Breeding Science 55 : 311–319 (2005)

Tolerance to Salt Stress of the Transgenic Tetrasomic Tetraploid Potato, *Solanum tuberosum* cv. Desiree Appears to be Induced by the *DREB1A* Gene and *rd29A* Promoter of *Arabidopsis thaliana*

Fevziye Celebi-Toprak[1,4], Babak Behnam[1], Gustavo Serrano[2], Mie Kasuga[3], Kazuko Yamaguchi-Shinozaki[3], Hiroshi Naka[5], Junko A. Watanabe[1,5], Shinsuke Yamanaka[1,6] and Kazuo N. Watanabe*[1,5]

[1] *Gene Research Center and Graduate School of Life and Environmental Sciences, University of Tsukuba*, 1-1-1 Tennoudai, Tsukuba, Ibaraki 305-8572, Japan

[2] *Graduate School for Biosystem Studies, University of Tsukuba*, 1-1-1 Tennoudai, Tsukuba, Ibaraki 305-8572, Japan

[3] *Biological Resources Division, Japan International Research Center for Agricultural Sciences*, 1-1 Oowashi, Tsukuba, Ibaraki 305-8686, Japan

[4] *Present address*: *Department of Biology, Division of Molecular Biology, Pamukkale University*, Denizli, Turkey

[5] *Present address*: *Department of Biotechnological Science, Kinki University*, 930 Nishi-Mitani, Uchita, Wakayama 649-6493, Japan

[6] *Present address*: *Genebank, National Institute of Agrobiological Resources*, 2-1-2 Kannondai, Tsukuba, Ibaraki 305-8602, Japan

Tetraploid potato cv. Desiree was transformed using an *Arabidopsis thaliana* stress-inducible promoter *rd29A* and the *DREB1A* gene, which confers multiple tolerances to abiotic stresses (e.g., dehydration and elevated soil salinity). Transformed Desiree lines showed a direct correlation between the *DREB1A* expression levels and the tolerance to salinity. By producing a filial progeny from the transgenic lines which was considered to harbor a single copy of the *DREB1A* gene, crossed with a non-transgenic cultivar, the genotype at the transgenic locus of these tetrasomic tetraploids was estimated by chi-square test, suggesting the existence of a Simplex mode. The results showed that low-copy heterozygous loci with the *DREB1A* gene could sustain the expression, and consequently confer a significant tolerance to salinity in tetrasomic tetraploid potatoes.

Key Words: *DREB1A*, potato, polysomic polyploid, *rd29A*, salt stress.

Introduction

Plants are exposed to a large number of environmental stresses, including drought, soil salinity and cold. Because of these stresses, plant growth and yield can be considerably reduced (Boyer 1982, LeRudulier *et al.* 1984, Skriver and Mundy 1990, Stockinger *et. al.* 1997, Thomashow 1993, Yancey *et al.* 1982). To overcome these stresses, plants respond with biochemical, physiological and developmental changes. For instance, drought-stressed plants produce metabolic compounds with a low molecular weight, such as sugar alcohols and specific amino acids (Artus *et al.* 1996, Greenway and Munns 1980, Maqbool *et al.* 2002, Sheveleva *et al.* 1997, Shinozaki and Yamaguchi-Shinozaki 2000, Yancey *et al.* 1982). Not surprisingly, under stress conditions, changes in gene expression patterns have been observed (Bray 1997, Ingram and Bartels 1996, Seki *et al.* 2002, Shinozaki and Yamaguchi-Shinozaki 1996, 1997), and it has also been suggested that abscisic acid (ABA) plays an important role in plant response to stress (Bray 1997, Skriver and Mundy 1990). However, not all the stress-related genes respond to ABA (Yamaguchi-Shinozaki *et al.* 1992). For example, Shinozaki and Yamaguchi-Shinozaki (1997) showed that at least four independent signal pathways are involved in dehydration-induced gene expression. Two of these responses appear to be ABA-dependent, while the other two appear to function independently of ABA.

As part of their search for stress signal transduction pathways in plants, Yamaguchi-Shinozaki and Shinozaki (1993) identified and characterized the expression of a desiccation-responsive gene (*rd29*), which is induced by desiccation, cold and high-salinity conditions in *Arabidopsis thaliana*. Yamaguchi-Shinozaki and Shinozaki (1994) analyzed the promoter of the *rd29A* gene in transgenic plants, and identified a novel *cis*-acting element now referred to as the dehydration-responsive element (DRE), which consists of the 9 bp sequence TACCGACAT. DRE is involved in the induction of the *rd29A* gene under dehydration, high salt and cold stress conditions, but acts independently of ABA. In addition, at least two other dehydration-responsive element genes, *DREB1A* and *DREB2* have been cloned. And, protein products from both genes appear to function like *trans*-acting factors in stress-induced transduction pathways (Liu *et al.* 1998).

Communicated by T. Mikami
Received November 2, 2004. Accepted February 20, 2005.
The first two authors contributed equally.
*Corresponding author (e-mail: nabechan@gene.tsukuba.ac.jp)

Previously, genes related to many different stresses were transferred using recombinant methods into various plants to improve stress tolerance (Goddijin *et al.* 1997, Holmberg and Bulow 1998, Huang *et al.* 2000, Kasuga *et al.* 1999, Kavi Kishor *et al.* 1995, Maqbool *et al.* 2002, Pilon-Smits *et al.* 1995, Xu *et al.* 1996). However, these plant species were mainly diploid, and these are few examples of polyploid crops like potato that had been successfully transformed to express stress tolerance genes. Furthermore, previous transgenic plants harboring *DREB* genes under the control of the commonly used 35S CaMV promoter produced stunted plants (Yamaguchi-Shinozaki and Shinozaki 1993). However, Yamaguchi-Shinozaki and Shinozaki (1993) were able to obtain normal size transgenic *Arabidopsis* plants with a normal shape when the *DREB1A* gene was placed under the inducible control of the *rd29A* promoter.

In the present study, we transformed tetrasomic tetraploid potatoes with the *DREB1A* gene under the control of the stress-inducible *rd29A* promoter to determine whether polyploid transgenic potatoes could display relevant expression of the gene and consequently increased tolerance to elevated levels of salinity. In addition, quantitative real-time reverse transcription PCR (TaqMan) was used to correlate the *DREB1A* mRNA levels with the apparent tolerance to salinity.

Materials and Methods

Production and identification of transgenic lines

Agrobacterium-mediated transformation was conducted using the tetrasomic tetraploid potato, *Solanum tuberosum*, cv. Desiree, and *A. tumefaciens*, LBA4404 according to the method of Inui *et al.* (2000). The binary vector contained the *rd29A* promoter and a *DREB1A* cDNA clone (Kasuga *et al.* 1999) that was constructed in pBE2113 at the *Not* I site (Mitsuhara *et al.* 1996). To construct *rd29A:DREB1A*, a *Bam* HI fragment of the *DREB1A* cDNA was cloned into the *Bam* HI site of the pBI29APNot vector, which was constructed by ligation of the *Hind* III site of the rd29A promoter into the *Hind* III site of the pBE2113 vector.

Polymerase chain reaction (PCR)

For the initial screening of the transgenic lines, the following PCR primers derived from *DREB1A* were used: forward, 5′-TGATTATATTCCGACGCTTG-3′ and reverse, 5′-TTCATGATTATGATTCCACT-3′ (Kasuga *et al.* 1999). The amplification reactions were performed in 25 µl of a reaction mixture containing 1x Buffer A, 3 mM of $MgCl_2$, 200 µM of dNTPs, 0.025 U/µl of AmpliTaq Gold DNA polymerase, and *DREB1A* reverse and forward primer concentrations were 200 nM. PCR was performed through 34 cycles using the following conditions: denaturation at 94°C for 1 min, annealing at 50°C for 30 sec, extension at 72°C for 2 min with final extension at 72°C for 5 min.

Refinement of the PCR profile was made to confirm *DREB1A* gene integration into selected transgenic potato lines. The same primer pairs and cocktails as those in the initial screening were employed, but, the profile was altered as follows: amplification was performed in an iCycler (BIORAD) programmed at 94°C for 5 min, followed by 35 cycles of denaturation at 94°C for 1 min, annealing at 52.9°C for 2 min, amplification at 72°C for 3 min, and primer extension at 72°C for 10 min. Existence of PCR product was checked by electrophoresis on 2% agarose gel using 1x TAE buffer.

Southern blotting

Southern blot hybridization was used to confirm the relative number of insertion sites in the transgenic potato plant genomes. Potato leaves from transgenic and non-transgenic plants were harvested for DNA extraction, as described by Bernatzky and Tanksley (1986), except that sodium bisulfite (3.8 g/l) was used instead of mercaptoethanol. Plant genomic DNAs were digested with the restriction enzymes *Hin*dIII and *Dra*I (Promega), and 7–10 µg of digested DNA was loaded and separated onto 1% agarose gels in 1x TAE buffer (40 mM Tris-acetate and 1 mM EDTA). Gels were then subjected to Southern analyses, as described by Sambrook *et al.* (2001). Probe from the 500 bp PCR product of *DREB1A* (Accession no. AB013815, +208 – +748) was used for hybridization to these filters.

All the filters were pre-hybridized for 15 min–2 hr in 28 ml hybridization buffer at 58°C. The probe was labeled according to the manufacturer's instructions (Amersham Pharmacia Biotech Inc.). Hybridization was carried out overnight at 58°C in a hybridization oven. Filters were washed in a primary washing buffer at 58°C and 55°C, respectively. Signals were detected using the AlkPhos kit (Amersham Pharmacia Biotech Inc.) and exposed to X-ray films.

Northern blotting

Northern blot hybridization was used to confirm the occurrence of gene expression in the transgenic potato plants. Chosen transgenic and control lines were exposed to salinity stress. Leaf samples were taken before the treatment, 30 min, 1 hr and 2 hr after the 2 M NaCl treatment. The samples were frozen in liquid nitrogen. Total RNA was extracted from frozen plant material using the Promega total RNA isolation system according to the manufacturer's instructions (Promega, Madison, WI, USA). RNA concentrations were measured using a spectrophotometer at OD 260.

For each plant analyzed, 10 µg of RNA was loaded per lane and separated on a 1% agarose gel in 1x MOPS buffer. Gels were then subjected to Northern analysis, as described by Sambrook *et al.* (2001). Probes from the 150 bp PCR product of *DREB1A* and rRNA 18S, as an internal control, were used for hybridization to each filter.

All the filters were pre-hybridized for 2 hr in 16 ml hybridization buffer at 58°C. The probes were labeled according to the manufacturer's instructions (Amersham Pharmacia Biotech Inc., MA, USA). Hybridization was carried out overnight at 58°C in a hybridization oven. Filters were washed with primary washing buffer first at 58°C and then at 55°C in a water bath on a 60 rpm shaker, and subsequently

twice with second washing buffer at room temperature. Hybridized membranes were analyzed by autoradiography using an X-ray film (Kodak).

Ploidy level analyses

Aliquots of approximately 0.5 cm^2 of fresh leaves from all the transgenic lines and non-transgenic control plant were collected and put into separate plastic petri dishes. All the solutions were supplied by and their contents corresponded to the description given by the manufacturer of Ploidy Analyser (Partech, Germany). Five hundred µl of extraction buffer was added to leaf samples which were chopped with a sharp razor blade. Then, the liquid was transferred with a disposable pipette into sample tubes through a 50 µm mesh filter (Partec, Germany). Staining solution (1.6 ml) was added to the test tubes and the samples were incubated for 30–60 sec, after which the samples were read on a Ploidy Analyser instrument (Partech, Germnay).

Salinity test

NaCl concentrations for the experiments were determined during preliminary experiments. Transgenic and non-transgenic control plants were grown in 10 cm pots filled with a 3 : 1 : 1 ratio of soil, peat moss and vermiculite, respectively. They were grown under 16/8 hour of illumination/darkness at approximately 150 µmol photon/m^2/sec at 25°C. Five sets of 4 cuttings from each of the 4–6 week old different transformed plants were put in 4 cm square size planting cells. Two-week-old plant cuttings were watered with 20 ml of a 2 M NaCl solution overnight and positive and negative control plant cuttings were watered with 20 ml tap water. On the following days, plants were watered with 20 ml tap water and observed for 6 days. Plants were scored each day using a scale of 0 to 5 (0 = plant standing, no damage on leaves or stems, 1 = plant standing, minimal damage on leaves or stems, 2 = plant standing, some damage on leaves or stems, 3 = plant weakly standing, some damage on leaves, 4 = plant not standing, some damage on leaves, 5 = plant not standing-death).

Data analysis

Physiological variables were tested by analysis of variance (ANOVA). Treatment means were compared using Fisher's protected LSD test.

Quantitative reverse transcrition polymerase chain reaction (RT-PCR)

All the reagents used were derived from the TaqMan Gold RT-PCR kit without control. Two sets of primers and probes were designed within the *DREB1A* gene coding region, according to the guidelines from the TaqMan probe and primer design protocol. The probes were labeled with FAM from the 5′end and TAMRA from the the 3′end. rRNA 18S was used as an internal control, and its probe was labeled with VIC from the 5′end and with TAMRA from the 3′end. Two-steps RT-PCR were performed in two separate reactions using the UNG enzyme. The relative standard curve method was used to quantify the *DREB1A* mRNA level (ABI PRISM 7900HT Sequence Detection System User Guide and User Bulletin No. 2) (updated 2001).

Reverse transcription PCR procedure for making the cDNA probe

The cDNA amplification reactions were performed in 50 µl of a reaction mixture containing 1x TaqMan Buffer A, 5.5 mM of $MgCl_2$, 500 µM of dNTP, 2.5 µM of Random hexamers, 0.4 U/µl of RNase inhibitor, 3.125 U/µl of MultiScribe Reverse Transcriptase. Thermal cycling conditions were: 25°C for 10 min, 37°C for 60 min and 95°C for 5 min.

Quantitative PCR procedure

The amplification reactions were performed in 384-well plates with 20 µl of a reaction mixture containing 1x TaqMan Buffer A, 5.5 mM of $MgCl_2$, 200 µM of dATP, dGTP, dCTP, 400 µM of dUTP, 0.01 U/µl of AmpErase (UNG) and 0.025 U/µl of AmpliTaq Gold DNA polymerase. Final rRNA 18S reverse and forward primers, and probe concentrations were 100 nM and 200 nM, respectively. Final *DREB1A* reverse and forward primers, and probe concentrations were 300 nM and 250 nM, respectively. Thermal cycling conditions were: 50°C for 2 min, 95°C for 10 min, and 95°C for 15 sec, and 60°C for 1 min.

Results

Transformation of potato plants with the DREB1A gene

One hundred regenerants were obtained after the *Agrobacterium*-mediated transformation. These regenerants were preliminarily evaluated by the polymerase chain reaction (PCR) and were tested in MS liquid media containing 1 M NaCl. Seventy-eight regenerants were identified as transgenic lines by PCR. As for the *in vitro* salt tolerance tests, the reaction to the salinity varied among the PCR-positive lines, and in the optimum category, no wilt was observed and the appearance was identical with that of the plantlets that had not been treated with salt. Nine lines that showed the highest tolerance to salt were chosen from the seventy-eight transgenic lines. In addition, one regenerant tolerant to salt, but lacking the *DREB1A* gene, based on the PCR analysis, was selected to act as a control. Thus, ten genotypes were selected for further studies and were designated as: DS-29AHS-6, -12, -47, -57, -78, -88, -91, -92, -93 with DS-29AHS-50 as non-transgenic salt-tolerant regenerant.

Southern analyses

Figure 1 shows the Southern analysis of the selected transformants using two independent treatments with the restriction enzymes, *Hin*dIII and *Dra*I, (the *DREB1A* gene lacks restriction sites for both enzymes). The results suggested that one or two genomic insertions of the *DREB1A* gene occurred in different transformed lines. Though high stringency conditions were used, weak homologue signals were observed in the non-transgenic control cv. Desiree, while clear

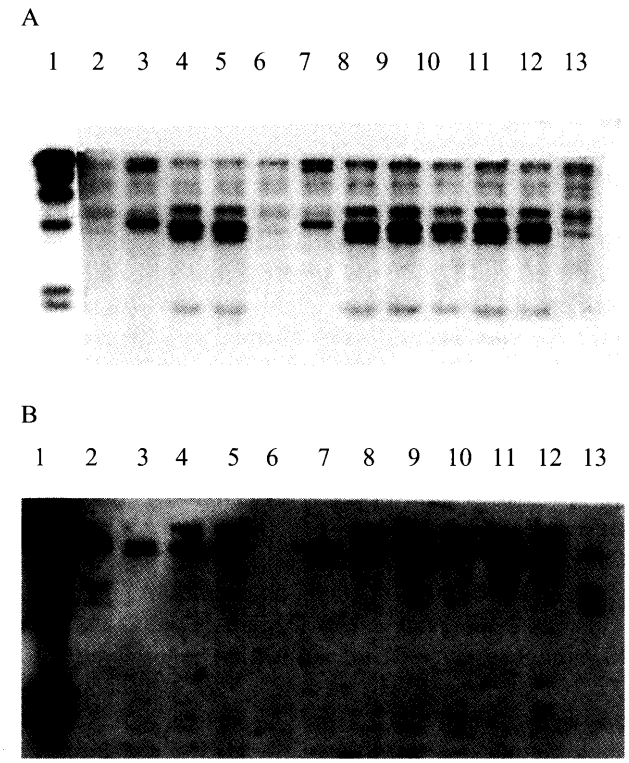

Fig. 1. Southern blot analysis shows the inserted *DREB1A* gene copy numbers. A *Hind*III and B. *Dra* I enzymes were used. Lanes 1 to 13 correspond to 1: Marker (λ-*Hind* III), 2: non-transgenic control , 3: DS-29AHS-6, 4: DS-29AHS-12, 5: DS-29AHS-47, 6: DS-29AHS-50 non-transgenic regenerant, 7: DS-29AHS-57, 8: DS-29AHS-78, 9: DS-29AHS-88, 10: DS-29AHS-91, 11: DS-29AHS-92, 12: DS-29AHS-93, and 13: non-transgenic control, respectively

differences were detected in the PCR-positive transgenic lines. Thus, it was assumed that a low copy number of transgenic insertions occurred in the transformed regenerants.

Ploidy level analysis

Ploidy levels of the transgenic lines were tested by flow cytometry. All the transgenic lines and non-transgenic plants were tetraploid. This was confirmed by chromosome counts from root tip samples.

Phenotypic observation for salinity stress

Five sets consisting of four cuttings/set were tested with 2 M NaCl for each of the 9 different transgenic lines, non-transgenic regenerant line DS-29AHS-50 and control Desiree. To determine whether the *DREB1A* gene conferred any beneficial effect on the survival of the trangenic plants under salinity stress conditions, a 0 to 5 scale was used to measure the plant responses. As outlined in the Materials and Methods section, "0" indicates full tolerance whereas "5" indicates complete plant death. Before averaging the results from 5 sets of cuttings for each day identical with those of the plants without salt treatment, ANOVA test was performed to determine the existence of any physiological differences among the 5 sets of 4 cuttings in each transgenic line. No statistical differences were detected among the sets.

Figure 2 shows the results from the cuttings for each transgenic line. ANOVA and Fisher's protected LSD tests were also performed to compare transgenic lines among themselves and with the control plants. The difference between the transgenic lines and control plants was statistically significant at the 1% level (Table 1). Comparison among the transgenic lines showed that the following lines were significantly more tolerant to salinity: on the first day, lines DS-

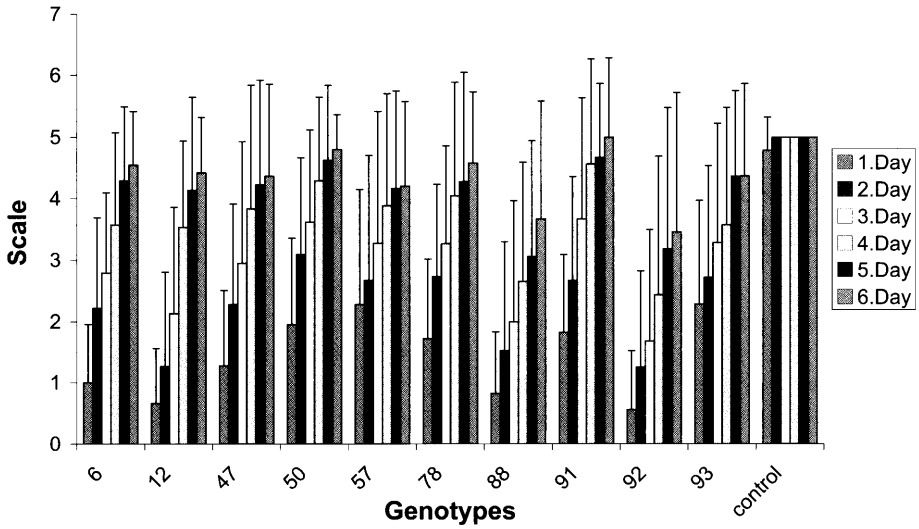

Fig. 2. Mean values of salinity test on 1–6 day cuttings. The lines on top of each bar indicate the respective standard deviation. The 6 bars for each genotype, from left to right, correspond to the first, second, third, fourth, fifth and sixth days, respectively. The abscissa refers to different transgenic lines (DS-29AHS) and control plants. The ordinate shows the scale 0–5: 0, no stress to 5 complete wilt, respectively.

Table 1. Fisher (F) test and least significant difference among genotypes in relation to salinity tolerance over six days

	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6
F	**	**	**	**	**	**
LSD	0.53	0.49	0.51	0.51	0.48	0.55

** indicates significance at the 1% level.
LSD = least significant difference

29AHS-6, -12, -47, -88, -91 and -92, on the second day, lines -12, -88, -91 and -92, on the third day, -12, -88 and -92, on the fourth, fifth and sixth days, only lines -88 and -92 (Fig. 3). Of salt stress tests, however, the correlation coefficient matrix indicated that there were discrepancies among the observations on different days (Table 2).

Northern hybridization

Total RNA was extracted from the stressed transgenic individuals of lines DS-29AHS-12, -88 and -92, the non-transgenic regenerant –50 and control cv. Desiree. Northern analysis suggested the existence of an association between the early-induced expression levels of *DREB1A* mRNA and the tolerance level among the transgenic lines (Fig. 4). Transgenic

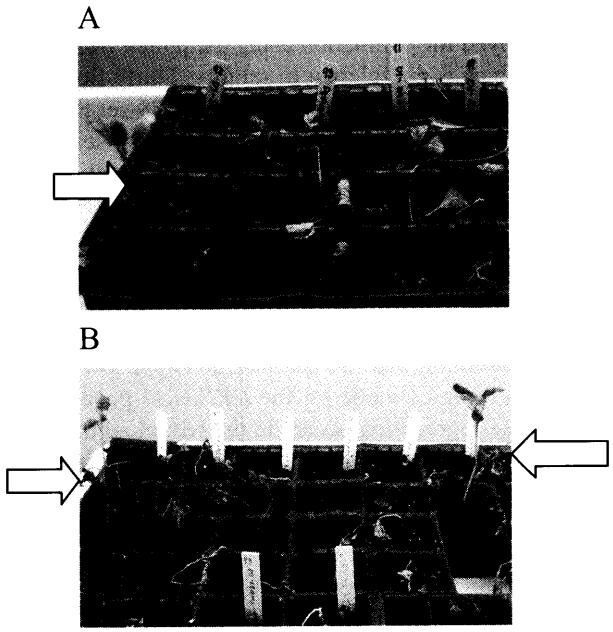

Fig. 3. Pictures A and B show the plants or 6 days after treatment with 2 M NaCl. Plants from the Lines DS-29AHS-92 and DS-29AHS-88 are still standing without any damage (arrows).

Table 2. Correlation coefficients from coefficient of variation in transgenic potato genotypes, the non-transgenic regenerant line 50 and Desiree control for salinity tolerance

	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6
Day 1		0.95**	0.93**	0.85**	0.83**	0.76**
Day 2			0.96**	0.82**	0.82**	0.77**
Day 3				0.92**	0.92**	0.89**
Day 4					0.95**	0.97**
Day 5						0.93**
Day 6						

** indicates significance at the 1% level.

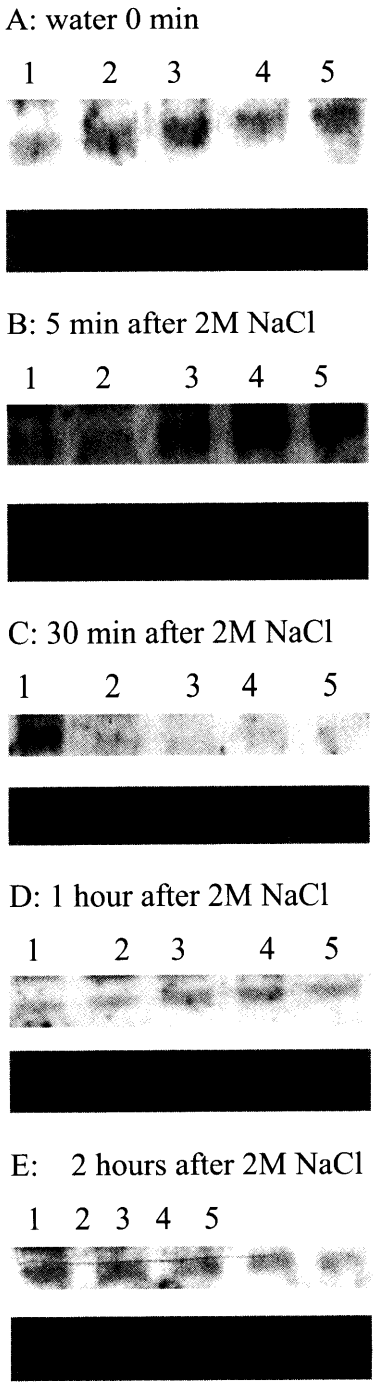

Fig. 4. Northern hybridization for leaf samples obtained from salt-stressed cuttings of transgenic potatoes with *rd 29A* promoter and *DREB1A1* gene. Upper photo: mRNA, Lower photo: rRNA. 1. Non-transgenic control Desiree, 2. Non-transgenic regenerant DS-29AHS-50, 3. DS-29AHS-12, 4. DS-29AHS-88, 5. DS-29AHS-92. A: water 0 min, B to E: 5 min, 30 min, 1 hr and 2 hr after 2 M NaCl treatment.

lines with salinity tolerance showed a substantially higher expression level at 5 min after exposure to salt stress, though afterwards the expression level appeared to decrease.

Quantitative reverse transcription PCR

Regular reverse transcription PCR was conducted for all the tested genotypes to confirm the existence of gene expression by the induction of salinity stress. Using phenotypic observation of salinity tests, lines DS-29AHS -12, -50, -88 and the non-transgenic control plants were selected to investigate any difference in the mRNA expression level among the transgenic lines. To determine the *DREB1A* mRNA expression levels, the selected lines and the non-transgenic control were treated with 2 M NaCl. Samples were taken before the treatment, 30 minutes, 1 hr and 2 hr after the 2 M NaCl treatment.

Relative standard curve method

Quantitative reverse transcription PCR analyses showed the existence of a correlation between the expression level of the *DREB1A* gene mRNA and the tolerance level among the transgenic lines (Fig. 2 and Fig. 5). Two standard curves were obtained from the amplification of total rRNA 18S and the *DREB1A* primers and probes. Unknown samples were also amplified by using rRNA 18S and the *DREB1A* primers and probes (in separate reactions). The relative amount of *DREB1A* mRNA was quantified by linear extrapolation of the Ct values using the equation to the line obtained from the *DREB1A* standard curve and ABI 7900HT software. Average values were recorded from 6-well replicates for each unknown sample. To normalize these average values, they were divided by the average values of their internal control.

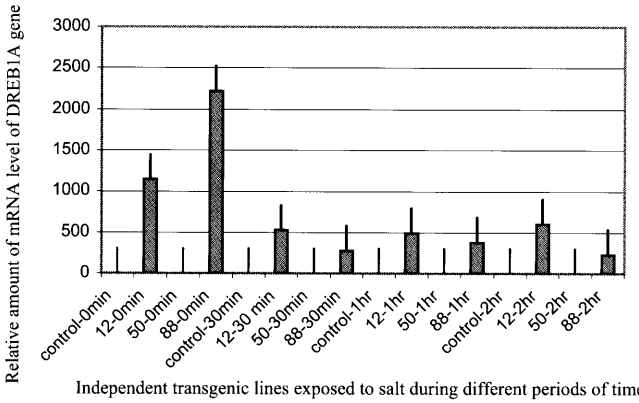

Fig. 5. Real time quantitative reverse transcription PCR results. The lines on top of each bar indicate the respective standard deviations. The abscissa refers to two transgenic lines, DS-29AHS-12 (12) and DS-29AHS-88 (88), a non-transgenic regenerant line (50), DS-29AHS-50 and Desiree control (C) plants treated with 2 M NaCl for different periods of time. (0) indicates the onset of the treatment, (30): 30 min after, (1): 1 hr after, and (2) : 2 hr after, respectively. The ordinate shows the relative amount of *DREB1A* gene in plants of different lines in relation to the control plants just before the treatment.

The normalized numbers represent the relative amount of mRNA in the unknown samples.

In order to compare the relative amount of *DREB1A* mRNA in different samples, we designated the non-transgenic control plant as a calibrator. Each normalized value of *DREB1A* from different samples was divided by the value in the normalized non-transgenic control plant. These data indicate that the lines in decreasing order of relative level of *DREB1A* mRNA expression were as follows: DS-29AHS -88, -12, - 50 and the control (Fig. 5).

Segregation of phenotypes with salinity tolerance in the filial progeny

It was recognized that the induction of flowering in cv. Desiree was rather difficult in Japan even under artificial conditions. However, using various techniques previously reported (reviewed in the report of Golmirzaie *et al.* 1994), attempts were made to obtain fertile flowers in the selected transgenic lines. Although three transgenic lines (DS-29AHS-12, -57 and -88) out of the nine selected genotypes flowered under the given treatment, however, all of them were male-sterile. All the three flowering transgenic lines were employed for pollination using the pollen from the cv. Atlantic, which is not tolerant to salinity. Thirty flowers per genotype were pollinated and DS-29AHS-88 set three berries which contained seeds, and a total of 122 seeds were collected. The seeds were germinated and 76 seedlings were transplanted into single cell planters. These plants were checked for ploidy by flow cytometry, and three cuttings per seedling were tested for tolerance to salinity at 2 M NaCl.

Chi-square test for goodness of fit was conducted to analyze the segregation mode in the filial progeny for salinity tolerance assuming that the parental genotype DS-29AHS-88 shows a Simplex mode for the *DREB1A1* gene and that chromosome segregation occurs in the tetraploid transgenic line. The expected tolerance ratio in the progeny was 1 : 1 for tolerant : susceptible, and the observed phenotype in the true seed progeny was 32 tolerant and 44 susceptible, respectively. Chi-square value which was 1.895, was significant at the 5% level. Therefore, a Duplex mode was rejected. Thus, it is likely that the genotype of the transgenic parental line DS-29AHS-88 shows a Simplex mode, namely that a single transgene allele is present. These findings suggest the induction of a significantly salt tolerant phenotype by a single transgene at the tetraploid level, and also in the filial tetraploid progeny.

Discussion

Phenotypic variation and association with copy number of the gene

In the present study, we were able to produce nine *DREB1A* transgenic lines that showed a relatively strong tolerance to 2 M NaCl out of seventy-eight transgenic lines. Interestingly, since one regenerant was salt-tolerant in spite of lacking the transgene, it was used as a control along with

parental Desiree. The nine transgenic potato plants and the non-transgenic regenerant line differed in their tolerances to 2 M NaCl. Two lines, DS-29AHS -88 and -92 showed the highest survival rates and some cuttings did not experience any damage from the exposure to 2 M NaCl. These variations among the lines could be attributed to the position effect of the *DREB1A* gene insertion and insert copy number. Also some transgenic lines showed the same banding patterns (Fig. 1), suggesting that there may be a hot-spots at the insertion positions followed by a high expression/tolerance in the genome.

As for the insertion copy number, Southern hybridization analysis indicated that there was no clear association between the insert copy number and the level of stress tolerance, which a low copy number i.e., one or two insertions per genome, appeared to be sufficient in polysomic tetraploid cv. Desiree to confer robust and durable salt tolerance. It was reported that transgene expression can be induced by gene silencing, and phenotype expression can be altered by posttranscriptional and posttranslational modifications (Angell and Baulcombe 1997). In our study, it was also observed that transgenic lines with multiple copy insertions did not display higher levels of salt tolerance. In general, position effects have not been elucidated in terms of the gene expression and consequently phenotype for stress tolerance.

Copy number and phenotype expression in tetrasomic tetraploid potatoes

The genetics of out-crossing polysomic polyploids is far more complex than that of self-pollinating homologous diploids (Watanabe *et al.* 1999). In tetrasomic tetraploid potatoes, four alleles are present per locus, and single allelic dominance for genotypes with a Simplex mode has been observed at specific loci, such as plant disease resistance genes (Celebi-Toprak *et al.* 2005). However, there are few examples of single-copy expression of specific phenotypes for diverse traits in potatoes in the case of genes induced abiotic stresses.

Requirements for sufficient expression of phenotype in potatoes may be more complex for gene regulation of the transgenes compared with those in diploid plants (Brown *et al.* 1991, Watanabe and Watanabe 2000). The Southern hybridization results which we obtained for the selected transgenic lines with a high salinity tolerance indicated the existence of transgene insertion of single or two copies in the tetraploid potato genome. The evaluation of the phenotypic segregation in the progeny from the transgenic genotype, DS-29AHS-88, indicated that the transgene locus for the DS-29AHS-88 genotype showed a Simplex mode. Thus, a single allele of the *DREB1A* transgene appeared to be enough to be sufficient the expression of a relatively high degree of salt tolerance at the tetraploid level, although this analysis would require the use of other transgenic lines.

In previous reports, all the transgenic lines with salinity tolerance derived from *Arabidopsis*, rice and tobacco, were homozygous. Thus, in the present experiment it was reported for the first time that a single transgenic allele could sustain relevant tolerance to salinity in polysomic polyploids. Also the association with the cis-trans relationships within the genome remains to be elucidated. The expression was correlated with the phenotype level for the salinity tolerance. Thus, low-copy heterozygous loci of *DREB1A* could sustain the expression, and confer a strong enough tolerance to salinity in tetrasomic tetraploid potatoes.

It should also be emphasized that our present report indicated that a high level of stable stress tolerance could be induced in higher plants with genetic complexity due to polyploidy, compared with previous studies on the production of transgenic plants conferring gene(s) inducing salt stress tolerance. While the expression profile of the transgene in highly heterozygous tetrasomic tetraploids remains to be elucidated, it is important to compare genetic characteristics between diploid and polyploid plants in reference to the reports on *Arabidopsis* (Seki *et al.* 2002) and rice (Rabbani *et al.* 2003), both of which are highly homozygous diploid plants.

Phenotype association with expression level of the DREB1A gene

Quantitative reverse transcription PCR analyses showed the existence of a correlation between the expression level of *DREB1A* gene mRNA and salt tolerance among the transgenic lines (Fig. 5), though some variation was observed among the transgenic lines. For example, in the non-transgenic line and in line DS-29AHS -50 gene expression was not revealed, while DS-29AHS-12 and -88 showed different expression levels at different treatment times. In the 0 min comparison between the lines DS-29AHS -12 and -88, line -88 was gauged a 1.94 fold higher *DREB1A* expression level than line DS-29AHS-12, while in the 30 min comparison, line DS-29AHS-12 showed a 1.89 fold higher *DREB1A* gene expression level. In the 1 hr treatment, line DS-29AHS-88 displayed a 4.08 fold higher expression level than line DS-29AHS-12. In the 2 hr treatment, line DS-29AHS-12 showed a 2.6 fold higher expression level than line DS-29AHS-88. There were fluctuations in the *DREB1A* gene expression level among the genotypes at different times. This may explain why line DS-29AHS-88 was more tolerant than line DS-29AHS-12. However, the overall gene expression level of line DS-29AHS-88 was higher than line DS-29AHS-12.

Genotype independence

Genotype independence of the tolerance expression was confirmed by testing transgenic regenerants derived from five different tetraploid potato cultivars with different genetic backgrounds: Atlantic (USA), Atzimba (Mexico), Dejima (Japan), Pito (Finland) and Yukon Gold (Canada) (data not shown). The transgenic individuals from each cultivar expressed a phenotypic tolerance to salt stress, but the level of the tolerance varied among the independent genotypes within a cultivar. Thus, quantitative assessment using clonal

cuttings is important for the evaluation of crop physiological characterisitcs in relation to the stability and use of transgenic potatoes for downstream application to potential crop utilization.

Comparative advantage of rd29A promoter and DREB 1A gene for inducing salt stress tolerance

Various transgenic approaches have been adopted using foreign genes of plant and non-plant origin to enhance salt stress tolerance in plants. Many studies have been conducted on plant-derived substances that are closely related to salt stress. The genes corresponding to these biological substances, such as heat-shock proteins, proline and glycinebetaine, have been employed in transgenic studies (Holmberg and Bulow 1998, Iba 2002, Shinozaki and Yamaguchi-Shinozaki 1997).

Genetic engineering work has been conducted on genes of non-plant origin, which drastically increased the level of salt tolerance, e.g., the choline oxidase gene from *Arthrobacter globiformis* (Sakamoto and Murata 2001) and the *Sodium2* (*SOD2*) gene from *Schizosaccharomyces pombe* (Gao *et al.* 2003). However, the present report dealt for the first time with the first use of the *rd29A* promoter and the *DREB 1A* gene to enhance salt stress tolerance in a polyploid potato cultivar, which could potentially lead to commercial utilization in areas with high salinity.

Acknowledgments

All authors thank Ms. Akiko Yamakawa and Mr. Motoya Ikegawa, Nara Advanced Institute for Science and Technology, Ikoma, Japan, and Mr. Takuya Yamada, Department of Biotechnological Science, Kinki University, Uchita, Wakayama, Japan for maintenance and preparation of the plant materials. They thank Dr. P. Russo for correction of the manuscript. This work was supported by an award from Univ. of Tsukuba given to F.C.-T., by RFTF-00L01602 of Japan Society for Promotion of Sciences (JSPS), by the Innovative Development Project under Ministry of Education, Culture, Sports, Science and Technology of Japan and by a Grant-in-Aid Kiban A #17208001 from JSPS.

Literature Cited

Angell, S.M. and D. Baulcombe (1997) Consistent gene silencing in transgenic plants expressing a replicating potato virus X RNA. EMBO J. 16: 3675–3684.

Artus, N.N., M. Uemura, P.L. Steponkus, S.J. Gilmour, C. Lin and M.F. Thomashow (1996) Constitutive expression of the cold-regulated *Arabidopsis thaliana* COR15a gene affects both chloroplast and protoplast freezing tolerance. Proc. Natl. Acad. Sci. USA 93: 13404–13409.

Bernatzky, R. and S.D. Tanksley (1986) Toward a saturated linkage map in tomato based on isoenzymes and random cDNA sequences. Genetics 112: 887–898.

Boyer, J.S. (1982) Plant productivity and environment. Science 218: 443–448.

Bray, E.A. (1997) Plant responses to water deficit. Trends Biol. Sci. 2: 48–54.

Brown, C.R., C.P. Yang, S. Kwiatkowski and K.D. Adiwilaga (1991) Insert copy number, chromosome number, pollen stainability, and crossability of *Agrobacterium*-transformed diploid potato. Am. Potato J. 68: 317–330.

Celebi-Toprak, F., J. Watanabe and K.N. Watanabe (2005) Chapter 10: Molecular Markers in Identification of Genotypic Variation. In Genetic Improvement of Solanaceous Crops, Vol. 2: "Potato" (Razdan, M.K. and A.K. Mattoo, eds.), Science Publishers Inc., Enfield, USA. p. 115–141.

Gao, X., Z. Ren, Y. Zhao and H. Zhang (2003) Overexpression of *SOD2* increases salt tolerance of Arabidopsis. Plant Physiol. 133: 1873–1881.

Goddijin, O.J.M., T.C. Verwoerd, E. Voogd, R.W.H.H. Krutwagen, P.T.H.M. de Graaf, P.K. van Dun, A.S. Ponstein and J. Pen (1997) Inhibition of trehalase activity enhances trehalose accumulation in transgenic plants. Plant Physiol. 113: 181–190.

Golmirzaie, A.M., P. Malagamba and N. Pallais (1994) Breeding Potatoes Based on True Seeds Propagation. *In*: "Potato Genetics", Bradshaw, J.E. and G.R. Mackay (eds.), C.A.B. International, Wallingford, UK. p. 499–513.

Greenway, H. and K. Munns (1980) Mechanism of salt tolerance in nonhalophytes. Annu. Rev. Plant Physiol. 31: 149–190.

Holmberg, N. and L. Bulow (1998) Improving stress tolerance in plants by gene transfer. Trends Plant Sci. 3: 61–66.

Huang, J., R. Hirji, L. Adam, K.L. Rozwadowski, J.K. Hammerlindl, W.A. Keller and G. Selvaraj (2000) Genetic engineering of glycinebetaine production towards enhancing stress tolerance in plants: Metabolic limitations. Plant Physiol. 122: 747–756.

Iba, K. (2002) Acclimative response to temperature stress in higher plants: approaches of gene engineering for temperature tolerance. Annu. Rev. Plant Biol. 53: 225–245.

Ingram, J. and D. Bartels (1996) The molecular basis of dehydration tolerance in plants. Annu. Rev Plant. Physiol. Plant. Mol. Biol. 47: 377–403.

Inui, H., T. Kodama, Y. Ohkawa and H. Ohkawa (2000) Herbicide metabolism and cross-tolerance in transgenic potato plants co-expressing human *CYP1A1*, *CYP2B6* and *CYP2C19*. Pestic. Biochem. Physiol. 66: 116–129.

Kasuga, M., Q. Liu, S. Miura, K. Yamaguchi-Shinozaki and K. Shinozaki (1999) Improving plant drought, salt, and freezing tolerance by gene transfer of a single stress-inducible transcription factor. Nature Biotech. 17: 287–291.

Kavi Kishor, P.B., Z. Hong, G. Miao, C.A. Hu and D.P.S. Verma (1995) Overexpression of Δ`-pyrroline-5-carboxylate synthetase increases proline production and confers osmotolerance in transgenic plants. Plant Physiol. 108: 1387–1394.

LeRudulier, D., A.R. Strom, A.M. Dandekar, L.T. Smith and R.C. Valentine (1984) Molecular biology of osmoregulation. Science 224: 1064–1068.

Liu, Q., M. Kasuga, Y. Sakuma, H. Abe, S. Miura, K. Yamaguchi-Shinozaki and K. Shinozaki (1998) Two transcription factors, DREB1 and DREB2, with EREBP/AP2 DNA binding domain separate two cellular signal transduction pathways in drought- and low temperature-responsive gene expression, respectively, in Arabidopsis. Plant Cell 10: 1391–1406.

Maqbool, B., H. Zhong, Y. El-Maghraby, A. Ahmad, B. Chai, W. Wang, R. Sabzikar and M.B. Sticklen (2002) Competence of oat (*Avena sativa* L.) shoot apical meristems or integrative trans-

formation, inherited expression, and osmotic tolerance of transgenic lines containing *hva1*. Theor. Appl. Genet. 105: 201–208.

Mistuhara, I., M. Ugaki, H. Hirochika, M. Oshima, T. Murakami, Y. Gotoh, Y. Katayose, S. Nakamura, R. Honkura, S. Nishimiya, K. Ueno, A. Mochizuki, H. Tanimoto, H. Tsugawa, Y. Otsuki and Y. Ohashi (1996) Efficient promotor cassettes for enhanced expression of foreign genes in dicotyledonous and monocotyledonous plants. Plant Cell Physiol. 37: 49–59.

Pilon-Smits, E.A.H., M.J.M. Ebskamp, M.J. Paul, M.J.M. Jeuken, P.J. Weisbeek and S.C.M. Smeekens (1995) Improved performance of transgenic fructan-accumulating tobacco under drought stress. Plant Physiol. 107: 125–130.

Rabbani, M.A., K. Maruyama, H. Abe, M.A. Khan, K. Katura, Y. Ito, K. Yoshiwara, M. Seki, K. Shinozaki and K. Yamaguchi-Shinozaki (2003) Monitoring expression profile of rice gene under cold, drought, and high-salinity stresses and abscisic acid application using cDNA microarray and RNA gel-blot analyses. Plant Physiol. 133: 1755–1767.

Sakamoto, A. and N. Murata (2001) The use of bacterial choline oxidase, a glycinebetaine-synthesizing enzyme, to create stress-resistant transgenic plants. Plant Physiol. 125: 180–188.

Sambrook, J., E.F. Fritsch and T. Maniatis (2001) Molecular cloning: A Laboratory Manual, 2nd edn. Cold Spring Harbor Press, Cold Spring Harbor, New York.

Seki, M., M. Narusaka, J. Ishida, T. Nanjo, M. Fujita, Y. Oono, A. Kamiya, M. Nakajima, A. Enju, T. Sakurai, M. Satou, K. Akiyama, T. Taji, K. Yamaguchi-Shinozaki, P. Carnici, J. Kawai, Y. Hayashizaki and K. Shinozaki (2002) Monitoring the expression profile of 7000 *Arabidopsis* genes under drought, cold and high-salinity stresses using full-length cDNA microarray. Plant J. 31: 279–292.

Sheveleva, E., W. Chmara, H.J. Bohnert and R.G. Jense (1997) Increased salt and drought tolerance by D-ononitol production in transgenic *Nicotiana tabacum* L. Plant Physiol. 115: 1211–1219.

Shinozaki, K. and K. Yamaguchi-Shinozaki (1996) Molecular responses to drought and cold stress. Current Opin. Biotechnol. 7: 161–167.

Shinozaki, K. and K. Yamaguchi-Shinozaki (1997) Gene expression and signal transduction in water-stress response. Plant Physiol. 115: 327–334.

Shinozaki, K. and K. Yamaguchi-Shinozaki (2000) Molecular responses to dehydration and low temperature: differences and cross-talk between two signaling pathways. Curr. Opin. Plant Biol. 3: 217–223.

Skriver, K. and J. Mundy (1990) Gene expression in response to abscisic acid and osmotic stress. Plant Cell 2: 503–512.

Stokinger, E.J., S.J. Gilmour and M.F. Thomasow (1997) *Arabidopsis thaliana CBF1* encodes an AP2 domain-containing transcriptional activator that binds to the C-repeat/DRE, a *cis*-acting DNA regulatory element that stimulates transcription in response to low temperature and water deficit. Proc. Natl. Acad. Sci. USA 94: 1035–1040.

Thomashow, M.F. (1993) Characterization of genes induced during cold acclimation in Arabidopsis thaliana. *In* "Plant Responses to Cellular Dehydration During Environmental Stress" Close, T.L. and E.A. Bray (eds.), Am. Soc. Plant Physiol., Rockville, MD. p. 137–143.

Watanabe, J.A., M. Orrillo and K.N. Watanabe (1999) Frequency of multiple quantitative pest resistance traits in 4x × 2x crosses of potato. Breed. Sci. 49: 53–62.

Watanabe, J.A. and K.N. Watanabe (2000) Pest resistance traits controlled by quantitative loci and molecular breeding strategies in tuber-bearing *Solanum*. Plant Biotechnol. 17: 1–16.

Xu, D., X. Duan, B. Wang, B. Hong, T.D. Ho and R. Wu (1996) Expression of a late embryogenesis abundant protein gene, HVA1, from barley confers tolerance to water deficit and salt stress in transgenic rice. Plant Physiol. 110: 249–257.

Yamaguchi-Shinozaki, K., M. Koizumi, S. Urao and K. Shinozaki (1992) Molecular cloning and characterization of 9 cDNAs for genes that are responsive to desiccation in *Arabidopsis thaliana*: Sequence analysis of one cDNA clone that encodes a putative trans-membrane channel protein. Plant Cell Physiol. 33: 217–224.

Yamaguchi-Shinozaki, K. and K. Shinozaki (1993) Characterization of the expression of a desiccation-responsive *rd29* gene of *Arabidopsis thaliana* and analysis of its promoter in transgenic plants. Mol. Gen. Genet. 236: 331–340.

Yamaguchi-Shinozaki, K. and K. Shinozaki (1994) A novel *cis*-acting element in an Arabidopsis gene is involved in responsiveness to drought, low temperature, or high-salt stress. Plant Cell 6: 251–264.

Yancey, P.H., M.E. Clark, S.C. Hand, R.D. Bowlus and G.N. Somera (1982) Living with water stress: evolution of the osmolyte system. Science 217: 1214–1222.