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Original Paper

The *Arabidopsis DREB1A* gene driven by the stress-inducible *rd29A promoter* increases salt-stress tolerance in proportion to its copy number in tetrasomic tetraploid potato (*Solanum tuberosum*)

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Abstract Transgenic potato lines of cv. Desiree containing the *DREB1A* gene driven by the *rd29A* promoter were generated using *Agrobacterium*-mediated transformation. The morphological appearance of the 120 transgenic lines was classified into three categories as determined by *in vitro* test-tube evaluation. Southern blot analyses of genomic DNA were conducted using the restriction enzymes *Hin*dIII and *Dra*I. There were significant differences between the transgenic lines and DSC in the quantitative salinity-tolerance evaluations at 1 M NaCl. Two transgenic lines were recognized as highly tolerant to salinity based on Duncan multiple range testing. Furthermore, there was a significant correlation between the mean tolerance level of the transgenic lines and the *DREB1A* copy number estimated from the Southern hybridization experiments. Northern hybridization experiments were subsequently done using a *DREB1A* cDNA probe and transgenic lines with different levels of salinity tolerance. Salt-tolerant transgenic lines expressed substantially more of the transgene at 2 to 5 h of salt treatment, after which the expression returned to basal levels. These observations suggest that the gene transfer of *rd29A::DREB1A* can be used to increase the salt tolerance of important agricultural crops, such as tetrasomic polyploid potatoes, as occurs in diploid model species, such as *Arabidopsis*.

Key words: Abiotic stresses, dehydration-responsive element (DRE), salt tolerance, tetrasomic tetraploid potato.

Drought, salt loading, and freezing conditions are stresses that have adverse effects on the growth of plants and on crop yields. The physiological responses to these stresses arise from changes in the cellular gene expression profile, and a number of genes are induced by exposure to such conditions (Thomashow et al. 1994; Shinozaki and Yamaguchi-Shinozaki 1996). The products of these genes can be classified into two groups: those that protect against environmental stresses directly and those that regulate gene expression and signal transduction during the stress response (Shinozaki and Yamaguchi-Shinozaki 1997). The first group includes proteins that are most likely to function by protecting cells from dehydration, including enzymes required for the biosynthesis of various osmoprotectants, late-embryogenesis-abundant (LEA) proteins, antifreeze proteins, chaperones, and detoxification enzymes (Ingram and Bartels 1996; Bray 1997; Shinozaki and Yamaguchi-Shinozaki 1997). The second group of gene products includes transcription factors, protein kinases, and enzymes involved in phosphoinositide metabolism (Shinozaki and Yamaguchi-Shinozaki 1997).

In nature, when plants are exposed to conditions of stress, they can develop tolerance, but this is a slow process. To overcome this time lag, many genes related to different stress responses have recently been transferred to various plants to improve stress tolerance (Pilon-Smits et al. 1995; Goddijn et al. 1997; Kasuga et al. 1999; Huang et al. 2000; Maqbool et al. 2002). Transgenic methods involving several different gene transfer approaches have been used to improve the stress tolerance of plants. To investigate the possibility of simultaneously enhancing tolerance toward multiple abiotic stresses using *Agrobacterium*-mediated gene

Abbreviations: DREB, dehydration-responsive element binding; DRE, dehydration-responsive element.
This article can be found at http://www.jspcmb.jp/

transfer, Kasuga et al. (1999) used this system to test a stress-inducible transcription factor that regulates many genes involved in stress tolerance in *Arabidopsis thaliana*. A *cis*-acting element has been identified in the promoter region of the *rd29A* gene and is responsible for both dehydration- and cold-induced expression (Yamaguchi-Shinozaki and Shinozaki 1994). This sequence (TACCGACAT), called the dehydration-responsive element (DRE), is essential for regulating dehydration-responsive gene expression (Yamaguchi-Shinozaki and Shinozaki 1994) and is found in the promoter regions of other genes induced by dehydration and cold stresses (Yamaguchi-Shinozaki and Shinozaki 1994; Wang et al. 1995).

The cDNAs encoding the DRE-binding proteins DREB1A and DREB2A have been isolated using yeast one-hybrid screening (Liu et al. 1998), and both proteins specifically bind to and activate the transcription of genes containing the DRE sequence in *Arabidopsis*. The overexpression of the *DREB1A* (*CBF3*) cDNA, under the control of the CaMV 35S promoter, was subsequently shown to result in the strong expression of target genes that are stress-inducible following conditions of drought, high salt, or freezing temperatures (Liu et al. 1998; Kasuga et al. 1999; Gilmour et al. 2000). Therefore, the stress-inducible *rd29A* promoter was used to drive the expression of *DREB1A*, with the aim of minimizing the negative effects on plant growth experienced with the use of the 35S CaMV promoter. Improvements in the stress tolerance responses of the transgenic plants and much improved growth under normal conditions were observed with this construct (Kasuga et al. 1999).

The potato is one of the most important crops in the world and is ranked fourth behind wheat, rice, and maize. The potato crop is not only an important food source but is also a vital raw material in the starch-processing industry, a source of animal feed that makes use of potato vines, and a potentially important resource in medicine owing to the compounds present in its seeds (Ortiz and Watanabe 2004). Most genetically modified plant species, however, are diploid, and gene-transfer techniques are far more challenging in polyploid crops such as potatoes, in which the gene expression profile may be altered with multiple transgene inserts, given the complexity of polysomic genetics (Celebi-Toprak et al. 2005).

The first objective of this research was to determine how transcription factors such as *DREB1A* act in enhancing the tolerance to salinity in the tetrasomic tetraploid potato. A large number of transgenic potatoes derived from *S. tuberosum* L. cultivar Desiree (2n=4x=48) were generated with this gene, driven by the *rd29A* promoter, and tested for salinity tolerance under growth-room conditions. Second, we examined the copy numbers of the inserted gene and determined their association with salinity tolerance using phenotypic evaluation. Finally, we assessed the relationship between the *DREB1A* copy numbers and gene expression at the RNA level.

Materials and methods

Plasmid transformation, selection, and plant regeneration procedures

Agrobacterium-mediated transformation was conducted using the tetrasomic tetraploid potato, *S. tuberosum*, cv. Desiree, with *A. tumefaciens* LBA 4404, according to Inui et al. (2000). The plasmid pBE2113 *Not* I (Mitsuhara et al. 1996) contained the neomycin phosphotransferase II (*NPT II*) gene as a selectable marker (conferring resistance to NPT II or kanamycin) under the control of the *NOS* promoter and a binary vector containing the *rd29A* promoter and *DREB1A* cDNA (Kasuga et al. 1999). Plantlets were further selected for kanamycin resistance by adding kanamycin at 35–50 mg l^{-1} in MS media. Surviving plantlets with sufficient rooting were then tested for the presence of the *DREB1A* gene using PCR.

Polymerase chain reaction

For the initial screening of transgenic lines, the following PCR primers derived from *DREB1A* were used to amplify the *DREB1A* gene: 5′-TGATTATATTCCGACG-CTTG-3′ (forward) and 5′-TTCATGATTATGATTCCA-CT-3′ (reverse), as described previously (Kasuga et al. 1999). The 25 μl amplification reaction contained 1× Buffer A, 3 mM $MgCl_2$, 200 μM dNTPs, and 0.025 U μl^{-1} AmpliTaq Gold DNA polymerase. The final concentration of each primer was 200 nM. The amplification reaction protocol was an initial denaturation at 94°C for 5 min, followed by 35 amplification cycles of 94°C for 1 min, 55°C for 1 min, and 72°C for 90 s, and primer extension at 72°C for 10 min. The PCR amplification products were separated in 1% (w/v) agarose gels using 0.5×TBE buffer.

Southern hybridization

Southern analyses of the *DREB1A* gene insert were performed on *Dra*I and *Hin*dIII digests of genomic DNA from 120 transgenic potato lines, probed with *DREB1A* cDNA, as these restriction sites are absent in the *DREB1A* cDNA (Liu et al. 1998). Total genomic DNA was isolated from 0.2–0.5 g fresh leaf mass using a DNA microprep method (Fulton et al. 1995). A 30 μg aliquot of DNA was digested overnight at 37°C with *Dra*I or *Hin*dIII, separated in a 1% agarose gel, and transferred to a positively charged nylon membrane (Hybond-N+, Amersham Bioscience) using a denaturing buffer. The *DREB1A* probe was labeled using the Gene Image AlkPhos direct labeling and detection system kit

(Amersham Bioscience), and the bands were visualized using CDP-Star™ (Amersham Bioscience) according to the manufacturer's instructions, followed by exposure to Hyperfilm™ (Amersham Bioscience) at room temperature in the dark for 3 h.

Phenotypic evaluation of salinity stress

Subsequent stress evaluations were conducted as a preliminary experiment using 27 representative transgenic plants from the T_0 family with various copy numbers of the inserted *DREB1A* gene. The salinity stress evaluation procedure was first calibrated by subjecting cuttings of non-transgenic cv. Desiree (DSC) at the four- to five-leaf stage (10–15 days growth) to various levels of salinity stress in small plastic cells (5×5 cm) containing 25 g of a dry soil mix composed of one-third commercial horticultural soil (Kureha Chemical Industry, Japan), one-third perlite, and one-third vermiculite. Evaluations were conducted in a growth room with a 16 h light : 8 h dark photoperiod, at temperatures between 22 and 25°C, and 65% humidity. In a preliminary experiment to optimize the concentration of NaCl for the salinity stress test to distinguish differences between transgenic lines and DSC, we found that 1 M NaCl solution could discriminate transgenic plants from DSC controls, as all of the controls died (data not shown). Four cuttings were tested per individual transgenic plant in four repeat experiments; for every three of these individual transgenic plants, four cuttings from DSC at the same developmental stage were tested in tandem. Our evaluation of these specimens under salinity stress conditions was based on a split plot against time using a randomized complete block design (RCBD). The experiment was conducted in trays, and the plants were rated on a scale from 0 to 5, where 0=100% survival without damage; 1=plant standing, little damage to leaves or stem; 2=plant standing, some damage to leaves and stem; 3=plant not standing upright, some damage to leaves; 4=plant not standing, damage to leaves; 5=plant not standing, complete wilting and death (Celebi-Toprak et al. 2005). These results were then tested using ANOVA (split plot against time with RCBD). The mean values obtained from the treatments were compared using Duncan's test at the 5% least squares regression (LSR) level.

Ploidy level analysis

Fresh leaves, approximately 0.5 cm^2, from transgenic and DSC plants were used to detect the ploidy level using a Ploidy Analyser (Partec, Germany) with a high-resolution DNA staining kit (Cystain UV Precise P, Partec, Germany) after filtering through 50 μm mesh filters (Partec, Germany). The results were standardized using the rice Taichun 65 variety as an internal control.

RNA extraction and Northern hybridization

Leaf samples of approximately 50 mg were ground in liquid nitrogen and total RNA was extracted using an RNAgents® total RNA isolation system (Promega), according to the manufacturer's instructions. RNA aliquots (20 μg) were fractionated on 1% agarose gels in 1×MOPS [3-(N-morpholino) propanesulfonic acid] buffer and then transferred to a positively charged nylon membrane (Hybond-N+, Amersham Bioscience) using 10×SSC. The *DREB1A* gene and rRNA 18S control probes were labeled as described for Southern blotting. The hybridization signal was detected following exposure to Hyperfilm™ (Amersham Bioscience) at room temperature for 1 h.

In addition, total RNA samples from plants stressed for 2 h were used in another Northern blot (with *DREB1A* gene and rRNA 18S control probes) for the transgenic lines most tolerant to and those sensitive to 1 M NaCl, along with one transgenic line (D24: Dsrd29A-24) that is tolerant to three abiotic stresses. The blots were visualized using a cooled CCD camera (LAS1000, Fujifilm, Tokyo, Japan), and the chemiluminescent bands were quantified using Image Gauge software (version 3.3, Fujifilm).

Results

Generation of transgenic lines

More than 300 regenerants were obtained using *Agrobacterium*-mediated transformation of cv. Desiree following the method of Suzuki et al. (1997). A total of 188 plantlets were selected by kanamycin resistance in MS media and tested by PCR using the *DREB1A* primers. One hundred twenty transgenic potato lines, the T_0 family, were positively identified as transgenic. The phenotypic appearance of the transgenic lines in test tubes was classified into three categories: normal morphology, similar to the non-transgenic cv. Desiree phenotype (DSC; 43.3%, 52/120; Figure 1A); normal growth characteristics, but phenotype different from DSC (intermediate shape; 30.8%, 37/120; Figure 1B); and abnormal formations (25.8%, 31/120; Figure 1C). The ploidy levels of the transgenic potato lines were examined using flow cytometry. Most were tetraploid, although 14/120 (12%) were identified as ploidy chimeric forms, and none of these had normal phenotypes (Table 1).

Genomic analysis of the DREB1A transgene by Southern hybridization

As the DREB1A cDNA contains no *Dra*I or *Hin*dIII restriction sites, Southern analyses of the *DREB1A* gene insert were performed using those restriction enzymes. The results showed different numbers of inserts within each genome. Representative results for *Dra*I digests

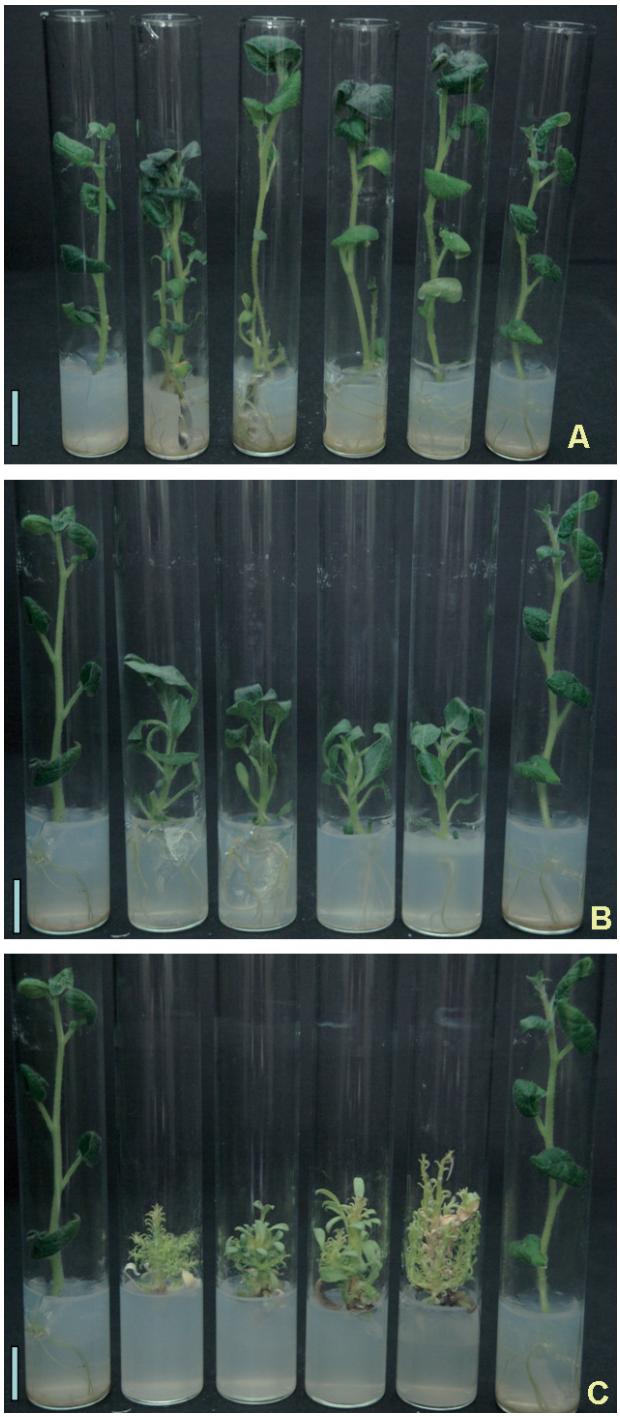

Figure 1. Morphological classification of transgenic lines based on *in vitro* culture. A: Transgenic lines that displayed normal growth and wild-type morphologies. Control plants (non-transgenic cv. Desiree) are shown at either end, and the transgenic lines shown from the left (second position) are Ds-rd29A-10, Dsrd29A-14, Dsrd29A-163, and Dsrd29A-164. B: Transgenic lines with normal growth characteristics but different aerial shapes compared with non-transgenic cv. Desiree controls (shown at either end of the row). The transgenic lines shown from the left (second position) are Dsrd29A-16, Dsrd29A-47, Dsrd29A-103, and Dsrd29A-129. C: Transgenic lines with abnormal aerial and root forms. Non-transgenic cv. Desiree controls are shown at either end of the row, and the transgenic lines shown from the left (second position) are Dsrd29A-5, Dsrd29A-29, Dsrd29A-41 and Dsrd29A-106. The scale bar represents 15 mm in 1A, 10 mm in 1B and 1C.

indicated between one and four fragments, whereas *Hin*dIII digestion resulted in one to eight bands (Figure 2A). The distribution of the numbers of *DREB1A* inserts in the 120 transgenic lines, using *Hin*dIII and *Dra*I, is shown in figure 2B. The most frequent number of inserts was three (37.5% and 36.7% for *Hin*dIII and *Dra*I, respectively).

Phenotypic evaluation of salinity stress

In preliminary experiments, four repeat sets comprising four cuttings per genotype were tested with 1 M NaCl treatment, using DSC and 27 transgenic lines. The salinity tolerances of these transgenic lines and the DSC, based on an arbitrary scale ranging from 1 to 5 as described in Materials and Methods, are shown in Figure 3A. To show the effects of time and to elucidate any association between time and genotype, we used an ANOVA method: a split-plot against time based on a RCBD. Table 2 summarizes these analyses and shows that the effects of the transgenic genotype were significant between the transgenic lines and DSC at the 5% level. A representative example, D22, and DSC are shown in Figure 3B.

Correlation between salinity tolerance and band number

There was no strong correlation between the copy number of the inserted gene and salinity tolerance among the 27 transgenic lines ($r=-0.17$ and -0.15 for *Hin*dIII and *Dra*I, respectively). However, when transgenic lines Dsrd29A-44 (D44) and Dsrd29A-51 (D51), which had high numbers of *DREB1A* inserts, were excluded, there was a significant correlation ($t_{cal}=3.87^{**}>t_{(5\%,\,24)}=2.064$) at a nearly high level for *Hin*dIII ($r=-0.62$) and a significant correlation for *Dra*I ($t_{cal}=2.138^{*}>t_{(5\%,\,24)}=2.064$) at a low level ($r=-0.40$), as shown in Figure 4A and B.

Selection of highly tolerant lines using Duncan's test

As the effect of the genotype in our transgenic lines was significant based on ANOVA, we compared the 27 transgenic lines that grew under salt stress in our preliminary study and DSC using Duncan's test. The results showed that the difference between transgenic and control plants was statistically significant. In Duncan's test classification, a typical grouping would be: a, ab, bc, cd, abcd, abcde,...and e, where 'a' is the group most sensitive to salinity stress and 'e' is the most tolerant. The transgenic lines and DSC were grouped based on Duncan's test, and the related average scores are shown as a bar graph (Figure 3A). Two transgenic lines, Dsrd29A-103 (D103) and Dsrd29A-132 (D132), had significantly higher tolerance to salinity than the remaining samples and constituted the 'e' group. D163

Table 1. Classification of the phenotypes of transgenic potato (cv. Desiree) lines containing the *DREB1A* transgene and the frequency of variation in ploidy.

Phenotype	Frequency of transgenic plants (%)	Ploidy variation (%)
Normal phenotype similar to non-transgenic Desiree (DSC)	52/120 (43.4)	0/52 (0.0)
Normal phenotype different from DSC (intermediate shape)	37/120 (30.8)	7/37 (18.9)
Abnormal phenotypes among Desiree transgenic lines	31/120 (25.8)	7/31 (22.6)

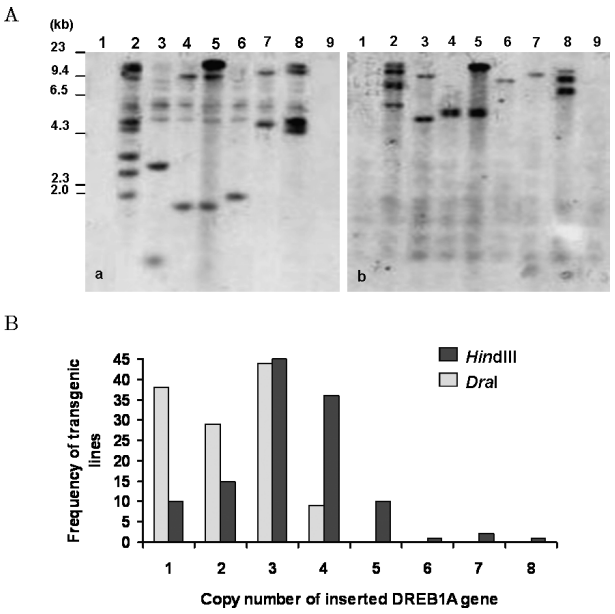

Figure 2. Representative results of Southern hybridization. A: Southern hybridization with a *DREB1A* probe following *Hin*dIII (a) or *Dra*I (b) restriction digestion of non-transgenic cv. Desiree as a control (DSC) and transgenic potato cv. Desiree genomic DNA. Lanes 1: DSC, 2: Dsrd29A-51, 3: Dsrd29A-53, 4: Dsrd29A-54, 5: Dsrd29A-125, 6: Dsrd29A-59, 7: Dsrd29A-118, 8: Dsrd29A-103, 9: DSC. *Hin*dIII-digested λ markers are in the first column on the left. B: Diagram of the frequency of band numbers based on restriction enzyme digestion. The bar graph shows the distribution of the band numbers obtained with both restriction enzymes (*Hin*dIII and *Dra*I) for 120 transgenic potato lines.

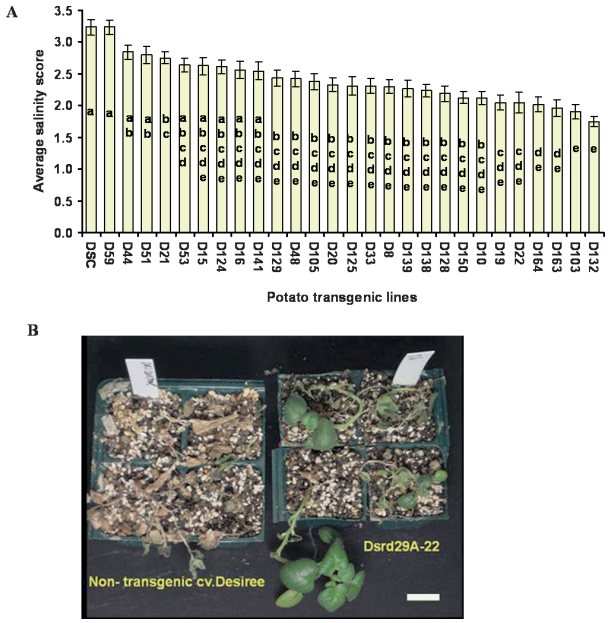

Figure 3. Comparison of the average visual scores for the transgenic lines using Duncan's test at the LSR level of 5%. A: Assessment of salinity tolerance in 27 *DREB1A* transgenic potato lines and non-transgenic cv. Desiree, with mean values of four cuttings, in the salinity test. The lines at the top of each bar indicate the respective standard error. The bar for each genotype corresponds to the average scoring data. The letters a, b, c, d, and e are used to divide the plants into recognizable groups. The abscissa refers to different independent transgenic lines (Dsrd29A), with non-transgenic cv. Desiree as a control (DSC). The ordinate shows the average salinity score based on a scale from 0 to 5, where 0 is almost no damage and 5 is complete wilt. B: Representative photos of the phenotypic evaluation of salinity stress for transgenic potato lines and non-transgenic cv. Desiree (DSC). A transgenic potato line (Dsrd29A-22) and non-transgenic cv. Desiree as a control were tested in 1 M NaCl stress for 7 days. The scale bar represents 16 mm.

and D164 (the 'de' group) and D19 and D22 (the 'cde' group) were tolerant transgenic lines (Figure 3A).

DREB1A gene expression analysis in transgenic potato using Northern hybridization

The expression of stress-inducible *DREB1A* transgene inserts under the control of the *rd29A* promoter was determined using Northern hybridization for the following transgenic lines with different levels of salt tolerance based on phenotypic evaluations (Figure 3B): highly tolerant (D103 and D132), tolerant (D22, D24, D19, D163, and D164), moderately tolerant (D138), and most sensitive (D44, D59, and DSC). Total RNA was extracted from these ten transgenic lines and DSC at the following intervals: no salt treatment and after 1, 2, 5, 10, 18, and 24 h of salt treatment. The expression profile of *DREB1A* in D103 is shown as a representative (Figure 5A). Transgenic lines with salinity tolerance showed substantially greater expression of *DREB1A* after 2 to 5 h of salinity stress, and the expression subsequently fell to low levels. In some cases, there was no expression signal, as with D19, or a low signal, as with D164. There was almost no signal with DSC.

Quantification of gene expression in the stressed transgenic lines and the association with phenotypic evaluation

Quantified data obtained using Image Gauge Software (Fujifilm) for samples of the selected transgenic lines stressed for 2 h showed varying expression among the transgenic lines and no expression in DSC. The maximum intensity was seen in D103, followed by

Table 2. ANOVA, based on a split plot against time design, for 28 selected genotypes during a 7-day salinity stress evaluation.

Source		Degrees of Freedom	Sum of Squares		Mean Squares	F[1]
Block (R)		3	5.263		1.754	
Genotype		27	98.177		3.636	4.141**
E (a)		81	71.121		0.878	
Day		6	821.070		136.845	709.041**
Day×Gen		162	33.000		0.204	1.057
E (b)		504	97.244		0.193	
	Day×R	18		1.499	0.083	
	Gen×Day×R	486		95.745	0.197	
Total		783	1023.400		1.307	

**: Indicates significance at the 1% level; [1] F=F (Fisher) standard table value for checking significance.

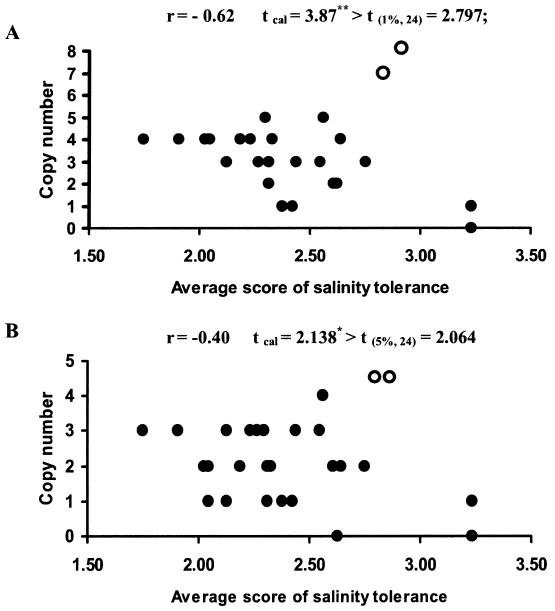

Figure 4. Diagrams of the correlation studies between the phenotypic evaluation and the number of inserted bands of the *DREB1A* gene in Southern blot analysis. Scatter diagram of the correlation between the phenotypic evaluation in the 1 M NaCl treatment for 24 transgenic lines and DSC, and the number of inserted *DREB1A* gene bands obtained using *Hin*dIII (A) and *Dra*I (B). In both cases, when all transgenic lines are included, there is no significant correlation (−0.18 and −0.15% with *Hin*dIII and *Dra*I, respectively). However, when Dsrd29A-44 (D44) and Drsrd29A-51 (D51) (the open circles with high band numbers) are excluded, the correlation rate becomes −0.62 and −0.40% with *Hin*dIII and *Dra*I, respectively, and these values are significant (*Hin*dIII: $t_{cal}=3.87^{**}>t_{(1\%,\ 24)}=2.797$; *Dra*I: $t_{cal}=2.138^{*}>t_{(5\%,\ 24)}=2.064$).

D132, with low expression in D22, D24, D138, D163, and D164, and no expression in D19, D44, D59, and DSC (Figure 5B). There was a significant correlation between the phenotypic evaluation and the quantitative gene expression among all 11 plants ($r=-0.67$, $t_{cal(df=9,\ 5\%)}=2.71^{*}>t_{tab(df=9,\ 5\%)}=2.26$), as shown in Figure 5C.

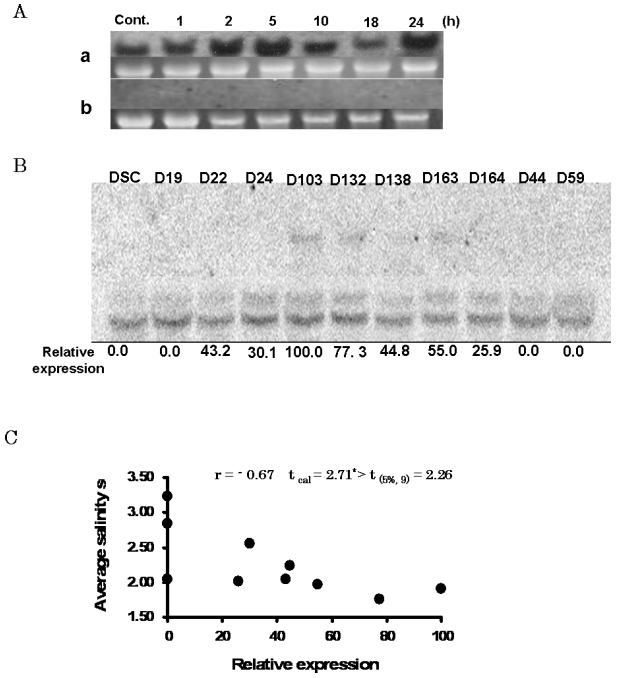

Figure 5. Northern hybridization in one representative tolerant transgenic line and non-transgenic cv, and quantification of the 2 h samples of the most tolerant and sensitive transgenic lines and DSC. A: Northern analysis of *DREB1A* expression, with ethidium bromide staining normalized to the control. a: *DREB1A* expression levels at different times following 1 M NaCl treatment of transgenic samples of Dsrd29A-103 (as a representative tolerant transgenic line). b: *DREB1A* expression levels at different times in non-transgenic cv. Desiree. No expression was detected in the control plant, whereas maximal expression was observed after 2–5 h of salt treatment. B: Northern analysis of *DREB1A* expression, with a related rRNA probe control. *DREB1A* expression levels after a 2-h exposure to 1 M NaCl for non-transgenic cv. Desiree (DSC) and transgenic lines (D19, D22, ..., etc.). No expression was detected in the control plant, whereas maximal expression was observed in the transgenic lines. C: Correlation scatter diagram of the phenotype salinity stress tolerance and Northern quantification of ten selected transgenic potato lines with *rd29A::DREB1A* and non-transgenic cv. Desiree. The X-axis shows the Northern band intensity, and the Y-coordinate is the average survival score under 1 M NaCl stress. There was a significant correlation between the phenotypic evaluation and the quantitative gene expression for all 11 lines ($r=-0.67$, $t_{cal(df=9,\ 5\%)}=2.71^{*}>t_{tab(df=9,\ 5\%)}=2.26$).

Discussion

Morphological variation in transgenic lines

Following the transformation, abnormalities were seen in some of the transgenic lines. Previous studies of stunted growth have shown the benefits of using the *rd29A* promoter instead of 35SCaMV, as it minimized the negative effects on plant growth in transgenic *Arabidopsis* (Yamaguchi-Shinozaki and Shinozaki 1993b; Kasuga et al. 1999), tobacco (Kasuga et al. 2004), and wheat (Pellegrineschi et al. 2004). To determine whether the abnormalities in the transgenic lines resulted from a transgene effect, ploidy tests were done (Table 1). The results showed that in the normally shaped plants (similar to DSC), there were no abnormalities in the ploidy level, whereas there was a genomic change in the abnormally shaped plants.

Regarding ploidy chimerism, there have been some reports of genomic changes in transgenic potato cv. Desiree (Stiekema et al. 1988). Importantly, these ploidy chimeric lines were associated with abnormal plant morphologies, such as stunt formation. Periclinal chimerism is a common occurrence in tetraploid potatoes (Wilkinson 1994) and involves further re-assortment of the initial cellular profiles in aneusomatic lines, leading to genetic changes and phenotypic abnormalities, albeit with low frequency. Therefore, the phenotypic abnormalities in our transgenic potato lines (with the *rd29A* promoter, which drives *DREB1A* gene) could be largely related to the influence of chimeras or regeneration events rather than transgene expression. Furthermore, the salinity tolerance was no higher in the anomalous-looking plants, and there was no obvious association between the transgene expression level and abnormal phenotypes.

Copy number of transgenes and phenotypic salt stress tolerance

Pellegrineschi et al. (2004) analyzed 12 transgenic lines of wheat by Southern blotting, using *Sac*I digestion and probing with a 428-bp fragment of the *DREB1A* gene, and found from five to ten bands, whereas we observed between one and four fragments for digestion with *Dra*I and between one and eight with *Hin*dIII (Figure 2). The Southern hybridization results indicate the complexity of the transgene integration among different independent transgenic lines (Pellegrineschi et al. 2004).

There is no clear reason for different band numbers using *Dra*I and *Hin*dIII. It may be because of incomplete introgression of the transgene, which means that the restriction enzyme can find more than one restriction site at which to cut the genome, especially in the case of *Hin*dIII. We demonstrated a correlation between the number of fragments and the average salt tolerance score (Figure 4). However, given that the fragment number based on restriction digestion roughly estimates the number of inserted copies, salinity tolerance depends on the number of inserted copies. The correlation between fragment number and salinity tolerance (Figure 4) suggests that one inserted copy of the *DREB1A* gene conferred salinity tolerance to transgenic potato lines and that increasing the *DREB1A* insert copy number increased the salinity tolerance in the transgenic potato lines, with some exceptions. A high copy number (7 or 8) based on *Hin*dIII digestion in the Southern blot analysis was associated with reduced salinity tolerance in D51 and D44. It may be that high-copy introgression causes gene silencing, as occurs in diploid plants like *Arabidopsis thaliana* (Yamaguchi-Shinozaki and Shinozaki 1993a). Various fragment patterns were obtained following *Dra*I and *Hin*dIII digestion of our transgenic lines, and these were not informative with regard to the region of insertion of the *DREB1A* gene in the tetraploid potato genome. Additional analyses, such as positional effect variegation studies, are needed to examine the association with copy number. Note that the positional effects of transgenes can cause phenotypic variation via altered gene expression with a single-copy insert (Celebi-Toprak et al. 2005).

The results of the phenotypic evaluation strongly suggest an association between tolerance to salinity and the *DREB1A* gene in the model polysomic polyploidy plant *S. tuberosum*. The phenotypic evaluation indicates that the differences in the response to salinity stress can be attributed to genotypic variation. Moreover, the significant difference in the tolerance levels of the transgenic lines and non-transgenic cv. Desiree further indicates that there is some induction of stress responses by the transgene in the transgenic lines.

Gene expression and association with phenotypic evaluation

In the gene expression studies, under the control of the *rd29A* promoter, low levels of *DREB1A* expression were evident during unstressed conditions in the transgenic lines, and these were induced rapidly to high levels during exposure to salinity stress. In the transgenic potato lines, the expression level became high within 2–5 h, decreased by 10–18 h, and increased markedly after 24 h (Figure 5A). This is consistent with previous studies (Kasuga et al. 1999). The data show that the *rd29A* promoter is effective with the *DREB1A* gene in transgenic potato. As the *rd29A* promoter contains DRE, a cis-acting element (Yamaguchi-Shinozaki and Shinozaki 1993a), it can activate the *DREB1A* gene and ultimately activate DREB1A protein, which acts as a transcription factor regulating the activation of other genes that confer tolerance against abiotic stresses to the transgenic plants. Recently, Maruyama et al. (2004) determined the cold-regulated genes downstream from

DREB1A in *Arabidopsis* and performed an array analysis using the approximately 7,000 RAFL cDNA microarray and the approximately 8,000 Affymetrix Gene Chip array. They selected 19 and 32 genes as candidate downstream genes of DREB1A protein using the respective microarrays. Previously, it was shown that DRE in the *rd29A* promoter also functions in response to stress in tobacco plants (Yamaguchi-Shinozaki and Shinozaki 1993b). DRE-related motifs have also been reported in the promoter regions of cold-inducible *Brassica napus* and wheat genes (Jiang et al. 1996; Ouellet et al. 1998). Hence, it is possible that similar mechanisms exist in transgenic potato lines, although additional analysis of both the dehydration and freezing stress responses in this plant are needed to confirm this.

Furthermore, quantification of the gene expression in the transgenic lines within 2 h of stress (Figure 5B), and the association of salinity stress with the phenotypic data showed a linear correlation between gene expression and phenotypic evaluation ($r = -0.67$), indicating that the phenotypic evaluation of salinity stress tolerance was supported by the gene expression data (Figure 5C).

We have reported tolerance to salinity stress (1 M NaCl) and some provisional results on the tolerances to dehydration and freezing stress (data not shown) in tetrasomic tetraploid potato. These results suggest that the gene transfer of *DREB1A* cDNA and the *rd29A* promoter could also be used to improve the dehydration, salt, and freezing tolerance of agriculturally important crops, such as the potato. As difficulties have been observed using the 35S CaMV promoter with the *DREB1A* gene construct in *A. thaliana* (Kasuga et al. 1999), the use of the stress-inducible *rd29A* promoter appears of cardinal importance in overcoming the problem of growth retardation, as shown in this study.

Some of the transgenic potato lines showed significantly higher resistance to salt stress than the controls, indicating that gene transfer has the potential to improve the growth and yields of this important crop. In addition, the stability of the *DREB1A* expression was evident over several propagation periods using independent transgenic lines, suggesting that such transgenic potato lines could be used in modern plant breeding programs and propagated as new varieties. Furthermore, previous reports have highlighted the importance of the use of the stress-inducible *rd29A* promoter to overexpress *DREB1A* for improving drought, salt, and freezing stress tolerance in transgenic *Arabidopsis* (Yamaguchi-Shinozaki and Shinozaki 1993a, b), tobacco (Kasuga et al. 2004), rice (Dubouzet et al. 2003; Rabbani et al. 2003), and wheat (Pellegrineschi et al. 2004), along with further efforts to test other crop species as part of an international consortium (GCP 2005. http://www.generationcp.org/index.php).

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