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GENETIC TRANSFORMATION AND HYBRIDIZATION

Arabidopsis rd29A::DREB1A enhances freezing tolerance in transgenic potato

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Abstract The freezing tolerance of 38 independent transgenic potato lines derived from the cultivar Desiree was tested in vitro using plantlets. The lines were transgenic for the *DREB1A* gene under control of the *rd29A* promoter, both of which were derived from *Arabidopsis thaliana*. The level of damage caused by freezing varied significantly among the transgenic clones and a non-transgenic control (cv. Desiree). Phenotypic evaluation indicated that the variable responses to freezing were attributable to genotypic variation, but freezing tolerance was not dependent on the number of insertions. Northern blot analysis using a *DREB1A* cDNA probe revealed high levels of *DREB1A* expression among the transgenic clones during the initial cold exposure at 4°C (after 2 h) and in the early stages of freezing (–20°C, 1–10 min). Furthermore, a linear correlation was detected between the level of expression and the phenotypic response for all lines except D138. Thus, in the case of potato, a significant increase in freezing tolerance was observed in vitro on a small scale following the introduction of *rd29A*::*DREB1A*. Additional testing will show whether this strategy can be used for tolerance breeding in potato and to increase the freezing tolerance of other agriculturally important crops.

Keywords Potato · Freezing tolerance · rd29A::DREB1A · Arabidopsis · Transgenic

Abbreviations

ANOVA	Analysis of variance
CBF	C-repeat-binding factor
CRT	C-repeat
DRE	Dehydration-responsive element
DREB	Dehydration-responsive element binding
DSC	Non-transgenic cv. Desiree as control
r	Correlation coefficient
RCBD	Randomized complete block design
rd29A	Responsive drought 29A
RT	Room temperature

Introduction

The potato, one of the most important crops worldwide, is not only an important food source but also a vital raw material in the starch-processing industry (Ortiz and Watanabe 2004). Since the potato is of highland origin and favors cool climates, it is often cultivated in cool areas or during cool seasons; frost damage, however, is a recurring problem in potato cultivation (Vayda 1994). Thus, it is important to clarify the molecular mechanism of cold acclimation and freezing tolerance in potato, and transgenic techniques are useful in this regard.

Recently, the *Arabidopsis* DREB/CBF (dehydration-responsive element binding/C-repeat-binding factor)

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proteins were identified as key transactivational factors against such environmental stresses as cold, drought, and salinity (Stockinger et al. 1997; Gilmour et al. 1998; Liu et al. 1998; Shinozaki and Yamaguchi-Shinozaki 2000; Thomashow 2001). DREB/CBF proteins, which contain an AP2/EREBP DNA-binding domain, are bound to a dehydration-responsive element (DRE)/C-repeat (CRT) (Yamaguchi-Shinozaki and Shinozaki 1994) and control the expression of many stress-inducible genes in *Arabidopsis*, including *rd29A* (Yamaguchi-Shinozaki and Shinozaki 1993). *Arabidopsis DREB1A* (*AtDREB1A*) has been shown to confer environmental stress tolerance in many plant species, including rice (Kasuga et al. 1999), tobacco (Kasuga et al. 2004), and wheat (Pellegrineschi et al. 2004), and DREB homologs have been identified in several types of plants, including *Brassica napus* (Gao et al. 2002), barley (Choi et al. 2002), rice (Dubouzet et al. 2003), wheat (Shen et al. 2003), and maize (Qin et al. 2004). However, no visible change with At*DREB1*A was observed in some plant species such as *Eucalyptus camaldulensis* (Hibino and Kawazu, Oji Paper Co., personal communication). Although no DREB homologs have been identified in the potato, increased salt tolerance was observed in transformants of *Arabidopsis rd29A::DREB1A* (Celebi-Toprak et al. 2005; Behnam et al. 2006). Thus, *Arabidopsis rd29A::DREB1A* will likely affect the response of potato plants to freezing.

The objective of this study was to determine whether *Arabidopsis rd29A::DREB1A* could induce freezing stress tolerance in tetrasomic tetraploid potato plants, similar to its effect on salinity tolerance. In addition, the relationship between freezing tolerance and the transgene was examined by correlation analysis. Finally, by comparing our current results with those in our previous study (Behnam et al. 2006), we examined the differences between salinity and freezing tolerance and the acquisition of freezing tolerance by the introduction of *AtDREB1A* into potato plants.

Materials and methods

Phenotypic evaluation of freezing stress

Previously, we used *Agrobacterium*-mediated transformation to generate several transgenic lines of potato, derived from the *Solanum tuberosum* L. cultivar Desiree ($2n = 4x = 48$) expressing the *Arabidopsis DREB1A* gene and driven by the *Arabidopsis rd29A* promoter, and we tested the salinity tolerance of the lines using an in vitro culture system (Celebi-Toprak et al. 2005; Behnam et al. 2006).

In this study, our experimental conditions were optimized so we could rapidly and reproducibly assess the tolerance level of the transgenic lines in tissue culture form in MS medium. The effect of freezing was evaluated in 38 transgenic clones at the T_0 generation (i.e., the lines contained variable numbers of insertions) and in non-transgenic cv. Desiree as a control (DSC).

The freezing tolerance evaluation procedure was as follows. The selected transgenic clones (ten 3-week-old plantlets per clone) were initially exposed to 4°C for 2 h, and then transferred to –20°C for 1 h. The plantlets were then returned to 4°C for 2 h before finally being returned to room temperature (RT). The survival rate, determined 48 h after the plantlets were returned to RT, was defined as the number of surviving transgenic lines divided by the total number of transgenic lines exposed to freezing stress. Four replicates consisting of 8–10 plants from each transgenic line were used for the analysis. We used a randomized complete block design (RCBD) analysis of variance (ANOVA) to compare the differences among the transgenic clones and the DSC. Mean values obtained from the treatments were compared using Duncan's test with the DSC.

RNA extraction and northern hybridization

Leaf samples (50 mg) were ground in liquid nitrogen, and total RNA was extracted using RNAgents® Total RNA Isolation System (Promega, Madison, WI, USA), according to the manufacturer's instructions. Aliquots (20 μg) of RNA were fractionated on 1% agarose gels in 3-(N-morpholino) propanesulfonic acid buffer, and then transferred to a positively charged nylon membrane (Hybond-N^+, Amersham Biosciences, Piscataway, NJ, USA) using 10× SSC. Labeling of the probes for *DREB1A* and 18S rRNA was performed as described for southern blotting (Amersham Biosciences). The hybridization signals were detected following exposure to Hyperfilm™ (Amersham Biosciences) at RT for 1 h.

The expression of *DREB1A* and 18S rRNA in the most tolerant and sensitive transgenic clones was quantified using total RNA from samples exposed to 4°C for 2 h by Northern blotting using a cooled charge-coupled device camera (LAS1000; Fujifilm, Tokyo, Japan), and the chemiluminescent bands were quantified using Image Gauge Software (version 3.3; Fujifilm).

Results

Freezing stress: morphological damage and phenotypic evaluation

The level of damage induced by freezing varied among the transgenic lines and was divided into three categories: Type 1, serious damage to all parts of the plant as well as the DSC (Fig. 1a, d); Type 2, serious damage with wilted

and brownish tissue at the upper parts of the leaves and stems, but a green and living collar and root system (Fig. 1b); and Type 3, minimal damage with an unstressed appearance and normal plant growth (Fig. 1c). Types 2 and 3 plants were considered to have survived the treatment. The tolerance levels of the transgenic lines were based on their survival rates (see ''Materials and methods''), which varied between 0 and 65%. A previous correlation was shown between copy number and salinity tolerance (Behnam et al. 2006); thus, in this study, a correlation analysis was performed for freezing tolerance. However, scatter diagrams derived from an analysis of the phenotypic response to freezing and the number of *DREB1A* insertions (evaluated by southern blotting with *Hin*dIII and *Dra*I) did not indicate significant correlation. At $df = 37$, the correlation coefficient (r) was –0.11 for *Hin*d III ($t_{cal\ (df = 37)} = 0.67$, ns < 1) and –0.06 for *Dra*I ($t_{cal\ (df = 37)} = 0.37$, ns < 1).

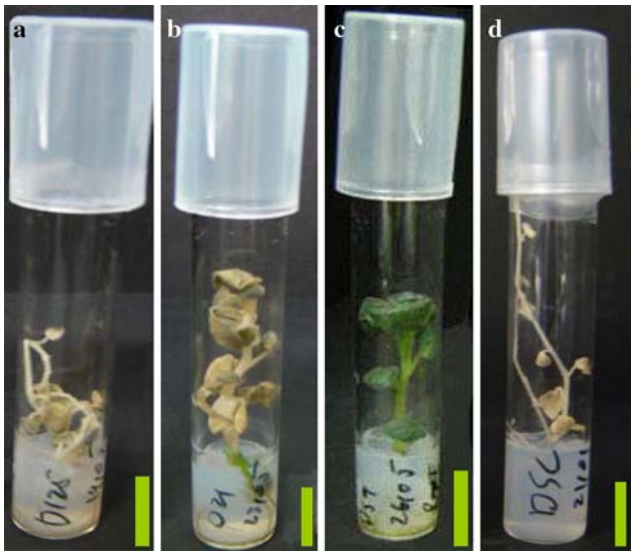

Fig. 1 Representative examples of plant phenotypes resulting from freezing stress among transgenic potato lines and the non-transgenic cv. Desiree (DSC). Freezing resulted in three types of physical damage. Type 1: **a** (D125) and **d** (DSC, control); complete damage with no recovery from freezing after the return to room temperature (RT). Type 2: **b** (D21); damage to the upper plant parts, but not to the collar or root system. These plants recovered after the return to RT. Type 3: **c** (D59); minimal damage to the entire plant and complete recovery after the return to RT. The *bar* indicates 15 mm

Identification of highly tolerant lines using Duncan's test

Although our in vitro system should have been uniform, replications were made by RCBD. Furthermore, as our analysis was based on a small variable number of plantlets in each plot, ''Arcsin'' transformation was used for the phenotypic data, followed by ANOVA (Table 1).

Significantly different freezing tolerances (Fig. 2; Table 1) were associated with specific genotypes, and these were classified into five groups: a, ab, abc, bc, and c. To determine which transgenic clones were highly tolerant to freezing, we considered two characteristics: the group classification from Duncan's test and the type of tolerance (Types 1–3). Based on these criteria, two Type 3 transgenic clones, D163 (group a) and D45 (group ab), were found to have significantly higher freezing tolerance than the other samples (survival rates of 66 and 54%, respectively; Fig. 2).

Moreover, the transgenic clones in group abc (Fig. 2) that had survival rates ≥40% showed increased freezing tolerance (i.e., faster recovery after their return to normal conditions) compared to the other transgenic clones in that group. The following transgenic clones, with survival rates of 40–48%, were considered to be tolerant: D22, D63, D33, D24, D16, D164, D17, D170, D118, D44, D172, and D53. While D13 had a survival rate of over 40%, it was classified as a Type 2 clone and therefore not considered freezing-tolerant.

DREB1A gene expression in transgenic potato: northern hybridization

Because no previous information on sampling intervals in transgenic potato was available, we assumed that optimizing the mRNA sampling time was important. Furthermore, the expression signals obtained from different time intervals of mRNA sampling suggested that sampling should be performed at 1 and 2 h during the first experi-

Table 1 Randomized complete block design analysis of variance for freezing tolerance among 38 in vitro transgenic clones and a non-transgenic control (DSC) after data conversion using ''Arcsin transformation''

SOV	*df*	SS	MS	F_{cal}	$F_{tab5\%}$	$F_{tab1\%}$
Treatment	37	30242.64	817.369	2.58**	1.51	1.7
Block	3	1819.60	606.533	1.92**		
Error	115	36396.99	316.496			
Total	155	68459.23				

** Significant at $P = 0.01$

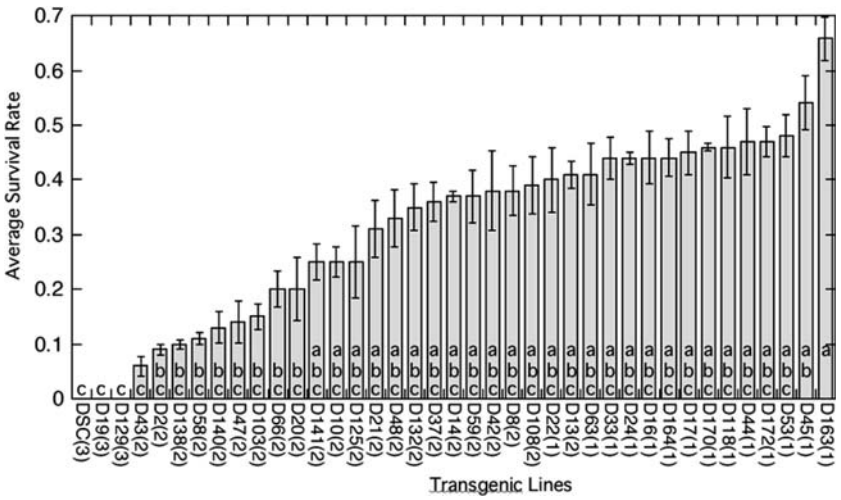

Fig. 2 Comparison of the average visual scores for the transgenic lines using Duncan's test at a 5% LSR level. Assessment of freezing tolerance in 38 *DREB1A*-transgenic potato clones and in the non-transgenic cv. Desiree. The *lines* at the *top of each bar* indicate the respective standard error of four replications. The *bar* for each genotype corresponds to the average of the scored data. The letters *a–c* are used to divide the plants into recognizable groups. The *abscissa* refers to independent transgenic lines with the non-transgenic cv. Desiree as a control (DSC). The *number* in parentheses for each line indicates the type of tolerance (i.e., D163(3) means that D163 has type 3 tolerance). The *ordinate* shows the average survival rate based on four replications

mental step (at 4°C), and then at 1, 5, and 10 min during the second step (at –20°C; data not shown). Expression of the transgene was assessed by Northern hybridization for the following transgenic lines, which were considered to be representative of the different levels of freezing tolerance: highly tolerant (D163), tolerant (D22, D24, D44, D59, D132, and D164), moderately tolerant (D103 and D138), and sensitive (D19 and DSC). Representative data for the expression of *DREB1A* during freezing stress in D24 is shown in Fig. 3. Prior to treatment and after 5 min during the initial exposure at 4°C, no expression of *DREB1A* was detected (Fig. 3a); however, expression was observed after 30 min of exposure at 4°C, and D24 showed its highest level of *DREB1A* expression after 2 h during the initial exposure at 4°C.

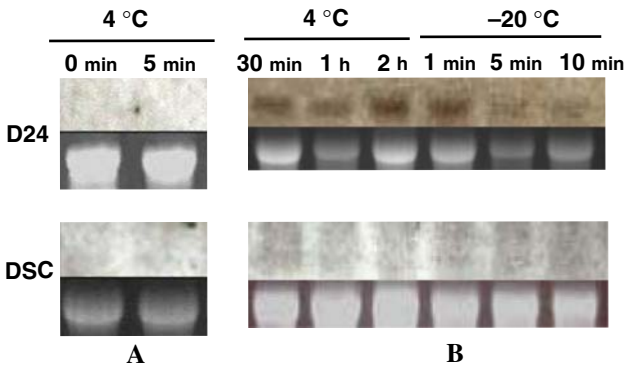

Fig. 3 Northern hybridization results for *DREB1A* expression, with the related ethidium bromide normalizing control for one representative freezing-tolerant transgenic clone (D24) and the non-transgenic control (DSC). **a** No signal was detected prior to cold treatment or after 5 min of exposure at 4°C. **b** No expression was detected in the DSC, whereas expression was detected in D24 after 30 min of exposure at 4°C; maximal expression was observed after 2 h at 4 °C and after 2 h at 4°C plus 1 min at –20°C

Based on these results, Northern hybridization was performed using the remaining lines at 30 min, 1, and 2 h during the initial exposure at 4°C, and 1, 5, and 10 min during the exposure at –20°C (Fig. 3b). Most of the transgenic clones showed relatively high expression during the initial exposure at 4°C and after 1 min at –20°C. No expression was detected in some of the transgenic lines (e.g., D19) or in the DSC. The reproducibility of our northern blot results was confirmed by repeating some samples at least twice.

Quantification of gene expression after 2 h of initial exposure at 4°C

Those transgenic clones that displayed phenotypic freezing tolerance had substantially higher levels of *DREB1A* expression after 2 h of the initial exposure at 4°C (Fig. 3). This indicates that the *rd29A* promoter rapidly induces high levels of gene expression during freezing stress (Fig. 3).

The level of *AtDREB1A* expression varied among the transgenic lines, and no expression was detected in the DSC after 2 h during the initial exposure at 4°C (Fig. 4a). The rRNA signal was normalized based on a related signal in the *DREB1A* probe for each transgenic clone (i.e., signal score from the *DREB1A* probe/signal score from the related rRNA band), and the relative intensity was calculated for each clone (Fig. 4b). Quantification of the bands in the

Northern blots confirmed that strong signals were obtained from D163 and D138. The calculations were made based on the correlation coefficient (r) between our quantitative gene expression data (i.e., relative band intensity) and our phenotypic evaluation data (i.e., survival rate). In the case of exclusion of D138, significant correlation was shown ($r = 0.76$, $t_{cal(df=8)} = 3.30$, * > $T_{tab(df=8,\ 5\%)} = 2.31$; Fig. 4c).

Discussion

The transgene conferred freezing stress tolerance to potato

Potato transformants of *Arabidopsis rd29A::DREB1A* have increased salinity stress tolerance (Behnam et al. 2006). Similarly, our morphological and statistical data show that the same construct effectively increases the freezing tolerance of tetrasomic tetraploid potato (Table 1; Fig. 1). Furthermore, a strong correlation exists between the average survival rate and the level of *AtDREB1A* expression (Fig. 4c). These results suggest that the AtDREB1A protein confers freezing tolerance to potato plants. The acquisition of freezing stress tolerance indicates that the *Arabidopsis rd29A* promoter can function in potato during freezing, and that *AtDREB1A* is able to control the downstream genes associated with freezing stress tolerance in potato plants. Our results indicate that freezing stress induces some kind of native transactivator in the potato that can bind the DRE of the *Arabidopsis rd29A* promoter (Yamaguchi-Shinozaki and Shinozaki 1994) and that the promoters of native genes associated with freezing stress tolerance containing a DRE-like element can be bound by AtDREB1A. Although the homolog of DREB in potato plants has yet to be identified, the potato may possess a DER-DREB-based stress response system similar to that in *Arabidopsis* (Liu et al. 1998), rice (Oh et al. 2005), and wheat (Shen et al. 2003).

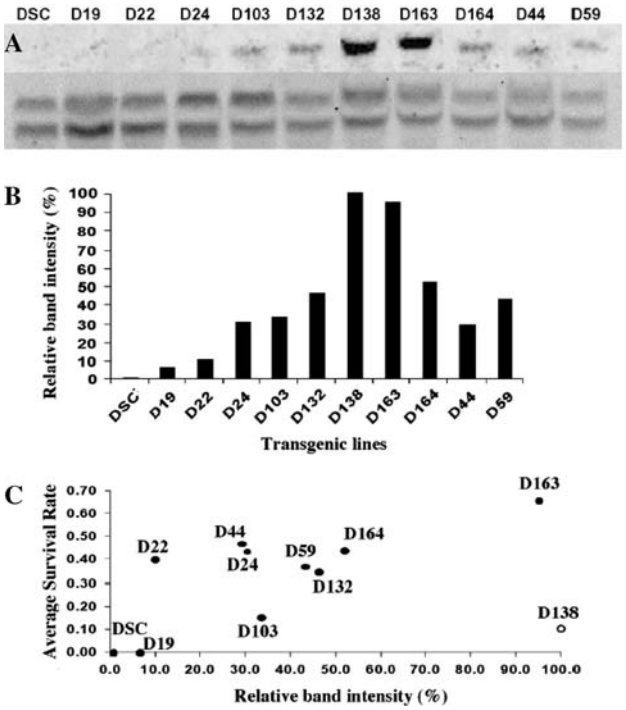

Fig. 4 **a** RNA blot (after 2 h at 4°C). No *DREB1A* expression was detected in the non-transgenic control (DSC), whereas maximal expression was observed among transgenic clones with different levels of freezing tolerance; the level of gene expression differed among the genotypes. D138 was an exception, with low physical freezing tolerance, but the highest level of gene expression. **b** Relative band intensity from a Northern blot of select transgenic clones and the DSC. **c** Correlation of survival rates after freezing with the level of gene expression for ten transgenic clones and the DSC (excluding D138, $r = 0.76$, $t = 3.30$)

Difference between tolerance to freezing and salinity

In terms of freezing, a strong correlation was demonstrated between the level of transgene expression and the average survival rate (Fig. 4c), while no significant correlation was detected between the copy number and the average survival rate. However, in terms of salinity, a moderate correlation between copy number and tolerance was identified (Behnam et al. 2006). We propose that the differences in the two sets of results may be attributable to the different types of stress. Since the transgene (*AtDREB1A*) encodes a transactivator, expression of the transgene does not control stress tolerance directly. Instead, stress tolerance results from the expression of multiple stress-tolerance-associated genes, and the expression of those genes was controlled by AtDREB1A in this study. It has been reported, however, that transgene expression does not always depend on the copy number, and expression may be influenced by such complicated mechanisms as DNA methylation or RNA interference (Jones et al. 1999; Dalmay et al. 2000). In other words, many factors and several steps lie between the introduction of the transgene and the expression of stress tolerance. Notably, not all of the transformants showed the same trait for both stresses. Some lines were tolerant to freezing or salinity while others were tolerant to both (Fig. 5). In *Arabidopsis*, some salinity and cold stress-inducible genes are controlled by AtDREB1A (Liu et al. 1998), while other genes are induced only by salinity or cold (Seki et al. 2001). In the potato, the mechanisms underlying stress tolerance against freezing and salinity may be slightly different, and some of the native inducers of the *rd29A* promoter may also be different for the two stresses.

The level of *AtDREB1A* expression was highest in D138, but this line did not exhibit increased tolerance to

freezing (Fig. 4). A strong correlation between *AtDREB1A* expression and the average survival rate was found by excluding the data for D138 (Fig. 4c). This may be explained by the creation of a deficient transcript or by disruption of a downstream pathway associated with freezing stress tolerance controlled by AtDREB1A. Since D138 showed increased salt tolerance, the transcript may also function as a transactivator during freezing (Fig. 5; Behnam et al. 2006). Some disruption may have occurred downstream in the pathway associated with freezing tolerance, such as a mutation caused by insertion of the transgene. As described above, many steps and many genes link the expression of *AtDREB1A* with the expression of stress tolerance, and a mutation caused by the introduction of the transgene might have influenced the traits of the transformant. The level of freezing tolerance varied among the transformants (Fig. 2), and not all transformant showed tolerance to both types of stresses (Fig. 5). Furthermore, variable levels of transgene expression are often observed among different lines produced using the same construct (Peach and Velten 1991; Longstaff et al. 1998). Though D138 is an extreme example, it may have been a natural occurrence that the physiological reaction of each transformant was different.

The mechanism of freezing tolerance and its application in molecular breeding

The mechanism of freezing stress tolerance has been studied from the viewpoint of membrane stabilizing proteins (St John et al. 1979; Graham and Patterson 1982) and cryoprotectans synthase (Hayashi et al. 1997; Nanjo et al. 1999; Taji et al. 2002). On the other hand, several stress-inducible genes have been identified that function in stress tolerance (Ingram and Bartels 1996; Bray 1997; Shinozaki and Yamaguchi-Shinozaki 1997, 2000), and several cold-inducible genes have been shown to be controlled by DERB1A/CBF3 (Maruyama et al. 2004). Furthermore, transformation of the *DREB1/CBF* genes has been found to improve the environmental stress tolerance of many plants (Kasuga et al. 1999; Dubouzet et al. 2003; Kasuga et al. 2004; Pellegrineschi et al. 2004). Still, little is known about how DREB1/CBF proteins protect plants against freezing without a COR15a (Artus et al. 1996; Steponkus et al. 1998), even in *Arabidopsis*. Nevertheless, DREB1/CBF regulatory proteins may be regarded as master switches that integrate the activation of multiple components of the cold acclimation response. To clarify the mechanism of freezing tolerance in plants, it will be important to establish a connection between these master switches and the expression of stress tolerance.

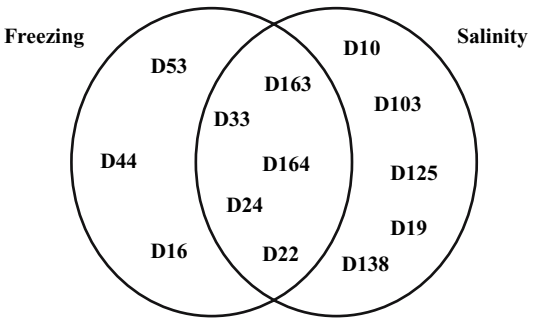

Fig. 5 Stress-tolerance testing of transgenic potato lines for salinity and freezing. The diagram shows our previous results for salinity (Behnam et al. 2006), and the current data for freezing from 22 transgenic potato clones derived from the cv. Desiree. Twelve clones were salt-tolerant and 11 were freezing-tolerant. Among them, seven clones expressed tolerance under both stress conditions (D8, D22, D24, D33, D132, D163, and D164)

Based on our results, we have selected 14 transgenic potato lines that were highly tolerant to freezing (Fig. 2) that can be used for future physiological and molecular studies. In particular, D163 and D138 are good candidates for molecular studies on freezing tolerance. Both lines showed high levels of *AtDREB1A* expression, but D138 did not exhibit freezing tolerance. Since D138 demonstrated strong salt tolerance (Behnam et al. 2006), the transgene (*AtDREB1A*) must function normally in that line. Thus, genes associated with freezing tolerance may be identified by comparing the genes induced by freezing stress in D138 and D163.

Several studies have focused on the physiological mechanism of low-temperature stress in potato plants (Seppanen and Coleman 2003; Vega et al. 2004; Bamberg et al. 2005; Mora-Herrera et al. 2005), and transgenic approaches have been employed to increase the level of tolerance (Cutler et al. 1989). However, little progress has been made in developing a strongly cold-tolerant variety for practical use. While our experiment was conducted in vitro using small plantlets, we observed significant tolerance to freezing and recovery from freezing stress in several lines transgenic for *Arabidopsis rd29A::DREB1A*. Thus, our results suggest that low-temperature tolerance can be further developed for use in potato breeding.

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