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Research Paper

Evaluation of the yield of abiotic-stress-tolerant *AtDREB1A* transgenic potato under saline conditions in advance of field trials

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Cultivated potato is a drought-, salinity-, and frost-sensitive species. The transgenic approach is one of the methods used to mitigate abiotic stress. The utility of transgenic potatoes that have abiotic stress tolerance should be judged from their yield under stress conditions. In order to establish transgenic potato lines with the *AtDREB1A* gene that could be used in practical applications, we screened candidate lines in a growth room with growth profiles under non-stress conditions rather than the expression level of transgene. After identifying better transgenic lines (D163 and D164), yield of those lines under stress conditions was evaluated in the special netted-house. Although the yield was lower than the yield under non-stress conditions, two selected transgenic lines were able to maintain their yield under high saline conditions (EC > 10 mS/cm). In this study, fertilizer was not added beyond what was already contained in the soil mix in order to evaluate the yield of the transgenic lines under saline conditions in as simple a manner as possible. In future studies, it will be necessary to evaluate their yield in a farming context in an isolated field after assessing the environmental biosafety of these transgenic potato lines.

Key Words: abiotic stress tolerance, *AtDREB1A*, yield evaluation, potato.

Introduction

Potato is the fourth largest staple crop in the world after wheat, rice, and maize. Potato is important as a food (raw and processed), as a source of starch in industry, as feed for farm animals, and as a potential resource for medicines (Ortiz and Watanabe 2004). Cultivated potato (*Solanum tuberosum*) is a drought-, salinity-, and frost-sensitive species. Drought stress is inimical to potatoes owing to their shallow root system. Furthermore, tuber production decreases at soil electrical conductivity (EC) levels above 1.7 mS/cm (Maas 1990). In a field evaluation, potato production was reduced by half at an EC of 5.9 mS/m (Kotuby-Amacher *et al.* 2000). Additionally, potato does not exhibit low temperature acclimation, and its maximum freezing tolerance is approximately –3°C (Chen and Li 1980). It is thus important to determine ways to maintain high potato yields even under abiotic stress conditions. For example, there is a cold-tolerant wild species, *Solanum commersonii*. It was thought that this species could become a genetic donor of cold tolerance for *S. tuberosum*. However, no cultivar with the tolerance trait from *S. commersonii* has been created by conventional breeding (Cardi *et al.* 1993, Estrada 1982, Estrada *et al.* 1993, Iovene *et al.* 2004, Pavek and Corsini 2001). It is thought that enhancing abiotic stress tolerance without altering cultivar traits is difficult since potato is autotetraploid. On the other hand, various crop species have been conferred with enhanced abiotic stress tolerance by means of transgenic techniques.

The transgenic approach is the one of the methods used to mitigate abiotic stress, because the direct introduction of genes by genetic engineering has proven to be an attractive and quick solution for improving stress tolerance in several species (Dunwell 2000, Wang *et al.* 2003). Stress response systems in plants are regulated by multiple signaling pathways (Knight and Knight 2001). Stress-inducible transcription factors regulate the expression of various downstream genes, and the introduction of transcription factors may be a more effective method of enhancing stress tolerance in

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plants (Pradeep *et al.* 2006). Among these transcription factors, the DREB/CBF (dehydration responsive element binding/C-repeat binding factor) protein of *Arabidopsis thaliana* is one of the most well studied, and it acts against abiotic stresses such as drought, salinity, and cold (Gilmour *et al.* 1998, Liu *et al.* 1998, Shinozaki and Yamaguchi-Shinozaki 2000, Stockinger *et al.* 1997, Thomashow 2001). Although over-expression of *AtDREB1A* produced a strong tolerance of drought, salinity, and cold stress in *Arabidopsis* (Gilmour *et al.* 2000, Kasuga *et al.* 1999, Liu *et al.* 1998), this over-expression caused growth retardation under non-stress conditions. It was reported, however, that use of the stress-inducible *rd29A* promoter minimized the negative effects on growth (Kasuga *et al.* 1999). We generated transgenic potato lines with *AtDREB1A* driven by the *rd29A* promoter and have evaluated their abiotic stress tolerance to factors such as salinity, cold, and drought (Behnam *et al.* 2006, 2007, Celebi-Toprak *et al.* 2005, Huynh *et al.* 2014).

Several recent studies report that abiotic stress tolerance has been enhanced in transgenic potatoes (see review Kikuchi *et al.* 2015). However, almost all the evaluation strategies examined only biochemical indicators, the growth profile, and viability under abiotic stress conditions, and did not consider tuber yield. The practical utility of transgenic potatoes that have abiotic stress tolerance should be judged from their yield under the stress condition. Only a few studies have evaluated the impact of stress tolerance on tuber yield in greenhouse or field (Gururani *et al.* 2012, Waterer *et al.* 2010). Under current Japanese regulations, the evaluation of transgenic plants must be carried out in a semi-confined greenhouse, such as the special netted-house, prior to field cultivation (Kikuchi *et al.* 2006, Tabei 1999). In order to establish transgenic potato lines with the *AtDREB1A* gene that could be used in practical applications, we screened candidate lines in a growth room and carried out yield evaluation in the special netted-house.

Materials and Methods

Plant materials and sample preparation

Transgenic potato lines (*Solanum tuberosum* L. cv. Desiree) with introduced *rd29A::AtDREB1A* (Kasuga *et al.* 2004) were generated by *Agrobacterium*-mediated transformation and evaluated for salinity stress tolerance in our previous study (Behnam *et al.* 2006, 2007).

Two-month-old non-transgenic (NT) and transgenic plantlets cultured on MS solid medium were transplanted into pots with a mixture of 9:1:1:1 soil (Kureha Engei Baiyoudo, Kureha Chemical Inc., Tokyo, Japan), peat moss (Kanuma Inc., Tochigi, Japan), vermiculite (Asahi Inc., Okayama, Japan), and parlight (Fuyou Parlight Inc., Nagano, Japan). After three weeks, apical shoots were cut approximately 5 cm below the top and dipped in root-inducing hormone (Rooton, Sumitomo Takeda Engei Inc., Tokyo, Japan). Shoots were planted in wet soil mix and acclimated for a week in plastic boxes. The shoots were then transferred to pots and grown for two weeks under an LD cycle of 16 h light/8 h dark at 25°C. These plant samples were used for further studies.

Screening of transgenic potato lines in a growth room

To select transgenic lines showing growth similar to non-transgenic potato from among the salinity stress tolerant lines (D19, D22, D103, D138, D163, and D164 in Behnam *et al.* 2006), four plants in each of these transgenic lines were grown in pots filled with soil mix under an LD cycle of 16 h light/8 h dark at 25°C. The pots were arranged in a completely randomized block design. After 30 days, plant height was measured and expression analysis of the transgene was performed on leaves from both the non-transgenic and transgenic lines. Each treatment was repeated three times.

Salinity test in the special netted-house

The special netted-house can confine the living modified organisms. It is a greenhouse with three main additional features: screens on the windows to exclude incoming insects carrying pollen, an anterior entrance chamber to prevent direct access to the outside, and a central ditch in the floor to collect discharged water (Yu *et al.* 2009). To assess the likely properties of the transgenic plants in the field, such as performance and risk to environmental biosafety, the plants must be cultivated under near field conditions, such as in the special netted-house.

Tuber production in two transgenic and non-transgenic potato lines was evaluated under salinity stress in the special netted-house in three independent cultivations. Four plants from each line were grown under non-saline and salinity stress conditions (100 and 150 mM NaCl) for 90 days. Young plants in 10.5-cm diameter pots were transferred from the growth room to the special netted-house. After acclimation for a week, plants were replanted in 10.5 L pots with 1 L of pumice (Karuishi, Makino Inc., Tochigi, Japan), clay soil (Akadamado, Kato Inc., Tochigi, Japan), and 7 L of pre-mix soil (Hana to yasai no engei baido, Kato Inc., Tochigi, Japan). The saline condition of each pot was adjusted before planting. Electrical conductivity (EC) of the pot soil was measured using an EC meter (Field Scout® Soil EC Probe and Meter, Spectrum Technologies, East Plainfield, IL, USA) before and after cultivation. The pots were arranged in a completely randomized block design. The test was conducted three times from February to May in Tsukuba, Japan. Because the Desiree cultivar requires approximately 10°C for an overnight temperature, the night temperature was kept above 10°C using a heater, and day length was controlled at 12 h using a sodium vapor lamp (Panasonic, Osaka, Japan).

The number and weight of all tubers having a diameter greater than 1 cm was measured after harvest. To confirm the expression of the *AtDREB1A* gene in the two transgenic lines, RT-PCR was performed. Total RNA was extracted from the leaves of non-transgenic and transgenic potato

lines before and one week after the start of salinity stress treatment.

RNA extraction, cDNA synthesis, and RT-PCR

We used leaves just before transplanting into the 10.5 L pots as before-stress-treatment samples, and we used leaves one week after transplanting into the 10.5 L pots as after-stress-treatment samples.

Total RNA was extracted from the leaf using the RNAqueouse® Small Scale Phenol-Free Total RNA Isolation Kit (Ambion, Austin, TX, USA). After DNase treatment, cDNA was synthesized with random 9mer primers using the Takara RNA PCR kit (AMV) Ver. 3.0 (Takara, Shiga, Japan). RT-PCR was performed with a GeneAmp® Taq Gold System (Applied Biosystems, Warrington, UK). The *AtDREB1A* primer sequences were 5′-GAT TAT ATT CCG ACG CTT G-3′ (forward) and 5′-TTC ATG ATT ATG ATT CCA CT-3′ (reverse). The *ubiquitin* primer sequences were 5′-GCA GTT GGA GGA CGG AC-3′ (forward) and 5′-GGC CAT CTT CCA ACT GTT TC-3′ (reverse). Real-time PCR was performed with a LightCycler® 480 System (Roche, Mannheim, Germany).

Results

Screening of transgenic lines in a growth room

In our previous studies, two transgenic lines (DS29-AHS-88 and DS29-AHS-92) that displayed salinity stress tolerance were selected as higher stress tolerant lines (Celebi-Toprak *et al.* 2005). Here, these two transgenic lines, along with non-transgenic potato lines, were grown under non-saline and salinity-stress conditions in the special netted-house (a semi-contained condition). Although the non-transgenic potato grew well under the non-saline condition, the two transgenic lines exhibited severe growth retardation (**Fig. 1**), with high expression of *AtDREB1A*. The properties of plants grown *in vitro* are often different from those grown in soil. To select transgenic lines that displayed high production in the special netted-house, we added growth profiles to our selection strategy for transgenic lines.

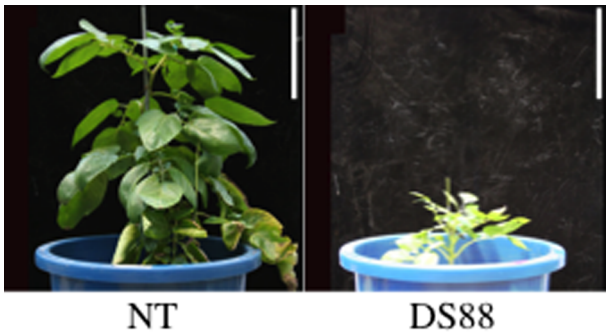

Fig. 1. Characteristics of selected lines grown under non-saline conditions in the special netted-house. Non-transgenic potato plants (NT) and transgenic lines (DS-29AHS-88; DS88) were grown in a pot for 90 days under non-saline conditions in the special netted-house. Features of the non-transgenic and transgenic lines 90 days after the start of cultivation. Scale bars represent 20 cm.

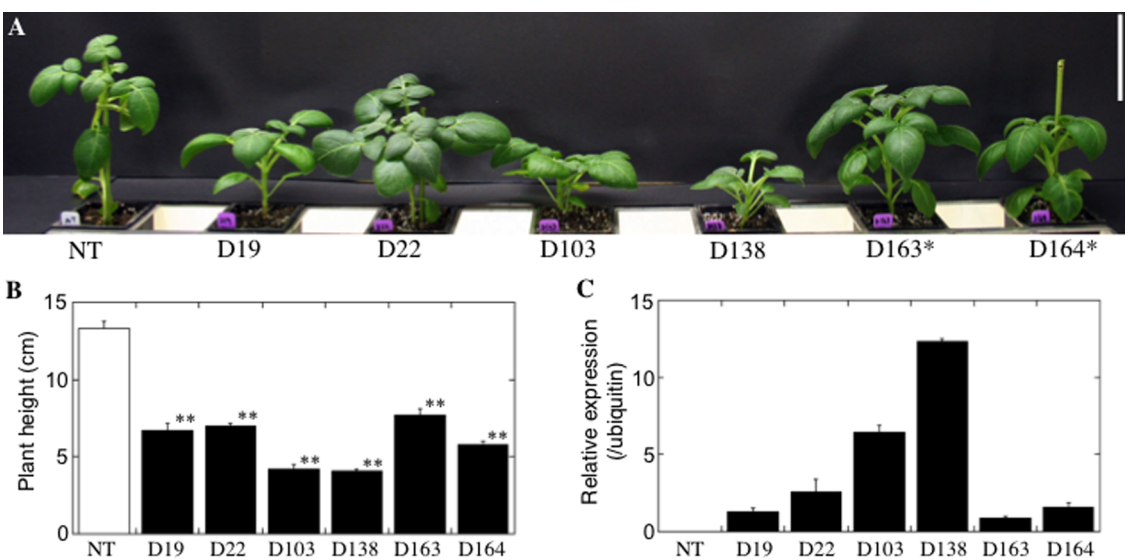

Fig. 2. Screening of transgenic lines in a growth room. To identify transgenic lines with the same growth parameters as those of the non-transgenic (NT) plants, six transgenic lines (D19, D22, D103, D138, D163 and D164) along with non-transgenic lines were kept in a growth room under non-saline conditions for 30 days. (A) Representative features of each potato line 30 days after the start of cultivation in the growth room. Scale bar represents 5 cm. Lines selected for further study are indicated with an asterisk. (B) The average shoot length of each potato line 30 days after the start of cultivation. Bars represent the means ± SE of three replications. Double asterisks indicate significant differences from non-transgenic potato at the 1% level by Tukey's test. (C) Expression level of the *AtDREB1A* gene. Total RNA was extracted from leaves of transgenic and non-transgenic potato plants 30 days after the start of cultivation in the growth room. The expression level was evaluated by real-time PCR. Bars represent the means ± SE of three replications.

In total, 120 transgenic potato lines were positively identified as a new *rd29A::AtDREB1A* transgenic series; among them, 89 individual transgenic potato lines showed normal growth *in vitro*, and 27 of these lines were evaluated for salinity stress tolerance (Behnam *et al.* 2006). To select transgenic lines displaying the same growth as non-transgenic potato, six high salinity stress tolerant lines (D19, D22, D103, D138, D163, and D164) were grown for 30 days under non-saline conditions in a growth room (**Fig. 2A**). All six of these transgenic lines showed growth retardation compared with the non-transgenic potato; two of them (D103 and D138) displayed strong growth retardation, while the other four (D19, D22, D163, and D164) showed slight growth retardation (**Fig. 2B**).

We analyzed the association between the growth retardation and the expression level of the *AtDREB1A* gene using real-time PCR. All transgenic lines expressed *AtDREB1A*, even under the non-stress condition. The expression of *AtDREB1A* was higher in D103 and D138 compared with the other four transgenic lines. The level of growth retardation corresponded to the *AtDREB1A* gene expression level (**Fig. 2B**, **2C**). Because transgenic lines D163 and D164 belonged to the higher-tolerance category than D19 and D22 (Behnam *et al.* 2006), these two lines (D163 and D164) were selected for further testing.

Salinity stress tolerance in the transgenic lines

Tuber production in the two transgenic lines and in non-transgenic potato lines was evaluated under salinity stress in the special netted-house in three independent cultivations. Four plants of each line were grown under non-saline and salinity stress conditions (100 and 150 mM NaCl) for 90 days in the special netted-house. After harvest, the number and weight of the tubers having a diameter greater than 1 cm was measured.

Under non-saline conditions (EC = 0.3 ± 0.1 mS/cm),

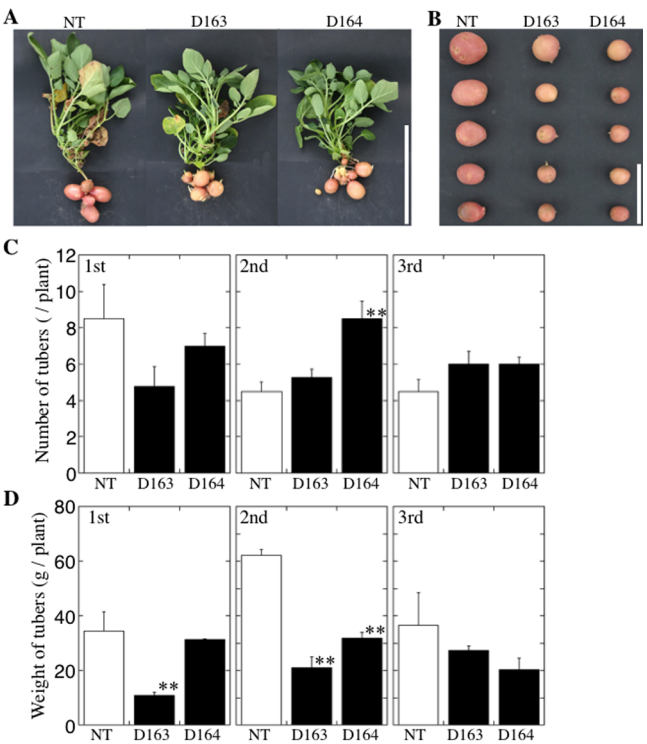

Fig. 3. Characteristics of each line under non-saline conditions. The number and weight of the tubers with diameters over 1 cm was measured after harvest; plants were grown with tap water irrigation (EC = 0.3 ± 0.1 mS/cm) in three independent cultivations. (A) Representative features of the non-transgenic potato plants (NT) and two transgenic lines (D163 and D164) 90 days after the start of cultivation in the special netted-house. Scale bars represent 10 cm. (B) The five largest tubers formed from four plants in each line at the third evaluation. Scale bar represents 5 cm. (C) The average number of tubers per plant in each line. (D) The average weight of the tubers per plant in each line. Bars represent means ± SE of four plants. Asterisks indicate significant differences from non-transgenic plants at the 5% level by Tukey's test.

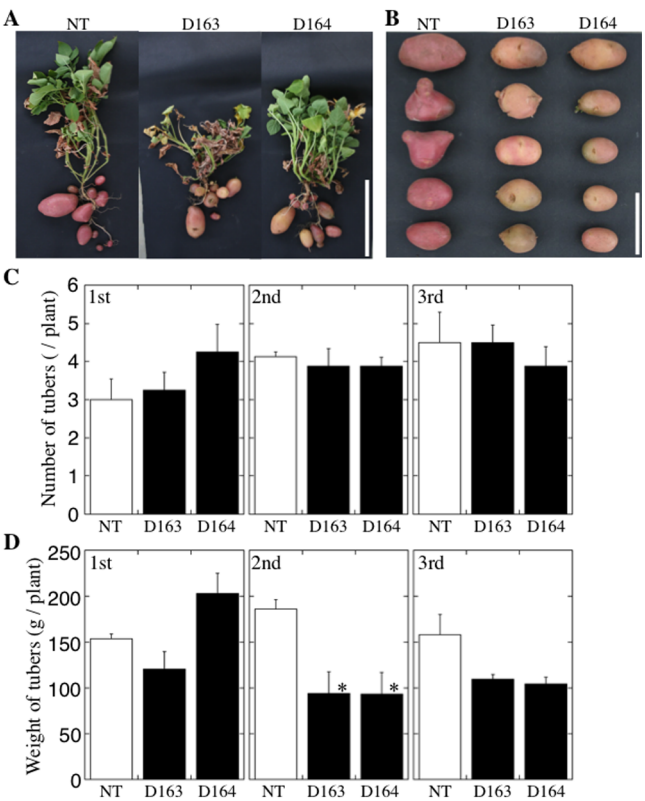

Fig. 4. Characteristics of each line under mild salinity stress conditions. The number and weight of the tubers with diameters over 1 cm were determined after harvest; plants were grown with saline water treatment (100 mM NaCl; EC = 5.8 ± 1.2 mS/cm) in three independent cultivations. (A) Representative features of the non-transgenic potato (NT) and two transgenic lines (D163 and D164) 90 days after the start of cultivation in the special netted-house. Scale bars represent 10 cm. (B) The five largest tubers formed from the four plants in each line at the third evaluation. Scale bar represents 5 cm. (C) The average number of tubers per plant in each line. (D) The average weight of the tubers per plant in each line. Bars represent means ± SE of four plants. Double asterisks indicate significant differences from non-transgenic potato at the 1% level by Tukey's test.

the non-transgenic potatoes showed higher tuber production than the two transgenic lines (**Fig. 3**). The number of tubers was not significantly different between the non-transgenic and transgenic potatoes (**Fig. 3C**), but the total yield of the non-transgenic potatoes was higher than that of the two transgenic lines (**Fig. 3D**). In particular, the yield of D163 was only two-thirds that of the non-transgenic lines. Under the 100 mM NaCl condition (EC = 5.8 ± 1.2 mS/cm), the tuber production of all lines decreased to one-quarter of that observed under the non-saline condition. Still, the non-transgenic potato showed higher tuber production than the two transgenic lines (**Fig. 4**). Under the high salinity stress condition (150 mM NaCl; EC = 10.5 ± 0.1 mS/cm) (**Fig. 5**), the two transgenic lines survived for 80 days, but the non-transgenic plants wilted after 40 days (**Fig. 5E**). The non-transgenic potatoes formed only one or two tubers among four plants, while both of the transgenic lines formed an average of 2.0~3.3 and 1.8~3.3 tubers in the D163 and D164 lines, respectively (**Fig. 5C**). Additionally, the yield was more than six-fold higher in the transgenic lines than in the non-transgenic plants (**Fig. 5D**). Under the high salinity condition, the two transgenic lines displayed stable tuber production, whereas tuber production in the non-transgenic plants was poor.

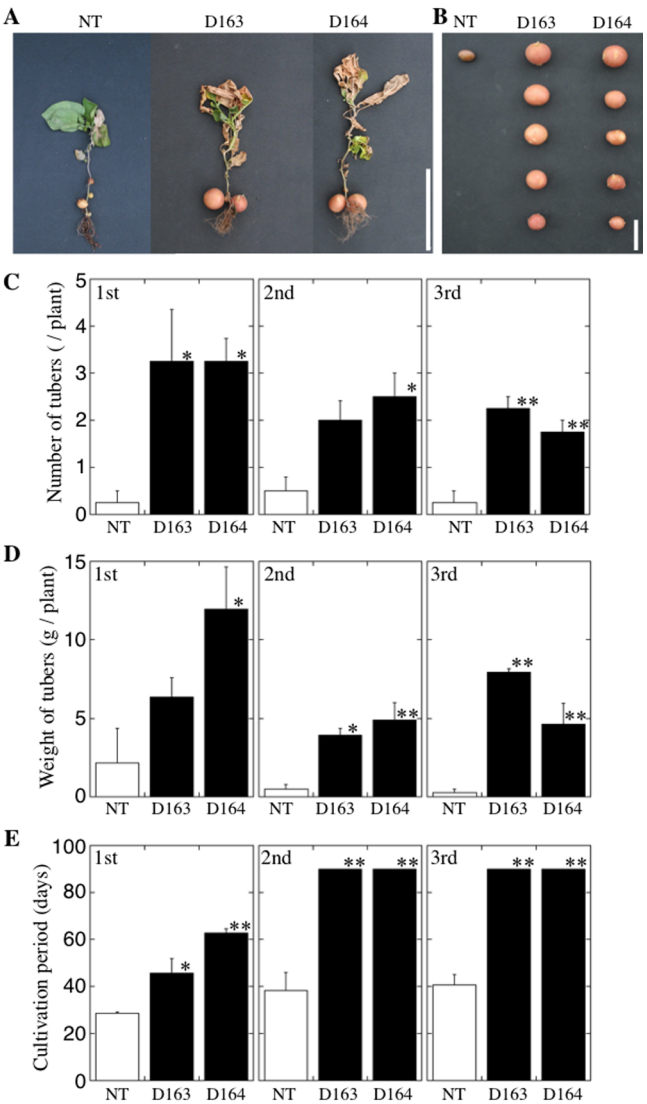

Fig. 5. Characteristics of each line under salinity stress conditions. The number and weight of the tubers with diameters over 1 cm was measured after harvest; plants were grown with saline water treatment (150 mM NaCl; EC = 10.5 ± 0.1 mS/cm) in three independent cultivations. (A) Representative features of the non-transgenic potato plants (NT) and two transgenic lines (D163 and D164) after harvest. Scale bars represent 5 cm. (B) The five largest tubers formed from four plants in each line at third evaluation. Scale bar represents 2 cm. (C) The average number of tubers per plant in each line. (D) The average weight of the tubers per plant in each line. (E) The average survival duration of each line. Bars represent means ± SE of four plants. Single and double asterisks indicate significant differences from non-transgenic plants at the 5% and 1% levels, respectively, by Tukey's test.

AtDREB1A *gene expression profile by RT-PCR*

RT-PCR was performed to confirm the expression of the *AtDREB1A* gene in the two transgenic lines. Total RNA was extracted from the leaves of the non-transgenic and transgenic lines under non-saline and salinity stress conditions (100 and 150 mM NaCl) both before and one week after the start of the salinity test in the special netted-house. There was little expression of the *AtDREB1A* gene in D164 before the salinity test. After the salinity test, both transgenic lines expressed the *AtDREB1A* gene under non-saline and saline conditions (**Fig. 6**). All treatments in the transgenic lines showed *AtDREB1A* gene expression, and the expression levels were almost the same among the treatments (**Fig. 6**).

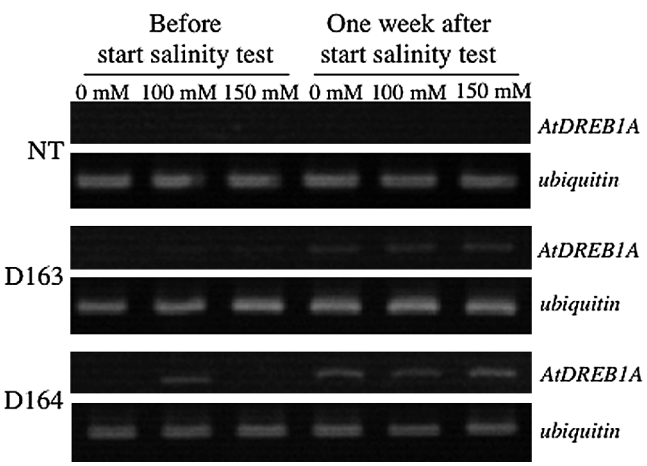

Fig. 6. Expression profiles of the *AtDREB1A* gene. Expression profiles of the *AtDREB1A* gene (upper panel), with *ubiquitin* as an internal control (lower panel), detected with RT-PCR. Total RNA was extracted from the leaves of non-transgenic potato (NT) and two transgenic lines (D163 and D164) under non-saline and salinity stress conditions (100 and 150 mM NaCl), both before and one week after the start of cultivation in the special netted-house.

Discussion

Screening of available transgenic lines for practical application in the special netted-house

Salinity is one of the major abiotic stresses that frequently restrict plant growth and crop yields in various agricultural situations. It is important to improve the stress tolerance of crop plants in order to maintain stable yields under saline conditions. In our previous study, DS-29AHS-88 and DS-29AHS-92 were selected as salinity stress tolerant lines (Behnam *et al.* 2006, Celebi-Toprak *et al.* 2005). Tuber production is the most important trait for potatoes as a crop, so we evaluated the tuber yield of transgenic lines under salinity stress in the special netted-house. Although the selected lines showed high salinity stress tolerance and grew normally in the growth room (Celebi-Toprak *et al.* 2005), they exhibited growth retardation with high expression of the *AtDREB1A* gene under non-saline conditions in the special netted-house (**Fig. 2A**). Such growth retardation has also been reported in transgenic plants when the *AtDREB1A* gene is driven by the constitutive CaMV 35S promoter in *Arabidopsis* (Kasuga *et al.* 1999) and tomato (Hsieh *et al.* 2002). The constitutive expression of stress-related genes has been thought to have serious implications regarding energy loss and other deleterious effects (Pradeep *et al.* 2006). To overcome this, the stress-inducible *rd29A* promoter has been used, and it has been shown to minimize the negative effects of growth retardation under control conditions in *Arabidopsis* (Kasuga *et al.* 1999), tobacco (Kasuga *et al.* 2004), and wheat (Pellegrineschi *et al.* 2004). Minimization of negative effects in the transgene has also been reported in *rd29A::CBF1/DREB1B* and *rd29A::CBF3/DREB1A* transgenic potato (Pino *et al.* 2007). Even if the stress-inducible *rd29A* promoter is used, the simple screening of high-tolerance lines under artificially controlled conditions might lead to the selection of lines with low performance in the special netted-house. Leaky expression of the *AtDREB1A* gene has been reported in *rd29A::AtDREB1A* transgenic tobacco (Kasuga *et al.* 2004). In addition, the conditions in the special netted-house are readily influenced by weather, and some transient stresses could have been encountered, possibly leading to the activation of the *AtDREB1A* gene during cultivation in the special netted-house. Because the *rd29A* gene is downstream of *AtDREB1A* (Seki *et al.* 2001), transient expression of *AtDREB1A* might affect the *rd29A* promoter, leading to the constitutive expression of *AtDREB1A*. The transiently activated *AtDREB1A* gene might, in this way, cause growth retardation in these transgenic lines.

To select some of the available transgenic lines for use as crop plants, we included growth profiles and measurements of *AtDREB1A* gene expression under non-stress conditions in our evaluation of those lines, and successfully identified two transgenic lines (D163 and D164) from the six stress-tolerant lines. All six transgenic lines selected as high salinity stress tolerant in the growth room expressed some level of *AtDREB1A* under the non-stress condition (**Fig. 2D**), and there was some association between expression level and growth retardation (**Fig. 2C**). It is important that growth profiles and/or expression of the *AtDREB1A* gene are included in the screening strategies under non-stress conditions. It is expected that these transgenic lines will show stable yields in salt-damaged farmland.

AtDREB1A *gene enhanced salinity stress tolerance in potato*

Two transgenic lines showed stable tuber production under high salinity conditions (150 mM NaCl; EC = 10.5 ± 0.1 mS/cm), whereas the non-transgenic lines did not survive sufficiently to be evaluated for yield in the special netted-house (**Fig. 5C–5E**). The *AtDREB1A* gene was expressed in all transgenic lines (**Fig. 6**). These results suggest that the expression of *AtDREB1A* enhanced salinity stress tolerance in potato. Similar results have been reported in tobacco (Kasuga *et al.* 2004), wheat (Pellegrineschi *et al.* 2004), and rice (Oh *et al.* 2005). The *AtDREB1A* gene acts as an important transcription factor against abiotic stresses such as drought, salinity, and cold in *Arabidopsis* (Gilmour *et al.* 1998, Liu *et al.* 1998, Shinozaki and Yamaguchi-Shinozaki 2000, Stockinger *et al.* 1997, Thomashow 2001). Overexpression of *AtDREB1A* in transgenic *Arabidopsis* plants activated the expression of many stress-inducible genes and resulted in higher salinity stress tolerance (Kasuga *et al.* 1999, Liu *et al.* 1998). In potato, microarray analysis revealed that the gene product of *AtDREB1A* may function as a transcription factor in abiotic stress (Watanabe *et al.* 2011). The acquisition of salinity stress tolerance in potato indicates that certain native genes may be up-regulated by *AtDREB1A* gene expression, and hence confer salinity stress tolerance on the transgenic lines.

Future applications

We successfully selected two transgenic lines that possessed salinity stress tolerance and exhibited stable tuber production under salinity stress in the special netted-house. This success resulted from having monitored growth profiles in our selection strategy. The conditions in the special netted-house are variable, similar to field conditions, and it is expected that these lines will display yield in the field similar to that observed in the special netted-house. After assessing the environmental biosafety of these transgenic potato lines, we are planning to cultivate them in an isolated field to evaluate their outdoor performance. In this study, fertilizer was not added beyond what was already contained in the soil mix in order to evaluate the yield of the transgenic lines under saline conditions in as simple a manner as possible. Potatoes demand proper amounts of fertilizer for successful tuber production, so in future studies it will be necessary to evaluate their yield in a realistic farming context.

Acknowledgements

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