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Note

In vitro evaluation of dehydration tolerance in *AtDREB1A* transgenic potatoes

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Abstract Abiotic stresses have negative effects on potato growth and production. To enhance the abiotic stress tolerance of the commercial potato cultivar, Desiree, *rd29A*::*AtDREB1A* transgenic lines have been developed. The salinity and freeze tolerance of these transgenic lines has previously been demonstrated; however, their dehydration tolerance remains to be elucidated. First of all, we have tackled the reproducible tolerance evaluation methodology, which have been the hurdle for selecting the stable dehydration tolerant phenotypes in potato. A novel *in vitro* method was developed using the rotary liquid culture combined with PEG. This method enhanced oxygen diffusion into the medium and diminished root damage/injury during plant transfer, thereby reducing the side effects caused by hypoxia and penetration of osmotica. In the present study, we evaluated the dehydration tolerance of twelve transgenic potato lines, and seven of the transgenic lines showed enhanced dehydration tolerance in comparison with the non-transgenic line. However, we observed growth retardation in some dehydration tolerant lines. Therefore, balance between the dehydration tolerance and growth retardation of the transgenic plants should be considered. Four of seven of the transgenic lines displayed enhanced dehydration tolerance without growth retardation, and may represent good candidates for practical application.

Key words: Dehydration tolerance, *in vitro* evaluation, *rd29A*::*AtDREB1A*, transgenic potato.

The current increase in abiotic stresses, such as dehydration, salinity, and cold, because of global warming and climate change, is having a severe impact on global crop production (De Vries 2000; Varshney et al. 2011). Abiotic stresses cause morphological, physiological, and biochemical alterations in crop plants, thereby negatively affecting their growth and productivity. Improvements in abiotic stress tolerance of crops have been carried out by using conventional breeding, but with limited success (Cominelli et al. 2012; Varshney et al. 2011). Abiotic stress tolerance represents one of the most difficult breeding targets, because of the intricate nature of the traits involved (Bohnert et al. 1995; Richards 1996). Considerable time and effort is required to maintain the quality and productivity of the original cultivar. Potato has a complex hereditary mode, because most cultivars are tetrasomic tetraploid (autotetraploid) (Iwanaga and Peloquin 1982; Watanabe et al. 1994). Furthermore, some sexual incompatibility also exists between wild and cultivated potatoes or among them (Spooner and Hijmans 2001). Therefore, introgression for abiotic stress tolerance in potato by using conventional breeding is extremely difficult and time-consuming. Genetic engineering offers the possibility of more rapidly obtaining new potato cultivars with enhanced tolerance to abiotic stresses, by directly transferring useful genes from different species without any barriers. Genetic engineering is expected not only to improve the desired trait, but also to maintain the original cultivar traits (Bhatnagar-Mathur et al. 2008).

Tolerance or physiological reaction to dehydration is influenced by various experimental conditions, including plant type (species, variety, or line), developmental stage, growth conditions, and method of dehydration application (Boyer 2010; Bruce et al. 2002; Poorter et al. 2012; Tardieu 2011; Verslues et al. 2006). Moreover, the side effects of experimental dehydration treatment must be considered (Cominelli et al. 2012; Poorter et al. 2012; Salekdeh et al. 2009; Verslues et al. 2006). Plants are frequently affected not only by dehydration stress,

Abbreviations: MS, Murashige and Skoog; PEG, polyethylene glycol.
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but also by the toxicity of the penetrated osmotica used such as mannitol, sorbitol, and sucrose (Fritz and Ehwald 2010; Hohl and Schopfer 1991; Lipavska and Vreugdenhil 1996; Verslues et al. 1998). Polyethylene glycol (PEG) has a high molecular weight (> 6000) and is therefore larger than the cell wall pores of various plant tissues (Carpita et al. 1979). High-molecular-weight PEG is commonly used to induce dehydration stress (Verslues et al. 2006). However, side effects of PEG penetration have been reported (Jacomini et al. 1988; Lawlor 1970; Yaniv and Werker. 1983), probably because of physical injury to the plant root system during transfer. On the other hand, the high viscosity of PEG solution can cause hypoxia in plants (Verslues et al. 1998). In the present study, we evaluated the dehydration tolerance of *rd29A::AtDREB1A* transgenic potato lines, by developing a new *in vitro* method with minimal side effects. We used our new method to select dehydration-tolerant lines and assess the contribution of *AtDREB1A* expression to dehydration tolerance.

The 12 transgenic potato (*Solanum tuberosum* L. cv. Desiree) lines with *rd29A*::*AtDREB1A* (D10, D19, D20, D21, D22, D44, D53, D108, D132, D141, D163, and D164) (Behnam et al. 2006, 2007) were selected for evaluation of dehydration tolerance. All of the potato lines, including the non-transgenic line, were pre-cultured in 250-ml glass bottles containing 10 ml of liquid MS medium (Murashige and Skoog 1962) with 30 g l^{-1} of sucrose for four weeks at 25±1°C with shaking at 80 rpm, under a 16-h light/8-h dark photoperiod and a light intensity of 80 μmol $m^{-2}\cdot s^{-1}$. Four days before dehydration treatment, the liquid medium was refreshed. All potato plants grew well and showed no abnormal phenotypes, such as hyperhydration or chlorosis (Figure 1A). Most of the lines, including the non-transgenic line, reached a height of approximately 10 cm in four weeks (Figure 2). Pre-cultured potato plants (each with 6–7 opened leaves) were subjected to dehydration stress by removal of the old medium, followed by addition of 10 ml of fresh medium with PEG 8000 (final osmotic potential −1.8 MPa). After the culturing with the dehydration stress treatment for 9 days, the recovery treatment for the following 3 days was applied by removal of the old medium, followed by addition of 10 ml of fresh medium without PEG. Each potato line showed leaf wilting with different level under dehydration treatment (Figure 1B) and partially recovered after dehydration stress releasing (Figure 1C). The vital score of each leaf was determined as follows: 0, leaf fully wilted or dead; 1, more than half of leaf wilted; 2, small part of leaf wilted; and 3, leaf not wilted. The dehydration tolerance of each line was evaluated on the basis of the whole-plant score. Since the youngest (i.e., first) leaf was often not fully expanded, while the oldest (i.e., sixth or seventh) leaf often showed senescence, the whole-plant score was calculated as the sum of the vital score of the second to fifth leaves. For each potato line, the dehydration stress and recovery treatment procedure was replicated three times, by using 3–4 bottles per replication. Lines that showed a higher vital score during dehydration stress treatment tended to show a higher vital score after recovery treatment (Figure 3). Especially, the transgenic lines D108, D132, and D141 maintained a vital score of >6 during dehydration stress treatment (Figure 3). On the basis of the vital score on the third day after recovery treatment, dehydration-tolerant lines were selected by using one-way ANOVA, and ranked into three groups (a–c) according to the Tukey–Kramer test ($p<0.05$; Figure 2). Transgenic lines D44 (c), D164 (c), D53 (bc), D21 (bc) and D163 (bc) were categorized into "c", same as non-transgenic line. By contrast, the remaining seven lines were regarded as conferring dehydration tolerance by *AtDREB1A* gene. From our previous evaluation of salinity or freezing tolerance (Behnam et al. 2006, 2007), these seven lines were categorized into either tolerant group. Especially, D10, D20, D22 and D132 belonged to both tolerance groups. However, D19 or D141 had no tolerance to freezing or salinity, respectively. Both salinity and freezing tolerant lines were less than 40% of total tolerant lines (Behnam et al. 2007). Transformant of *AtDREB1A* showed not always all three type of tolerance. It might be caused by positional effect of transgene and so on.

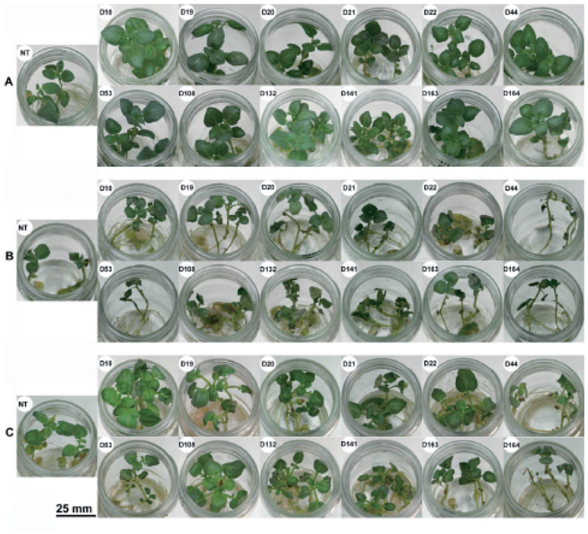

Figure 1. Phenotypic responses of non-transgenic (NT) and transgenic (D) potato lines under conditions of dehydration stress and recovery. A, before dehydration stress; B, at the end (ninth day) of dehydration stress; and C, after three days of recovery from dehydration stress. The plants were cultured in 250-ml glass bottles containing 10 ml of liquid medium (MS + 30 g l^{-1} sucrose). Stress or recovery treatment was carried out with or without PEG solution (−1.8 MPa). The scale bar represents 25 mm.

To elucidate the association the tolerance and transgene, the expression level of *AtDREB1A* was quantified by RT-PCR using a LightCycler® 480 System

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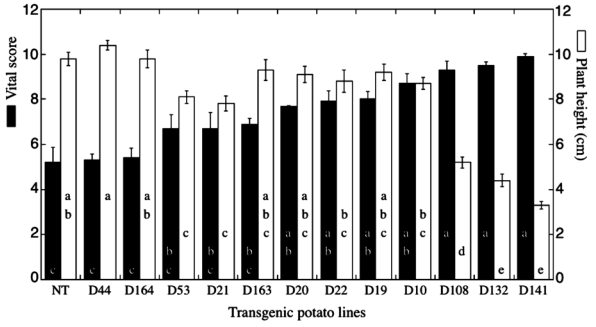

Figure 2. Dehydration tolerance and plant growth of non-transgenic (NT) and transgenic (D) potato lines grown under *in vitro* conditions. Black bars indicate vital score of each line. Dehydration tolerance was represented by the mean vital score of leaves of cultured plants under the dehydration stress by PEG for 9 days followed by the recovery treatment for 3 days. White bars indicate the plant height that was cultured without stress for 28 days. Each bar represents mean±standard error. Differences between means were analyzed by using one-way ANOVA, and ranked according to the Tukey–Kramer test ($p<0.05$). Lines not sharing the same letter differ significantly. Each bar represents mean±standard error.

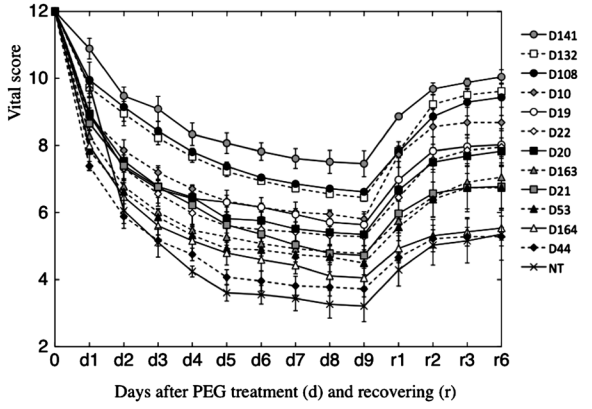

Figure 3. Vital score of potato plants grown under *in vitro* conditions of dehydration stress for 9 days followed by recovery treatment for 6 days. A non-transgenic line and 12 *rd29A::AtDREB1A* transgenic lines of the of the commercial potato cultivar, Desiree, were treated with −1.8 MPa PEG solution and recovered by using normal culture medium. Each point represents the mean of whole-plant leaf-wilting resistance scores derived from three experimental replications. Whole-plant leaf-resistance score=total leaf wilting resistance score of four leaves (second to fifth leaf). Each experimental replication included 3–4 bottles per line and three plants per bottle. Each bar represents mean±standard error.

(Roche, Mannheim, Germany). The *AtDREB1A* primer sequences were 5′-GAT TAC GAG TCT TCG GTT TCC TC-3′ (forward) and 5′-CTA ACC TCA CAA ACC CAC TTA CC-3′ (reverse). The *ubiquitin* primer sequences used as an internal control were 5′-CTG GAA AGC AGC TCG AGG AT-3′ (forward) and 5′-CCT GGA TCT AGC CTG GAC ATT A-3′ (reverse). For each sample collection, three independent experimental replications were conducted. The expression level just before and two days after dehydration stress treatment were quantified (Figure 4). Our results showed that all transgenic potato

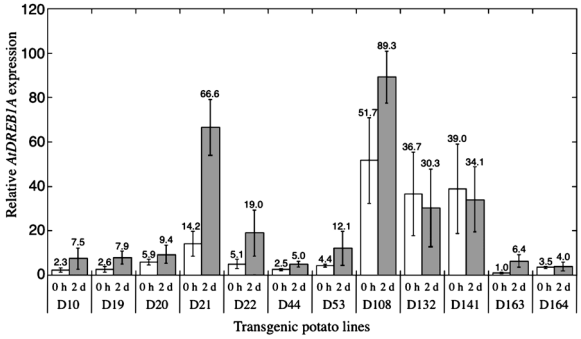

Figure 4. *AtDREB1A* expression profiles. Quantitative *AtDREB1A* expression in transgenic potato lines before (0 h) and after two days (d) of dehydration stress. The relative *AtDREB1A* expression of each transgenic line was derived from three experimental replications, and normalized against constitutive expression of *ubiquitin*. The *AtDREB1A* expression level of line D164 before dehydration stress was set at one. Each bar represents mean±S.E. of three independent experiments.

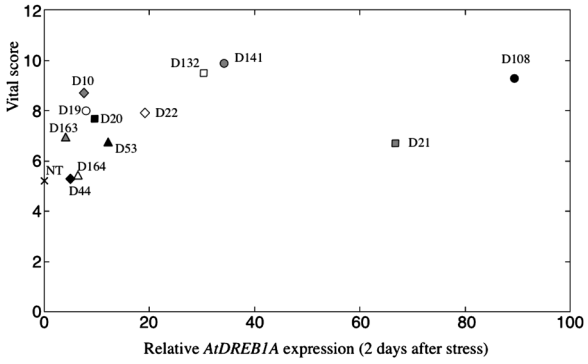

Figure 5. Relationship between dehydration tolerance and *AtDREB1A* expression. Tolerance level and expression level of *AtDREB1A* were plotted. Dehydration tolerance was represented by the vital score after three days of recovery in non-transgenic potato line and 12 transgenic potato lines (Figure 2). Expression level of *AtDREB1A* in transgenic potato lines was represented after two days of dehydration stress (Figure 4).

line showed *AtDREB1A* expression before dehydration stress treatment (Figure 4). A significant negative correlation ($p<0.01$, $r=-0.92$) between plant height (Figure 2) and *AtDREB1A* expression before stress (Figure 4) was observed. Our results indicate that growth retardation of transgenic potato plants may be derived from leaky *AtDREB1A* expression before stress. On the other hand, all lines showed *AtDREB1A* induction during stress treatment with exception of lines D132 and D141. To clarify the involvement of *AtDREB1A* expression in dehydration tolerance of these transgenic potatoes, we evaluated the association between the vital score on the third day of recovery and the relative *AtDREB1A* expression after two days of stress treatment (Figure 5). It seems to be a positive association between them, but it is not clear. In comparison with the other transgenic potato lines, lines D21 and D108 showed markedly high *AtDREB1A* expression (< 40 versus 89.3 and 66.6, respectively; Figure 5). Interestingly, these high

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expression levels did not correspond to dehydration tolerance, and may therefore, be beyond the threshold of conferring tolerance. We subsequently excluded lines D21 and D108 from our association analysis, and determined a significant positive correlation ($p<0.01$, $r=0.802$) between dehydration tolerance and *AtDREB1A* expression under conditions of stress. Various factors, including positional transgene effects, RNA interference, and DNA methylation (Meyer 1995; Wilson et al. 2006), are independent in all transgenic lines. These factors may reduce transgene activity. Hence, *AtDREB1A* may contribute differently to dehydration tolerance in lines D21 and D108 than in the other transgenic lines. Our results might suggest that transgenic potato lines show enhanced dehydration tolerance by induction of *AtDREB1A* expression under conditions of stress.

In vitro evaluation of dehydration has suitable choices for experimental objective. We can select the low or high molecule osmotica and the media type (liquid or solid). Low molecule osmotica such as mannitol and sorbitol are known to penetrate to plant. On the other hand, high molecule osmotica is apt to show viscosity and tends to induce hypoxia. In the case of whole plant evaluation via physiological status, low molecule osmotica should be avoided since their penetration property. When the evaluation condition needs to be changed, liquid medium has the advantage of preventing amount of injury and/or damage to the plant roots. We employed the rotary liquid culture with PEG 8000 to enhance the oxygen diffusion into the medium and diminish root damage/injury during medium exchange. Furthermore, pre-culture for 28 days is enough to cure the initial injury and reestablish the root system. Our method can reduce the side effects such as hypoxia and penetration of osmotica. Using this evaluation method, we selected seven tolerant lines for dehydration. Four transgenic lines (D22, D20, D10, and D19) of them showed significantly high dehydration tolerance without growth retardation (Figure 2), and may represent good candidates for practical application. This evaluation was performed under precisely controlled conditions of dehydration treatment and other experimental variables. By contrast, in the field, plants encounter a complexity of biotic and abiotic stresses. Expression of plant traits is highly dependent on the growing environment (Sinclair 2011), and therefore dehydration tolerance profiles are manifested differently under various conditions of dehydration (timing, duration, intensity, and location) and environmental variables (Boyer 2010). Hence, in order to select suitable transgenic lines for practical application, further evaluation under inconsistent environmental conditions is required. On the other hand, to accomplish a practical use for transgenic plants, step-by-step evaluations from confined conditions (growth room) to field conditions, via semi-confined conditions (special netted-house) (Hilbeck et al. 2011; Kikuchi et al. 2008), are required in accordance with Japanese regulation. Thus, further screening of the drought-tolerant transgenic potato lines by using pot cultivation in growth rooms and special netted-houses, combined with environmental biosafety assessment, is required. In future studies, we aim to perform field trials with these transgenic potato lines by using step-by-step evaluations.

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