoint (50% frozen seedlings, T₅₀) at −10.8 °C, which is 6.2 °C warmer than that of the non-transformed seedlings. Seedlings that were heterozygous for the inaZ gene (progeny of 1–6 × SRI cross) froze with a T₅₀ of −13.2 °C, which is 2.4 °C colder than their homozygous counterparts (lines 1–6) and 3.8 °C colder than the non-transformed control seedlings (Fig. 1). None of the seedlings recorded as frozen recovered after the assay.
The leaf disc freezing assay proved to be more effective in distinguishing between transformed and non-transformed (control) plants (Fig. 2). As with the MS agar control, in the seedling assay, water-filled wells froze well below the freezing temperatures of wells with leaf discs. The standard errors in the leaf disc freezing curves are smaller than those in the seedling freezing curves, particularly for the transformed plants. By contrast to the seedling freezing assay, transformed plants, heterozygous and homozygous for the \textit{inaZ} gene had almost identical freezing curves in the leaf disc freezing assay \((T_{50} \text{ at } -12 ^\circ\text{C and } -11.5 ^\circ\text{C, respectively})\), whereas there was a marked difference, of 6.4 \(^\circ\text{C}\) and 6.9 \(^\circ\text{C}\), respectively, between them and the \(T_{50}\) of the control (non-transformed) plants (Fig. 2). Leaf discs of the tobacco primary transformants described by Baertlein et al. (1992) (considered comparable to the heterozygous line used here) contain 10–100 ice nuclei mg\(^{-1}\) active between \(-9 ^\circ\text{C}\) and \(-11 ^\circ\text{C}\) (Baertlein et al., 1992). This value is well above the theoretical threshold of 1 nucleus leaf disc\(^{-1}\), which explains the identical freezing curves obtained from the heterozygous and homozygous plants with this assay.

In preliminary tests, an attempt was made to grow tobacco seedlings in Petri plates and freezing them \textit{in situ}. This proved to be insufficient for measuring the freezing survival of tobacco seedlings, although a similar approach had been applied for the identification of the \textit{Arabidopsis eskimo 1} mutant (Xin and Browse, 1998). The main problem that was encountered was that a freezing event was ‘propagated’ from each seedling to neighbouring ones, even though there was no physical contact among the seedlings. The microtitre plates provide a convenient system to isolate the individual seedling freezing event, in addition to providing uniform freezing conditions.

Freezing in microtitre plates had been reported earlier as a \textbf{Bacterial Ice Nucleation Diagnostic (BIND)} assay system (Andrews et al., 1992). Using the described assay, transformed (ice nucleation-active) seedlings were clearly distinguished from non-transformed seedlings and it was shown that the \textit{inaZ} gene is expressed at the 2–4 leaf developmental stage.

The leaf disc freezing assay is well suited for the validation of the results of the first seedling freezing assay. Though it is more labour-intensive and time-consuming than the seedling freezing assay, it can be applied in a second round of selection, when the number of plants that need to be tested has been reduced significantly. Surviving plants are transplanted in soil or potting mix and are allowed to develop to any desirable stage.

The assays described above are used to detect ice formation in plants and not the resultant freezing damage, which is dependent on the tolerance of the plant to freezing. They may be useful for assessing freezing survival in frost-tolerant species, where ice formation does not necessarily lead to freezing damage. Furthermore, they may be used for the genetic dissection of the ice nucleation process in plants (e.g. screening mutants blocked for the production of ice nucleation). In its current form the seedling freezing assay may only be applied to seeds/seedlings that have similar size to tobacco and, therefore, ‘fit’ in the microtitre plate well. Further development of the assay may lead to a more generalized use, including plant cell suspension cultures. A significant part of the procedure is amenable to automation (e.g. ‘loading’ the microtitre plates, transferring them to a low temperature unit), facilitating the evaluation of the genetic potential of various crops to freezing.

**Acknowledgements**

We are grateful to two anonymous reviewers for the valuable comments and corrections. This work was supported by grants from the Secretariat for Research and Technology, Greece and by the Institute of Molecular Biology and Biotechnology (IMBB), Foundation for Research and Technology (FORTH)—Hellas.

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