Assessments on Drought Tolerance in Transgenic Potato Lines under Confined Conditions

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Assessments on Drought Tolerance in Transgenic Potato Lines under Confined Conditions

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LIST OF ABBREVIATIONS

cDNA: complementary DNA

DNA: deoxyribonucleic acid

DRE: dehydration-responsive element

DREB1A: dehydration-responsive-element binding protein 1A

MS: Murashige and Skoog

NT: non-transgenic potato cv. Desiree

PEG: polyethylene glycol

PCR: polymerase chain reaction

rd29A: responsive drought 29A gene

RNA: ribonucleic acid

RT-PCR: reverse transcription PCR

ABSTRACT

Assessments on Drought Tolerance in Transgenic Potato Lines under Confined Conditions

Due to effects of climate change, drought is predicted has negatively impacts on potato production. To maintain and increase potato production, it is necessary to produce new potato cultivars with drought tolerance. Genetic engineering offers a possible solution to establish new potato cultivars for drought tolerance by directly introducing useful genes to commercial elite potato cultivar. AtDREB1A transcription factor can up-regulate many stress responsive genes, therefore confers abiotic stress tolerance in *Arabidopsis thaliana* and many kinds of plant species. In our previous studies, transgenic potato lines (*Solanum tuberosum* L. cv. Desiree) harboring *rd29A::DREB1A* construct for abiotic tolerance were developed. These lines have been evaluated for salinity and freezing tolerance and some good performance lines were selected. However, their potentials for drought tolerance are still not known.

To assess drought tolerance of *rd29A::AtDREB1A* transgenic potatoes, twelve transgenic lines were conducted under *in vitro* and growth room conditions. Rotary liquid culture combined with PEG was applied as drought treatment method for *in vitro* evaluation. Soaking the soil pot with plant to PEG solution periodically was

applied as drought treatment method for growth room evaluation. In both *in vitro* and growth room evaluations, the same drought stress strength was precisely controlled at -1.8 MPa to all potato lines throughout the drought treating. The other environmental conditions such as light, temperature, relative humidity were also be controlled and managed in both evaluations. Based on leaf wilting symptom, drought response was represented, and consequently drought tolerance of each potato line was clarified. Seven of the transgenic lines – D10, D19, D20, D22, D108, D132, and D141 – showed enhanced drought tolerance in comparison to non-transgenic line under *in vitro* condition. Six of the transgenic lines - D10, D19, D20, D53, D163 and D164 – were identified more drought tolerant than the non-transgenic line under growth room condition. Thus, based on *in vitro* and growth room evaluations, the enhanced drought tolerance in some *rd29A::AtDREB1A* transgenic potato lines were identified.

The expression profiles of *AtDREB1A* in *rd29A::AtDREB1A* transgenic potatoes were performed under both *in vitro* and growth room conditions. The associations between expression of *AtDREB1A* and drought tolerance was analyzed. There was a significant positive correlation between *AtDREB1A* expression and drought tolerance under *in vitro* conditions ($p < 0.01$; $r = 0.82$) while no correlation between *AtDREB1A* expression and drought tolerance was found under growth room conditions ($p = 0.45$; $r = -0.23$). These result reflected

the changing of drought tolerance in some *rd29A::AtDREB1A* transgenic potato lines from *in vitro* to growth room conditions.

The leaky expression of *AtDREB1A* under non-stress *in vitro* and growth room conditions was observed in all in transgenic potato lines. The significant negative correlations between expression of *AtDREB1A* under non-stress condition and plant growth in the transgenic lines were identified under both *in vitro* ($p < 0.01$; $r = -0.92$) and growth room ($p < 0.01$; $r = -0.84$) conditions. However, only D108, D132, and D141 showed severe growth retardation due to highly expression of *AtDREB1A* under non-stress conditions.

The promotion level of *AtDREB1A* expression in twelve transgenic lines was analyzed under both *in vitro* and growth room conditions. Transgenic lines D10, D19, D21, D22, D53, and D164 showed high promotion values (> 2.5) while D20, D44, D108, D132, D141 and D163 showed low promotion values (< 2).

In this study, the conferring drought tolerance of *AtDREB1A* in *rd29A::AtDREB1A* transgenic potatoes was indicated under both *in vitro* and growth room conditions. The leaky expression of *AtDREB1A* in these transgenic lines was observed, however, only D108, D132, and D141 showed severe growth retardation. In consideration on drought tolerance trait, growth retardation problem and expression pattern of *AtDREB1A*, six of the transgenic lines – D10, D19, D20, D22, D53 and D164 – may represent good candidates for practical uses.

ABSTRACT (in Japanese)

閉鎖系での遺伝子組換えジャガイモ系統の乾燥耐性の評価

地球規模の気候変化に伴い、乾燥地域が拡大している。このような環境変化に適応できる乾燥耐性ジャガイモの育種は、重要な課題になってくる。このため、遺伝子組換え技術を用いて、ジャガイモの優良栽培品種に関する研究が行われている。そこで、乾燥耐性遺伝子である *AtDREB1A* は、シロイヌナズナなどの植物で発生でき、乾燥耐性の効果が見られている。先行研究により、遺伝子組換えジャガイモ（*Solanumtuberosum* L. cv.Desiree）では、すでに *rd29A::AtDREB1* の発現を実現し、耐塩性と耐冷性が見られていたが、乾燥耐性はまだ研究されていない。

本研究では、*rd29A::AtDREB1A* 遺伝子を導入したジャガイモを評価するため、12 系統のジャガイモを *in vitro* と栽培室の条件で検討した：*in vitro* での評価では、液体振動培養及び PEG 入りの培地で検討した；栽培室での評価では、鉢植えの植物体を断続的に PEG 溶液に浸けることで検討した。

その結果、環境要素（光、温度、湿度など）を保ちながら、乾燥ストレスを-1.8 MPa の条件で、両方の評価で組換え体の乾燥耐性が見られた。組換え体葉の萎える具合により、それぞれの系統を評価した結果、*in vitro* の培養では、D10、D19、D20、D22、D108、D132、D141 の７系統において、非組換え体より乾燥耐性を示した；栽培室での培養では、D10、D19、D20、D53、D163、D164 の６系統において、非組換え体より乾燥耐性を示した。これに基づいて、*rd29A::AtDREB1A* 遺伝子導入したジャガイモでは、乾燥耐性を示したと考えた。

それから、*rd29A::AtDREB1A* 遺伝子の発生と乾燥耐性間の関連性を分析してみた。*in vitro* の培養条件では、関連性が見られ（$p < 0.01$；$r = 0.82$）、栽培室の培養では、関連性が見られなかった（$p = 0.45$；$r = -$

0.23）。このように、*in vitro* から栽培室に移ることで、組換え体乾燥耐性の変化を見た。

rd29A::AtDREB1A 遺伝子の欠損発見では、環境ストレスなしの条件で、in vitro と栽培室の両方で見られた。グラフにより、*in vitro* 条件では（$p < 0.01$；$r = -0.92$）、栽培室条件では（$p < 0.01$；$r = -0.84$）の結果だった。しかし、D108、D132、D141 系統では、成長遅れが見られた。

さらに、*AtDREB1A* 遺伝子の促進レベルについて、12 系統の *in vitro* と培養室条件で分析してみた。D10、D19、D21、D22、D53、D164 系統では、高い促進値（>2.5）が見られ、D20、D44、D108、D132、D141、D163 系統では、低い促進値（<2）が見られた。

以上のように、本研究では *AtDREB1A* 遺伝子で、*rd29A::AtDREB1A* 組換えジャガイモに関する耐乾燥性評価を行うことで、*AtDREB1A* 遺伝子の欠損発生を見つけた。D108、D132、D141 系統の成長の遅れが見られた。乾燥耐性の特徴及び植物の成長などを総合的に考え、D10、D19、D20、D22、D53、D164 の 6 系統の実用が期待される。

CHAPTER 1

General introduction

1.1 Drought tolerance in plants

1.1.1 Climatic change and its impacts

Global climate models predict increases over time in average temperature worldwide with significant impacts on local patterns of temperature and precipitation (Ganopolski 2008). Accordingly, it is expected that the frequency of extreme climate events such as floods and droughts are increased (Alcamo et al. 2007; Mirza 2007). Moreover, Climate change impacts significantly on annually stream flow as well as spatial distribution of water availability (Ma et al. 2008; Wurbs et al. 2005). As a result, the scarcity of water occurs more often in various land area, and those area are enlarging (Dai 2011).

Crop production is highly dependent on weather conditions and/or water availability. With these effects of climate change, weather will become more extreme and unpredictable, and water availability for crop production will decrease (Kang et al. 2009). Crop production models predict a decreasing in yield of maize, wheat, rice, and soybean in several cultivated areas due to the lack of water availability (Aggarwal et al. 2006). Furthermore, climate change increases the crop plants encountering to various environmental stresses. These stresses induce

numerous alteration to crop plants physiologically and biochemically, and then cause to change chemical composition of crop plants. As a result, the quality of the harvested products is influenced (Wang and Frei 2011).

1.1.2 Plant response against drought stress

In agriculture, drought can be defined as insufficient moisture supply which is reduced crop production (Blum 2011). For plant, drought is the gap between supply and demand for water. Based on the interrelation between supply and demand for water, the response of plants to drought stress can be displayed delineated into three distinct stages of soil dehydration. In stage I, plants grow and develop normally because soil moisture is supplied sufficiently. When the water uptake from the soil of plant does not match the potential transpiration rate, plants start suffering the drought stress in stage II. During stage II, plants maintain the water balance by reducing transpiration rate to appropriate to the limited water uptake. Stage III is reached when the water uptake of plant is unable to meet its transpiration demand. At stage III, the closure of stomata and inhibition of photosynthesis occur. The strategy of plants is shifted from productivity to survival to conserve water (Sinclair & Ludlow 1986). Therefore, response of plants change depends on the stress strength. In addition, the demand and usage efficiency of water are different among plant species or cultivar. For these reasons, drought

condition should be set according to the material plant in drought tolerance evaluation.

On the other hand, strategy of plant against to drought stress has been divided into drought escape, dehydration avoidance and dehydration tolerance (Levitt 1972; Turner 1986). Drought escape displays a high degree of developmental plasticity. Some plants can sense initial drought stress, and possess their life cycle to complete before exposing severe stress. Dehydration avoidance is defined as the capacity to sustain the high water status or cellular hydration under drought condition. Plants avoid dehydration by enhancing soil water uptake or limiting water loss. Dehydration tolerance is involved in the capacity of plant cells and/or tissues to withstand dehydration. Plants withstand dehydration by synthesizing and accumulating protective proteins and solutes, and antioxidants to stabilize cell membrane and/or to prevent cell damage. Each of those strategies requires different physiological reaction, morphological feature, and/or phenological character in plant (Deikman et al. 2011). In addition, plants seem to change kinds of response types depending on developmental stage and drought environment (Chaves et al. 2003, Mullet 2009). Therefore, plant response to drought stress might be composed of multiple strategies. Each strategy might contribute to the response depending on plant stage and environment to a lesser or greater degree.

Drought affects on development, growth, and survival of plant. To cope with drought, plants have evolved a wide spectrum of molecular programs to sense and adapt to drought (Sakuma et al. 2006). At the cell level, the plant response to drought stress is a complex signal cascade that is composed of four basic steps: signal perception, signal transduction, gene induction, and expression of tolerance to drought stress (Ingram and Bartels 1996; Shinozaki and Yamaguchi-Shinozaki 2000: Bartels and Sunkar 2005). The initial stress signals trigger the downstream signaling process and transcription controls, which activate mechanisms to protect the plant from drought stress. These signaling pathways constitute a complex network, interconnected at many levels through several pathways (Bohnert and Sheveleva 1998; Knight and Knight 2001). Accordingly, drought stress signals activate stress responsive genes which are expressed at various levels of plant organization, and are involved in many functional processes of plant growth and development (Ingram and Bartels 1996; Chaves and Oliveira 2004). Therefore, response and tolerance in plant to drought stress is a result from the combination activities of many genes, and all of them need to contribute in concert.

1.1.3 Selection criteria for drought tolerance evaluation

In order to establish drought tolerant plants, setting appropriate selection criteria is primary tasks in drought tolerance evaluation. The criteria should associate to

the vitality or damage of plant under stress (Reynolds et al. 2001). Selection criteria should reflect the plant response and/or physiological processes that are involved in the drought tolerance (Araus 1996). Moreover, selection criteria should be chosen appropriately according to the drought evaluation method (Salekdeh et al. 2009). In addition, selection criteria should be a comparable and measurable trait since the plant response to drought stress depending on plant stage and environmental condition.

Leaf wilting is a prominent symptom of plant under drought stress. Leaf is wilted when plants become water shortage and reduce their turgor in each cell under stress. Depending on drought stress intensity and duration, leaf shows different wilting level from partial to fully. Besides, leaf wilting is a visual and not acute lethal symptom, it can be easily measured and monitoring as indicator of plant damage during the stress. Therefore, leaf wilting or rolling is being used as a reliable indicator for drought symptom and tolerance level in cereals (Banzinger et al. 2000; Fisher et al. 2003), soybean (Fletcher et al. 2007; Sadok and Sinclair 2010).

1.2 Potato

Potato is one of the most important food crops in the world with an annual production of 368 million tons and cover over 19 million hectares (FAOSTAT

2012). However, almost modern potato cultivars are considered highly susceptible to drought stress (Van Loon 1981; Weisz et al. 1994; Deblonde and Ledent 2001), subsequently, potato productivity and cultivation can be impacted highly in case of increasing of drought frequency and severity. Climate models have indicated that drought episodes or drought events will become more frequent and extreme because of the long-term effects of global warming (Wilhite 2005; Salinger et al. 2004; Cook et al. 2004). On the other hand, the sparse and shallow root system, and the low ability in water uptake of root are mainly contributed to the drought susceptibility in potato (Miller and Martin 1987; Jefferies 1995; Iwama 2008). Drought impacts on potato growth by reducing photosynthesis and leaf expansion (Jefferies 1993a and b; Schapendonk et al. 1989; Deblonde and Ledent 2001; Ta et al. 2003). Drought inhibits physiological and biochemical activities such as photosynthesis, nitrogen uptake, and nitrate reductase activity (Basu et al. 1998; Schafleitner et al. 2007). Drought affects on tuber initiation and development (Haverkort et al. 1990; Walworth and Carling 2002; Schafleitner et al. 2007), and also tuber quantity and quality (Levy 1983; Deblonde et al. 1999; Porter et al. 1999). Furthermore, it is predicted that potato yield can be lost from 18 to 32% during next decade years due to climate change (Hijmans 2003). Hence, to maintain and increase potato production, it is necessary to produce new potato cultivars with drought tolerance.

Potato has a complex hereditary mode, because most cultivars are tetrasomic tetraploid (autotetraploid) (Iwanaga and Peloquin 1982; Watanabe et al. 1994, 1995). Furthermore, some sexual incompatibility also exists between wild species and cultivated potatoes or among them (Spooner and Hijmans 2001). On the other hand, drought tolerance is derived from complex quantitative traits that are controlled by many genes that interact with each other (Richards 1996; Edmeades et al. 2004; Boyer 2010; Sinclair 2011). Therefore, introgression for drought tolerance in potato by conventional breeding is extremely difficult and time-consuming.

Genetic engineering offers a possible solution for establishing new potato cultivars with drought tolerance rapidly, by directly introducing useful genes to commercial elite potato cultivar from different species. Genetic engineering is expected that not only introduce the desired trait but also maintain the original traits of cultivar (Bhatnagar-Mathur et al. 2008).

1.3 Role of *AtDREB1A* in drought stress tolerance in plants

Abiotic stresses cause morphological, physiological, and biochemical alterations in crop plants, thereby negatively affecting their growth and productivity. Plants respond to these stresses by operating various genes to protect physiological and biochemical activities, and subsequently to maintain growth and

development (Yamaguchi-Shinozaki and Shinozaki 2005). These stress-responsive genes contribute not only to cell protection but also to signal transduction and regulation of gene expression.

Transcription factors play critical roles to signal transduction and gene regulation. In *Arabidopsis*, many transcription factors, including bZIP, MYC, MYB, NAC, WRKY, and DREB, are involved in signal transduction and gene regulation under various abiotic stress conditions (Shinozaki and Yamaguchi-Shinozaki 2007). Among these transcription factors, DREBs have been indicated as key regulators for various abiotic stress responses (Sakuma et al. 2002; Shinozaki and Yamaguchi-Shinozaki 2007). In *Arabidopsis*, DREB transcription factors can specifically bind DRE (dehydration-responsive element) sequences, are classified into two groups; DREB1 and DREB2 (Yamaguchi-Shinozaki and Shinozaki 2005). It is known that *DREB1s* genes are induced by cold stress (Jaglo-Ottosen et al. 1998; Liu et al. 1998; Kasuga et al. 1999). On the other hand, over-expression of *DREB1* genes in *Arabidopsis* enhanced tolerance not only to cold but also to drought and salinity stresses (Stockinger et al. 1997; Gilmour et al. 1998; Liu et al. 1998). DREB2s genes are induced by drought, salinity and heat stresses (Liu et al. 1998; Nakashima et al. 2000). Over-expression of *DREB2* genes in *Arabidopsis* did not improve stress tolerance (Liu et al. 1998; Sakuma et al. 2002) since DREB2 proteins need post-translational activation (Liu et al. 1998). DREB1

proteins can activate many downstream genes that are responsible for drought tolerance without modification, it is expected that *DREB1* genes improve drought stress tolerance in plants with simple manner.

Microarray analyses showed that over-expression of *AtDREB1A* caused up-regulation of various stress-responsive genes in *Arabidopsis* (Seki et al. 2001; Fowler and Thomashow 2002; Maruyama et al. 2004). Many of these stress-inducible genes, encoding LEA proteins, antifreeze proteins, hydrophilic proteins, RNA-binding proteins, antioxidant enzymes, and protease inhibitors, contribute to cell-protective functions. Therefore, the over-expression of *AtDREB1A* enhanced tolerance to drought in *Arabidopsis*. *AtDREB1A* also conferred drought tolerance improvement in other plant species such as tomato (Hsieh et al. 2002; Zhang et al. 2004) and rice (Dubouzet et al. 2003; Ito et al. 2006; Oh et al. 2005), by activating many stress-responsive genes. However, constitutive over-expression of *AtDREB1A* caused severe growth retardation under non-stress conditions in *Arabidopsis* (Liu et al. 1998; Kasuga et al.1999; Gilmour et al. 2000, 2004), tobacco (Kasuga et al. 2004), and tomato (Zhang et al. 2004). To overcome this problem, the stress-inducible *rd29A* promoter was employed to improve abiotic stress tolerance without the negative effects on plant growth (Jia et al. 2012). Thus, it is expected that *AtDREB1A* gene driven by *rd29A* promoter is a good candidate for improving drought tolerance of plants.

1.4 Objectives

Due to effects of climate change, drought is predicted has negatively impacts on potato production. To maintain and increase potato production, it is necessary to produce new potato cultivars with drought tolerance. Genetic engineering offers a possible solution to establish new potato cultivars for drought tolerance by directly introducing useful genes to commercial elite potato cultivar.

In *Arabidopsis*, *DREB1A* up-regulates various stress-responsive genes which encoding LEA proteins, antifreeze proteins, hydrophilic proteins, RNA-binding proteins, antioxidant enzymes, and protease inhibitors (Maruyama et al. 2004). *AtDREB1A* also can activate many stress-responsive genes in other plant species such as tobacco (Kasuga et al. 2004), tomato (Rai et al. 2013), soybean (Polizel et al. 2011), peanut (Bhatnagar-Mathur et al. 2007), rice (Ito et al. 2006), and wheat (Pellegrineschi et al. 2003). As a result, *AtDREB1A* conferred tolerance for abiotic stresses such as salinity, freezing, and drought. In previous studies, the strong correlations between *AtDREB1A* expression and freezing or salinity tolerance in transgenic potatoes carrying *rd29A::AtDREB1A* were indicated (Behnam et al. 2006, 2007). From these results, *AtDREB1A* might up-regulate many cell-protective genes in potato, and consequently conferred those stresses tolerance. It was hypothesized that drought tolerance of *rd29A::AtDREB1A* transgenic potatoes

depend on the expression level of *AtDREB1A* under drought stress same as under salinity and freezing stresses.

Objective of this study was the screening of drought tolerant candidates for practical uses *via* the evaluation of drought tolerance in *rd29A::AtDREB1A* transgenic potatoes *in vitro* and at growth room. From the analyses of transgene expression and tolerance evaluation of transgenic potato lines, it was indicated that the leaky expression before stress treatment caused their growth retardant and that expression during stress associated to their tolerance. Selection was made on six tolerant lines as candidates for practical uses. Furthermore, the screening strategy for drought tolerance to *rd29A::AtDREB1A* transgenic potatoes combined with *in vitro* and growth room evaluation was recommended.

CHAPTER 2

In vitro evaluation of drought tolerance in *rd29A::AtDREB1A* transgenic potatoes

2.1 Introduction

A key task in genetic engineering and molecular studies of drought tolerance is the determination of appropriate experimental conditions. The most relevant factors are the strength of the stress, method for imposing the stress, portion of the plant to use for experimentation, and evaluation criteria. Tolerance or physiological reaction to drought is influenced by various experimental conditions, including plant type (species, variety, line), developmental stage, growth conditions, and method of drought application (Boyer 2010; Bruce et al. 2002; Poorter et al. 2012; Tardieu 2011; Verslues et al. 2006). Moreover, the side effects of experimental drought treatment must be considered (Cominelli et al. 2012; Poorter et al. 2012; Salekdeh et al. 2009; Verslues et al. 2006). Published literature reports regarding the expression analyses of stress-related genes have frequently failed to describe the experimental conditions used (Bhatnagar-Mathur et al. 2008; Herve and Serraj 2009). The results of previous studies cannot accurately be compared, because researchers have employed differing methods (Verslues et al. 2006). Thus, a suitable combination of experimental factors for the evaluation of drought tolerance remains to be elucidated. When developing a

new method, each variable must clearly be described; further, possible side effects should be considered.

In this section, twelve *rd29A::AtDREB1A* transgenic potato lines were evaluated drought tolerance by using a new *in vitro* method with minimal side effects, rotary liquid culture combined with PEG. Based on this method, drought-tolerant lines were selected, and the contribution of *AtDREB1A* expression to drought tolerance was assessed.

2.2 Materials and methods

2.2.1 Plant materials

Transgenic potato (*Solanum tuberosum* L. cv. Desiree) lines with introduced *rd29A*::*AtDREB1A* (Kasuga et al. 2004) were generated by using *Agrobacterium*-mediated transformation (Celebi-Toprak et al. 2005). In previous studies, these transgenic lines were evaluated for salinity and freezing tolerance (Behnam et al. 2006, 2007). In the present study, twelve transgenic potato lines (D10, D19, D20, D21, D22, D44, D53, D103, D132, D141, D163, and D164) were selected for evaluation of drought tolerance. All of the potato lines, including the non-transgenic line, were maintained in test tubes, on solid MS medium (Murashige and Skoog 1967) supplemented with 3% sucrose. A total of 3 node cuttings (each with a single opened leaf) were cultured in 250-ml glass bottles containing 10 ml of liquid medium (MS + 30 $g \cdot l^{-1}$sucrose). The cuttings were grown for 4 weeks at

25 ± 1 °C with shaking at 80 rpm, under a 16-h light/18-h dark photoperiod and a light intensity of 80 μmol·m^{-2}·s^{-1}.

2.2.2 Drought tolerance evaluation

Four days before drought treatment, the liquid medium was refreshed. Next, the 4-week-old potato plants (each with 6–7 opened leaves) were subjected to drought stress by removal of the old medium, followed by addition of 10 ml of fresh medium with or without polyethylene glycol 8000 (PEG). After the drought stress treatment, the recovery treatment was applied by removal of the old medium, followed by addition of 10 ml of fresh medium without PEG.

The osmotic potential of the MS medium plus PEG was fixed on the basis of the osmotic potential calculation for PEG 8000 (Michel 1983). To determine a suitable osmotic stress condition for drought tolerance evaluation, various levels of osmotic stress (final osmotic potential −0.2 MPa, −0.6 MPa, −1.0 MPa, −1.4 MPa, −1.8 MPa, and −2.2 MPa) was applied, and subsequently the appropriate stress strength, and durations of the drought and recovery treatments were assessed. After 9 days of drought treatment at −1.8 MPa, almost all of the plant leaves were wilted, but plants remained alive after the recovery treatment. At the end of 3 trials, the selection was fixed on stress strength at −1.8 MPa, and the durations of the drought and recovery treatments at 9 days and 6 days, respectively.

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 drought 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. To reduce the effects of *in vitro* culture on the evaluation, plants that exhibited physiological abnormalities, such as callus formation, chlorosis, or hyperhydricity were excluded from the evaluation. For each potato line, the drought stress and recovery treatment procedure was replicated 3 times, by using 3–4 bottles per replication.

2.2.3 RT-PCR and real-time PCR

Total RNA was extracted from the leaf sample by using an RNAqueous® Kit (Ambion, Austin, TX); cDNA was synthesized by using a PrimeScript RT Reagent Kit with a gDNA Eraser (Takara Bio Inc., Siga, Japan).

The transgene expression profiles were obtained by using RT-PCR, based on a PCR Amplification Kit with Takara Taq (Takara Bio Inc., Siga, Japan). The *AtDREB1A* primers were as follows: forward 5′-GAT TAT ATT CCG ACG CTT G-3′; and reverse 5′-TTC ATG ATT ATG ATT CCA CT-3′. The *ubiquitin* primers

were as follows: forward 5′-GCA GTT GGA GGA CGG AC-3′; and reverse 5′-GGC CAT CTT CCA ACT GTT TC-3′.

AtDREB1A was quantified by using a LightCycler® 480 System (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 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, 3 independent experimental replications were conducted.

2.2.4 Statistical analysis

Differences in drought tolerance among potato lines were analyzed by using one-way ANOVA, and ranked according to the Tukey–Kramer test ($p < 0.05$). Correlations between experimental parameters were established by using the Pearson correlation. All statistical analysis was conducted by using Minitab software.

2.3 Results

2.3.1 Relative growth of transgenic potato lines in the absence of stress treatment

Before the stress treatment in liquid culture, all of the transgenic potato plants grew well and showed no abnormal phenotypes, such as hyperhydration or chlorosis (Figure 2.1A). Most of the lines, including the non-transgenic line, reached a height of approximately 10 cm in 28 days. However, some transgenic lines showed a dwarfing phenotype; in particular, D141 and D132, which were less than half the height of the non-transgenic line (Figure 2.2). The growth difference was evaluated by using one-way ANOVA, and lines were ranked into 5 groups (a–e) according to the Tukey-Kramer test ($p < 0.05$; Figure 2.2). In comparison with the non-transgenic line (ab), the transgenic lines D141 (e), D132 (e), D108 (d), D21 (c), and D53 (c) were significantly smaller. By contrast, the heights of the transgenic lines D10 (bc), D22 (bc), D20 (abc), D19 (abc), D163 (abc), D164 (ab), and D44 (a) did not differ significantly from that of the non-transgenic line.

2.3.2 Drought stress response of *AtDREB1A* transgenic potato lines

The feature of non-transgenic and transgenic potato lines under drought stress and after recovery treatment was observed. Each potato line showed leaf wilting with different level under drought treatment (Figure 2.1B) and partially recovered

after drought stress releasing (Figure 2.1C). The drought tolerance was evaluated by measuring the leaf vital score. For all potato lines, the vital score decreased during drought stress, but gradually increased during recovery treatment (Figure 2.3). Lines that showed a higher vital score during drought stress treatment tended to show a higher vital score after recovery treatment. For all lines, the vital score decreased at the start of stress treatment, and reached a plateau after approximately 5–9 days of stress treatment. After recovery treatment, the vital scores of all lines increased after 2 days, and stabilized after 6 days (Figure 2.3). Especially, the transgenic lines D141, D132, and D108 maintained a vital score of >6 during drought stress treatment (Figure 2.3).

On the basis of the vital score on the third day after recovery treatment (Figure 2.3), the strongest and weakest lines were D141 and the non-transgenic line, respectively (Figure 2.4). Drought-tolerant lines were selected by using one-way ANOVA, and ranked into 3 groups (a–c) according to the Tukey-Kramer test ($p < 0.05$; Figure 2.4). 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 lines were regarded as conferring drought tolerance by *AtDREB1A* gene. Especially, D141 (a), D132 (a) and D108 (a) were defined as tolerant lines in this evaluation.

2.3.3 *AtDREB1A* expression profiles

On the basis of the vital score profile, four transgenic lines (D164, D163, D132, and D141) were selected for expression analysis of *AtDREB1A*. The results of RT-PCR showed that all 4 lines expressed *AtDREB1A* during drought stress (2 h, 2 days, and 9 days), recovery (2 h, 2 days, and 6 days), and control (non-stress) conditions (2 h and 2 days) (Figure 2.5A). Before stress treatment (0 h), *AtDREB1A* was strongly expressed in lines D132 and D141, but weakly expressed in lines D164 and D163. However, lines D163 and D164 showed upregulation of transgene expression after 2 h of stress treatment. The expression level decreased at 2 days and remained unchanged until 9 days. Further, lines D163 and D164 showed increased *AtDREB1A* expression after recovery treatment. On the other hand, lines D132 and D141 showed constitutive expression under all conditions.

The RT_PCR results indicate that the *AtDREB1A* expression level stabilizes after 2 days of stress treatment. Therefore, the before and 2 days after drought stress treatment were selected to clarify the quantitative expression of *AtDREB1A* in each transgenic potato line (Figure 2.5B). The results showed that most of the transgenic potato lines showed low *AtDREB1A* expression before drought stress treatment (Figure 2.5B); by contrast, lines D21, D108, D132, and D141 showed higher expression levels (>10-fold the relative level for *ubiquitin*). Further, with

exception of lines D132 and D141, all lines showed *AtDREB1A* induction during stress treatment.

2.4 Discussion

2.4.1 Screening of tolerant lines by using *in vitro* exposure to PEG

When evaluating drought tolerance, determination of a suitable method is crucial. However, plant tolerance and response to drought is complex, and adversely affected by various experimental conditions. Further, although many drought evaluation methods have been applied (Bhatnagar-Mathur et al. 2008; Herve and Serraj 2009; Verslues et al. 2006), each method involves differing experimental conditions. In addition, the side effects of experimental drought treatment must be considered. In the present study, the drought tolerance of 12 transgenic potato lines was evaluated by developing a novel method, namely, rotary liquid culture combined with PEG.

4-week-old intact potato plantlets with root systems were used at the beginning of drought treating. The plants were subjected to drought stress and recovery, by removal of the old medium, followed by addition of fresh medium with or without PEG 8000. Then the drought tolerance level of transgenic potato lines was evaluated on the basis of leaf wilting at the whole plant level.

The use of liquid culture for drought stress evaluation has a number of advantages. The *in vitro* plants and medium are easily separated, and therefore

drought or recovery treatment can readily be manipulated, thereby minimizing injury and/or damage to the plants. By contrast, when using conventional *in vitro* culture such as semi-arid agar, the drought and recovery treatments involve a certain amount of injury and/or damage to the plant roots (Lawlor 1970).

Drying of the whole plant or aerial portion (detached leaf or shoot) is simple and rapid, and has frequently been employed in previous studies (Bray 2004; Seki et al. 2001). In addition, soil drying (Poorter et al. 2012; Verslues et al. 2006) is a simple conventional method. However, because drought strength is dependent on relative humidity and plant transpiration, these evaluation methods are influenced by plant size or leaf area, and experimental environment. By contrast, this *in vitro* evaluation was carried out in a closed environment, thereby enabling precise control of osmotic conditions. Further, by using PEG solution to induce drought stress, the applying accurate stress strength to all potato lines, independently of the plant, could be performed.

In contrast to osmotica such as mannitol, sorbitol, and sucrose, PEG 8000 is unable to penetrate plants. Plants are frequently affected not only by drought stress, but also by the toxicity of the penetrated osmotica used (Fritz and Ehwald 2010; Hohl and Schofer 1991; Lipavska and Vreugdenhil 1996; Verslues et al. 1998). 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 drought stress (Verslues et al. 2006).

However, side effects of PEG penetration have been reported (Jacomini et al. 1988; Lawlor 1970; Yaniv et al. 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, the use of rotary liquid culture enhanced oxygen diffusion into the medium and diminished root damage/injury during plant transfer, thereby reducing the side effects caused by hypoxia and PEG penetration.

In general drought tolerance evaluation, determination of evaluation criteria is one of the most importance tasks. However, it is difficult to determine evaluation criteria for drought tolerance, because plant drought tolerance and drought response are influenced by the plant type (species, variety, line), growth conditions, and developmental stage (Poorter et al. 2012; Tardieu 2011). Further, different drought stress treatments (duration and intensity) cause varying plant responses (Lawlor 2013; Verslues et al. 2006). On the other hand, evaluation criteria must clearly reflect drought tolerance, and accurately represent the tendency of plant response. When part of a plant is exposed to drought stress, each organ or tissue may respond differently. However, plant response and tolerance to drought are derived from integrated regulation at the whole-plant-level (Tardieu 1996), and therefore, drought tolerance criteria must also be integrated at the whole-plant-level. In the present study, potato plants were maintained under *in vitro* conditions until completion of the evaluation.

Therefore, it is impossible to evaluate directly the response and tolerance to drought via physiological parameters; instead, indirect evaluation by observing plant features through the bottle glass was applied. Under severe drought stress (−1.8 MPa), all treated potato lines showed differing levels of leaf damage, including wilting, falling, and death (Figure 2.1). These leaf symptoms represent an emergent reaction to water loss, and a typical plant response under conditions of natural drought stress. After recovery treatment, the leaves of each treated line showed partial or full recovery. This tendency decreased from the top of the plant to the basal part (Figure 2.1). On the basis of a vital score (0–3) for each leaf and the whole-plant score for each plant, the drought tolerance of each transgenic potato line was determined (Figures 2.3 and 2.4). In addition, by examining the correlation between this criterion and the transgene expression level, the contribution of *AtDREB1A* expression to drought tolerance was investigated (Figure 2.7).

In summary, by using rotary liquid culture combined with PEG, the drought tolerance levels of twelve transgenic potato lines were determined. By enabling precise manipulation of the drought stress strength with minimal side effects, this newly developed method can eliminate plant injury and/or damage, thereby establishing appropriate evaluation criteria. However, a number of side effects must be considered, in particular, high sugar concentration and high relative humidity (90–100%). High sugar concentrations are known to inhibit

plant photosynthetic activity (Desjardins 1995; Nakayama et al. 1991). On the other hand, high relative humidity reduces the plant transpiration rate, and also the nutrient uptake rate (Kozai et al. 1995). Hence, plants cultivated *in vitro* may differ physiologically from those cultivated under natural conditions. Nevertheless, *in vitro* evaluation represents a simple and effective first screening method for tolerant lines. Prior to practical application of the twelve transgenic potato lines investigated in the present study, further evaluation is required.

2.4.2 Relationship between plant height and leaky *AtDREB1A* expression

Before stress treatment, all transgenic potato lines except D53, D21, D108, D132, and D141 showed similar growth to that of the non-transgenic line (Figure 2.2). On the other hand, all transgenic lines showed leaky expression of *AtDREB1A*, especially it was strong in D108, D132, and D141 lines (Figures 2.5A and B). In addition, a significant negative correlation ($p < 0.01$, $r = -0.92$) was recognized between plant height and *AtDREB1A* expression (Figure 2.6B). My results indicate that growth retardation of transgenic potato plants may be derived from leaky *AtDREB1A* expression before stress. Plant growth inhibition derived from constitutive *AtDREB1A* expression has previously been shown to occur in many transgenic plants, including *Arabidopsis*, tobacco, tomato, rice, and wheat (Lata and Prasad 2011; Mizoi et al. 2012).

DREB gene-induced plant growth inhibition in *Arabidopsis* is known to occur through the regulation of plant-growth-related proteins and phytohormone activity. DELLA proteins and gibberellins markedly influence plant growth in response to the environment (Harberd et al. 2009). Deactivation of gibberellins was strongly correlated with growth retardations in transgenic soybean (Suo et al. 2012) and tobacco (Cong et al. 2008), via *AtDREB1A* over-expression. In addition, expression of the *DREB1B* gene was positively correlated with plant growth inhibition, via accumulation of DELLA proteins (Achard et al. 2008). Moreover, over-expression of *DDF1*—belonging to the *DREB1/CBF* gene subfamily—caused a dwarf phenotype, by reducing the amount of bioactive gibberellins (Magome et al. 2008).

In the transgenic potato lines used in the present study, *AtDREB1A* is controlled by the abiotic-stress-inducible *rd29A* promoter, but showed leaky expression under non-stress conditions. This leaky expression may have been caused by the sensing of the *rd29A* promoter to some *in vitro* evaluation variables. Since node cuttings were used as explants, the wounding of these explants might trigger transgene expression through activation of the sensing of the *rd29A* promoter. In *Arabidopsis*, the *rd29A* gene is strongly activated by mechanical wounding (Cheong et al. 2002). Moreover, the addition of sugar ($30 g \cdot l^{-1}$) and inorganic nutrients to MS medium decreases the water potential to -0.44 MPa (Kozai and Kubota 2005). It is possible that the transgenic potato lines

used in my present study were subjected to mild drought stress. Similar to my study, Kasuga et al. (2004) reported that leaky *AtDREB1A* expression in *rd29A::AtDREB1A* transgenic tobacco plants was associated with growth retardation under non-stress conditions. Therefore, leaky expression in potato and tobacco may occur because of a heterogeneous promoter. On the other hand, leaky expression of the *GUS* gene in *rd29A::GUS* transgenic *Arabidopsis* was observed even under control conditions (Msanne et al. 2011). For these reasons, the *rd29A* promoter might be active without stress in *Arabidopsis*.

2.4.3 Relationship between drought tolerance and *AtDREB1A* expression

In comparison with the non-transgenic line, all of the *rd29A::AtDREB1A* transgenic potato lines showed reduced damage during drought stress and enhanced recovery (Figure 2.3). Moreover, under conditions of stress, *AtDREB1A* expression was high in all of the transgenic lines (Figure 2.5A). These results are in accordance with those of previous studies, in which enhanced tolerances to salinity (Behnam et al. 2006; Celebi-Toprak et al. 2005) and freezing (Behnam et al. 2007; Pino et al. 2007) were observed in transgenic potatoes carrying *rd29A::AtDREB1A*. Enhanced abiotic stress tolerance (salinity, freezing, and drought) has been observed in many transgenic plants carrying *rd29A::AtDREB1A*, for example, *Arabidopsis* (Gilmour et al. 2000; Kasuga et al. 1999; Liu et al. 1998), tobacco (Kasuga et al. 2004), tomato (Rai et al. 2013),

soybean (Polizel et al. 2011), rice (Ito et al. 2006), and wheat (Pellegrineschi et al. 2003).

The results of microarray analyses previously revealed that *AtDREB1A* over-expression activated a large number of stress-inducible and DREB-related genes in *Arabidopsis* (Maruyama et al. 2004), tomato (Zhang et al. 2004), and rice (Ito et al. 2006). Many of these stress-inducible genes, including LEA proteins, antifreeze proteins, hydrophilic proteins, RNA-binding proteins, antioxidant enzymes, and protease inhibitors, have a cell-protective function. Therefore, to clarify the involvement of *AtDREB1A* expression in drought tolerance of transgenic potatoes, the association between the vital score on the third day of recovery and the relative *AtDREB1A* expression after 2 days of stress treatment was assessed. A positive but non-significant correlation ($p = 0.104$, $r = 0.471$; Figure 2.7) was recognized. The results suggest that *AtDREB1A* expression under conditions of drought stress contributes to drought tolerance of transgenic potato lines, but that this contribution can be heterogeneous.

In comparison with the other transgenic potato lines, lines D108 and D21 showed markedly high *AtDREB1A* expression (<40 versus 89.3 and 66.6, respectively; Figure 2.5B). Interestingly, these high expression levels did not correspond to drought tolerance, and may therefore, be beyond the threshold of conferring tolerance. Unexpected and unintended traits are present in some transgenic events (Wilson et al. 2006). The effect of a transgene is known not

simply to be an effect of fixed magnitude, and high-expressing lines may result in undesirable phenotypes (De Wolf et al. 2008). Various factors, including positional transgene effects, RNA interference, and DNA methylation (Meyer 1995; Wilson et al. 2006), are independent in all transgenic lines, because each transgenic line is derived from an individual transformation event. These factors may reduce transgene activity. Hence, it was proposed that *AtDREB1A* may contribute differently to drought tolerance in lines D108 and D21 than in the other transgenic lines. When D108 and D21 transgenic lines were excluded from the association analysis, a significant positive correlation ($p < 0.01$, $r = 0.802$) between drought tolerance and *AtDREB1A* expression under conditions of stress was observed. These results suggest that transgenic potato lines show enhanced drought tolerance by induction of *AtDREB1A* expression under conditions of stress.

Abiotic stress sensing of the *rd29A* promoter has previously been determined in *Arabidopsis* via activation of the *rd29A* gene under conditions of salinity, cold, and drought stress (Kasuga et al. 1999; Liu et al. 1998). The *rd29A* promoter contains DRE and AREB cis-elements (Yamaguchi-Shinozaki and Shinozaki 1994). These cis-elements are present in the promoters of many abiotic stress-responsive genes (Yamaguchi-Shinozaki and Shinozaki 2005), and are also targets of many abiotic-stress signal transductions (Shinozaki and Yamaguchi-Shinozaki 2007). The *rd29A* promoter can also function in potato during

conditions of freezing and salinity stress (Behnam et al. 2006, 2007; Celebi-Toprak et al. 2005; Pino et al. 2007). In the present study, most transgenic potato lines showed induced transgene expression during drought stress treatment (Figures 2.5A and B). The results suggest that, similar to *Arabidopsis*, the *rd29A* promoter functions in the potato signal transduction system. Furthermore, the increased drought tolerance of *AtDREB1A* transgenic potato lines indicates that some downstream *AtDREB1A*genes function in drought tolerance. The results of microarray analyses with *35S::AtDREB1A* transgenic lines of two different potato species (*S. tuberosum* and *S.commersonii*) revealed the presence of many upregulated genes (Carvallo et al. 2011). Thus, the drought tolerance of *rd29A::AtDREB1A* transgenic potatoes may be derived from native downstream *AtDREB1A* genes, via sensing of drought stress by the *rd29A* promoter.

2.5 Proposed practical applications

On the basis of the novel *in vitro* evaluation method, drought tolerance levels of *rd29A::AtDREB1A* transgenic potato lines were classified. In comparison with the non-transgenic line, lines D20, D22, D19, D10, D108, D132, and D141 showed reduced damage under drought stress and enhanced recovery (Figures 2.3 and 2.4); however, at higher transgene expression levels, some of these lines showed a tendency for growth retardation (Figures 2.5 and 2.6). Four of the transgenic lines—D22, D20, D10, and D19—showed

significantly high drought tolerance (Figure 2.4) without growth retardation (Figure 2.2), and may represent candidates for practical application.

2.6 Figures

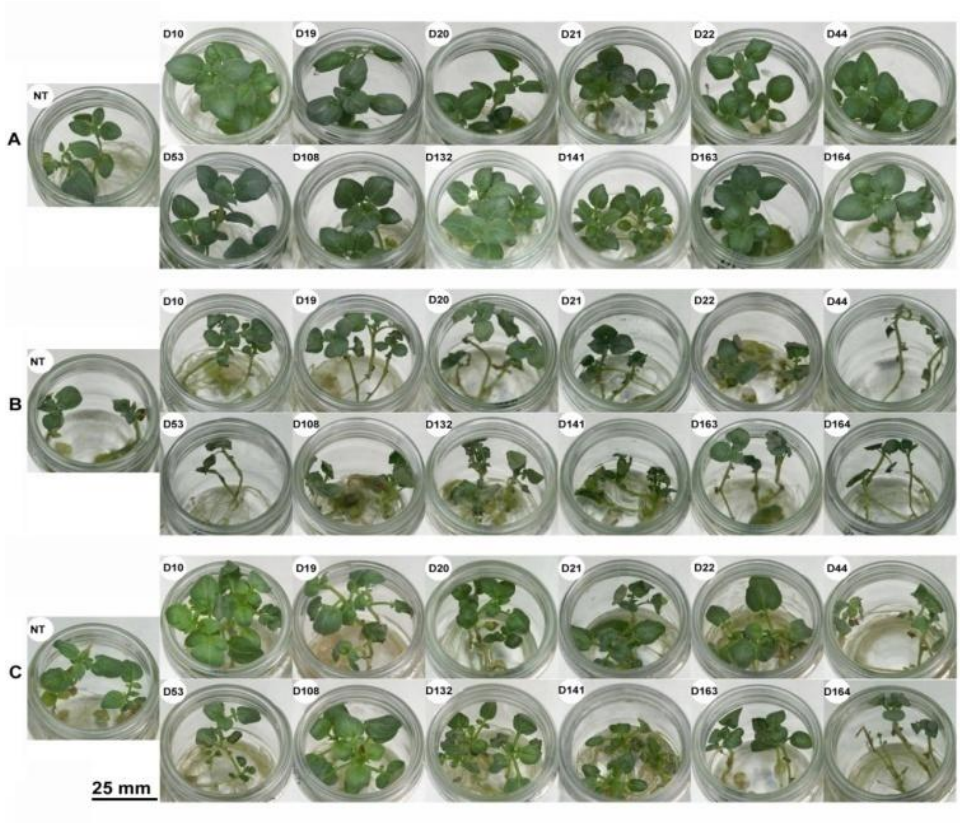


Figure 2.1 Phenotypic responses of non-transgenic (NT) and *rd29A::AtDREB1A* transgenic (D) potato lines under conditions of drought stress and recovery. A, before drought stress; B, at the end (ninth day) of drought stress; and C, after 3 days of recovery from drought stress. The plants were cultured in 250-ml glass bottles containing 10 ml of liquid medium (MS + 30g·l^{-1} sucrose). Stress or recovery treatment was carried out with or without PEG solution (−1.8 MPa). The scale bar represents 25 mm.

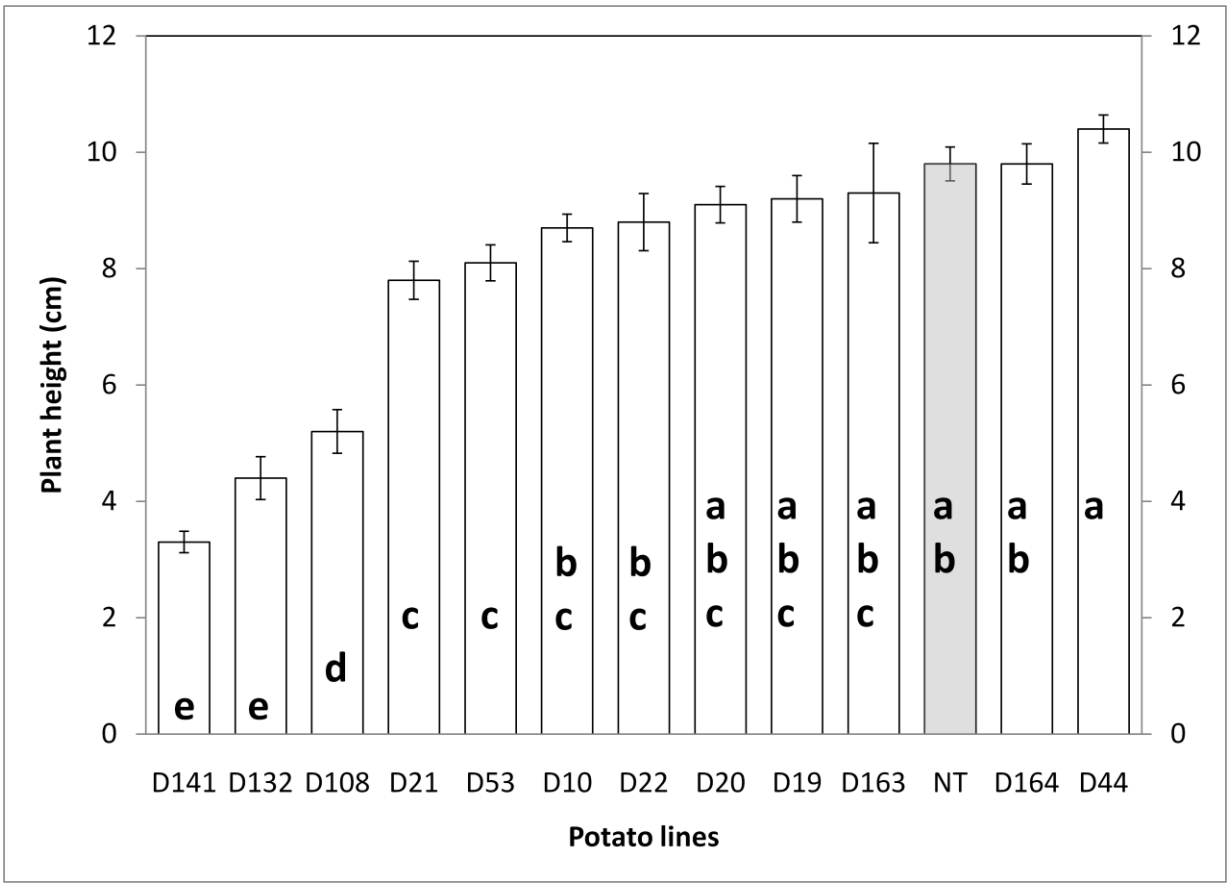


Figure 2.2 Plant growths under *in vitro* conditions. A non-transgenic potato line and twelve *rd29A::AtDREB1A* transgenic potato lines were cultured for 28 days without stress treatment. The plant height was measured immediately before stress treatment. Each bar represents mean ± standard error.

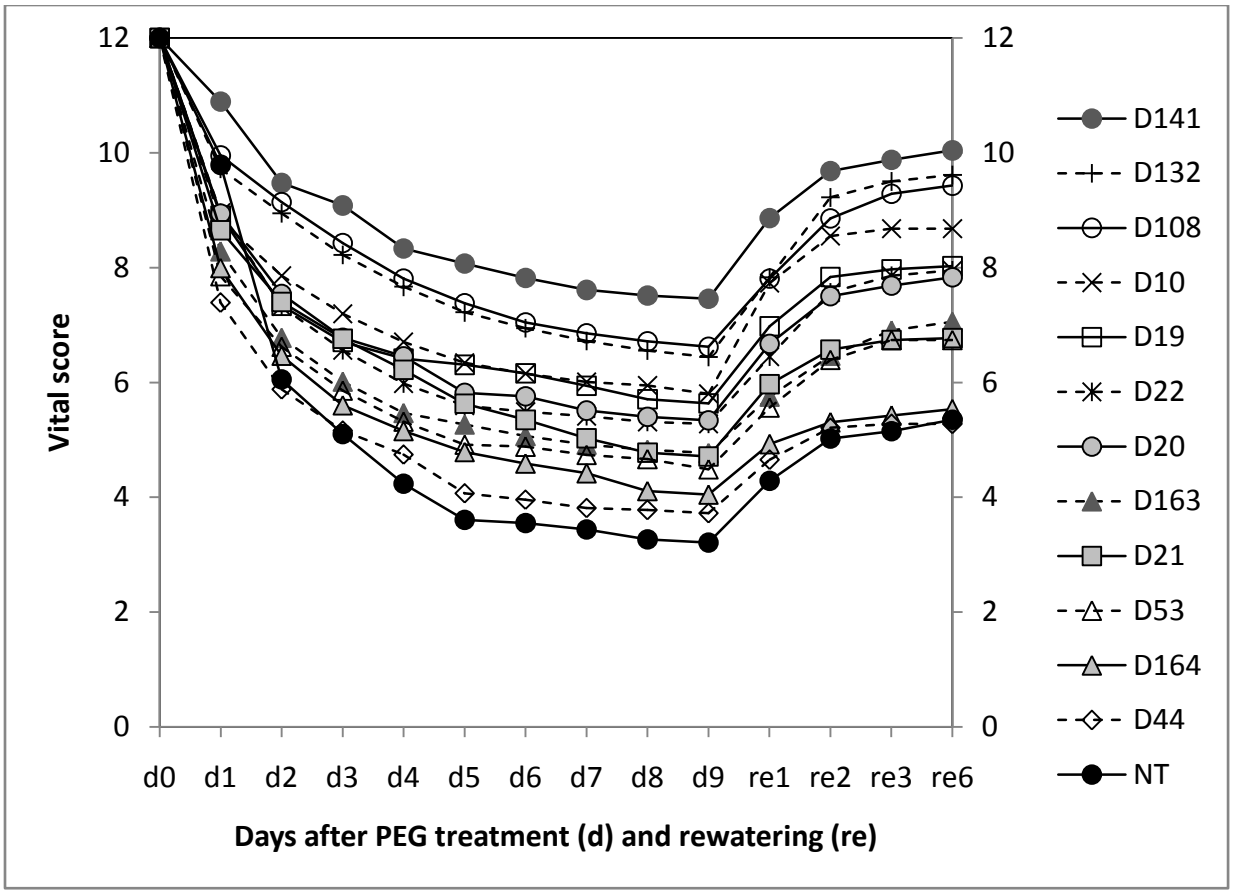


Figure 2.3 Drought stress and recovery responses of potato plants grown under *in vitro* conditions. A non-transgenic line and twelve *rd29A::AtDREB1A* transgenic lines of the of the commercial potato cultivar, Desiree, were treated with −1.8 MPa PEG solution and recovered by using normal culture medium. Each point represents the mean of whole-plant leaf-wilting resistance scores derived from 3 experimental replications. Whole-plant leaf-resistance score = total leaf wilting resistance score of 4 leaves (second to fifth leaf). Each experimental replication included 3–4 bottles per line and 3 plants per bottle.

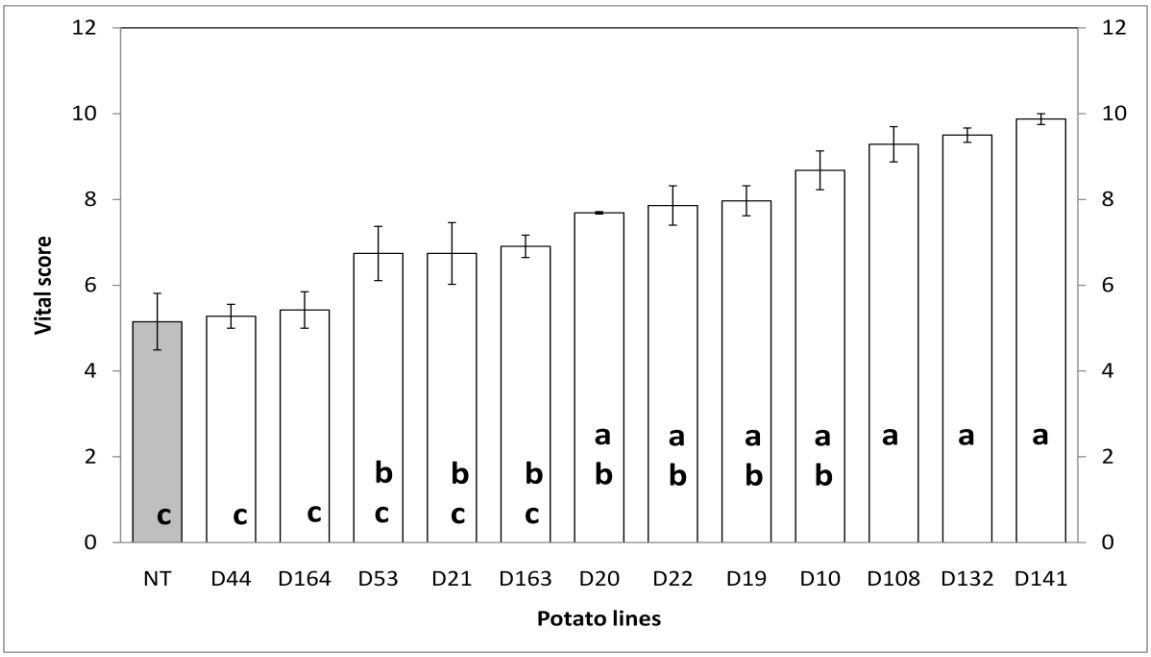


Figure 2.4 Drought tolerances of non-transgenic (NT) and *rd29A::AtDREB1A* transgenic (D) potato lines grown under *in vitro* conditions. Drought tolerance was represented by the mean vital score after 3 days of recovery. Differences between means were analyzed by using one-way ANOVA, and ranked according to the Tukey-Kramer test ($p < 0.05$). Lines not sharing the same letter differ significantly. Each bar represents mean ± standard error.

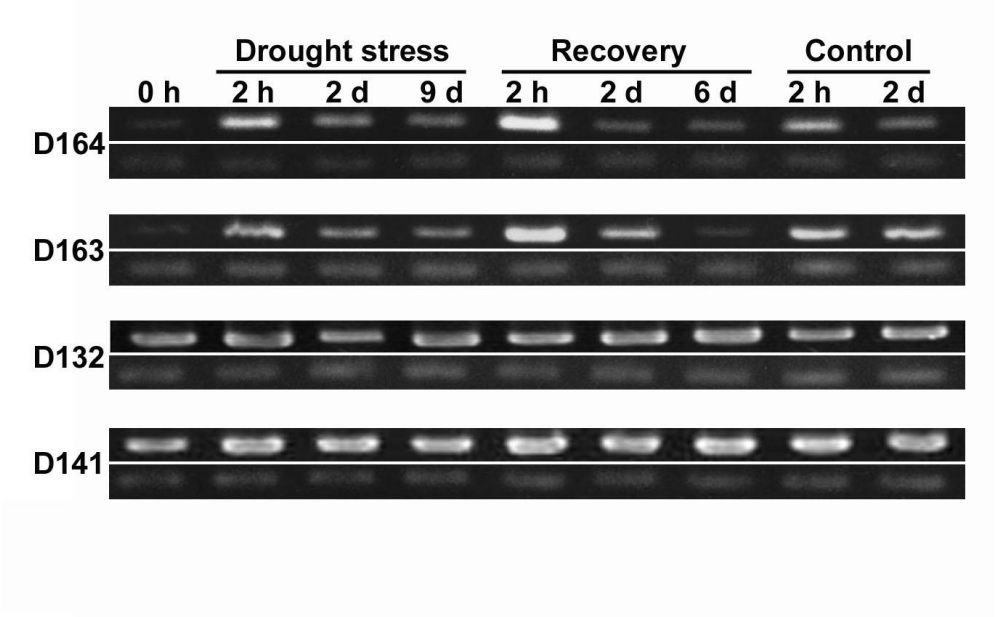


Figure 2.5A RT-PCR analysis of *AtDREB1A* expression in transgenic potato lines. For each transgenic line, the upper row represents *AtDREB1A* expression, and the lower row represents expression of reference gene, *ubiquitin*.

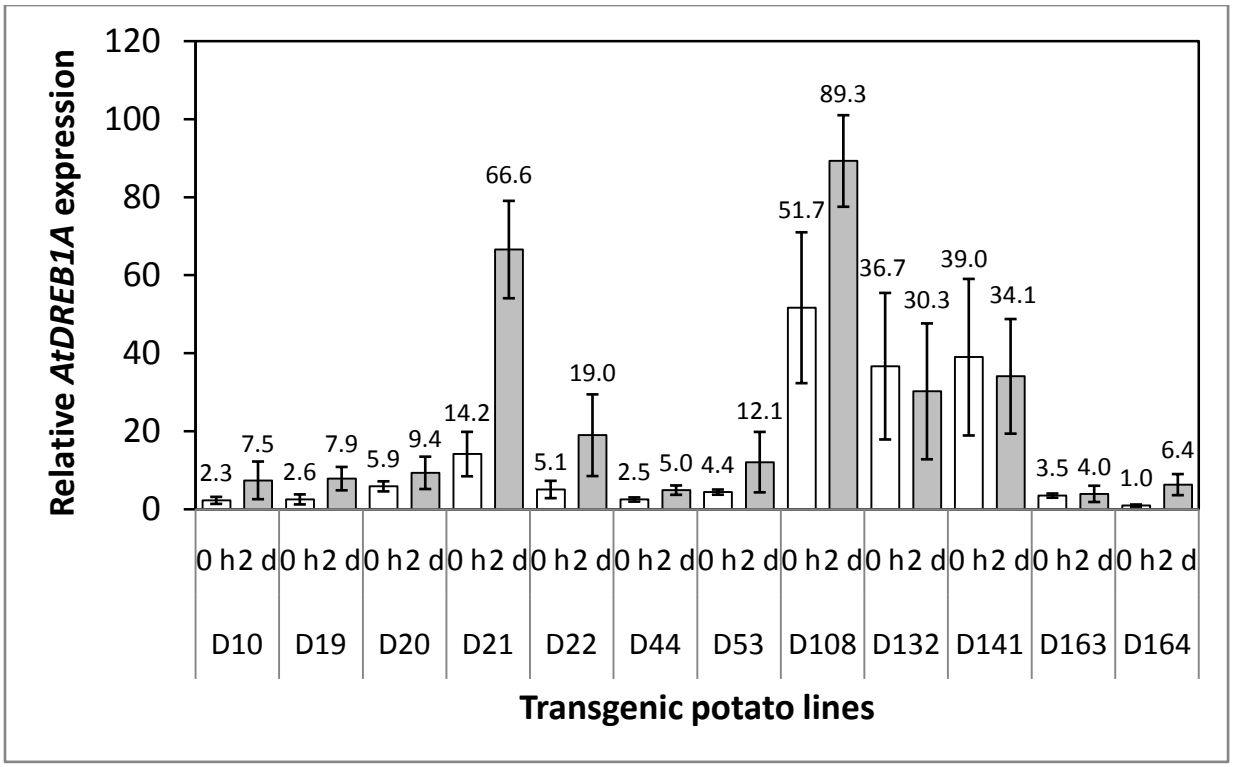


Figure 2.5B Quantitative *AtDREB1A* expression in transgenic potato lines before (0 h) and after 2 days (d) of drought stress. The relative *AtDREB1A*expressionof each transgenic line was derived from 3 experimental replications, and normalized against constitutive expression of *ubiquitin*. The *AtDREB1A*expression level of line D164 before drought stress was set at 1. Each bar represents mean ± SE of 3 independent experiments.

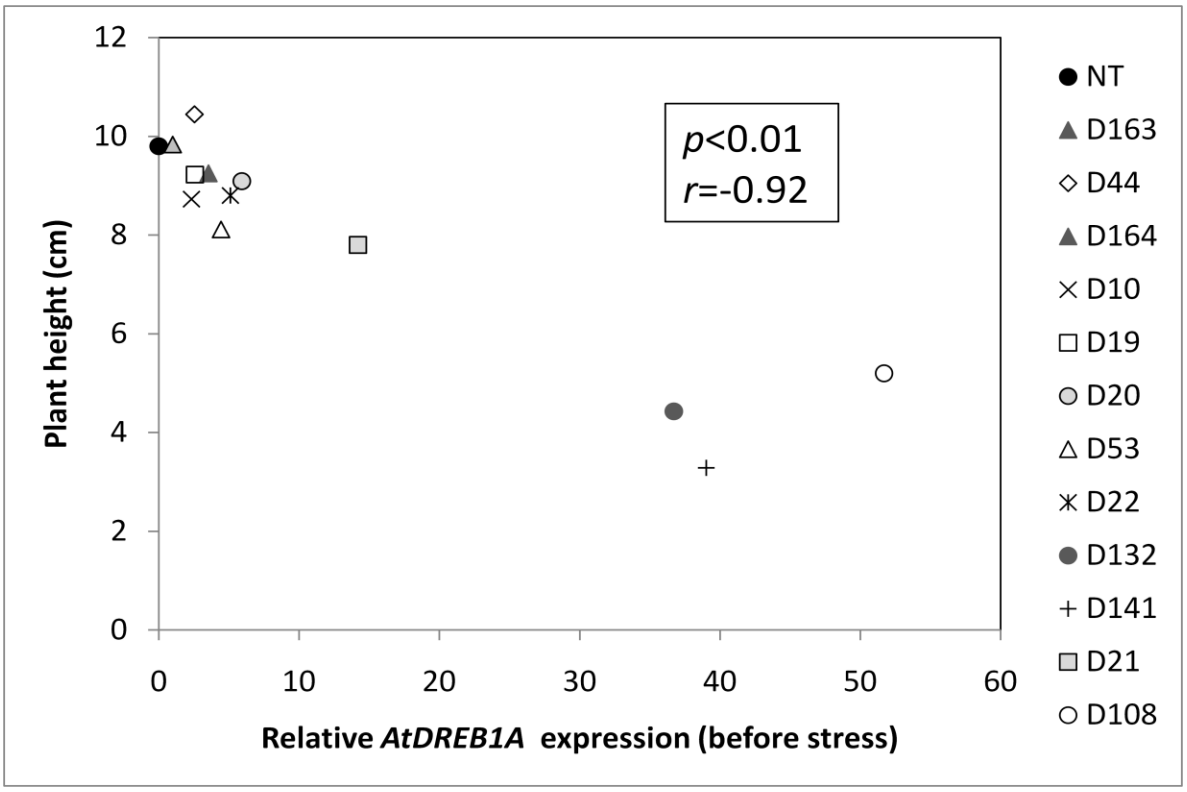


Figure 2.6 Relationships between *AtDREB1A* expression (0 h) and plant height in non-transgenic and *rd29A::AtDREB1A* transgenic potato lines. Each point represents the mean of 3 experimental replications.

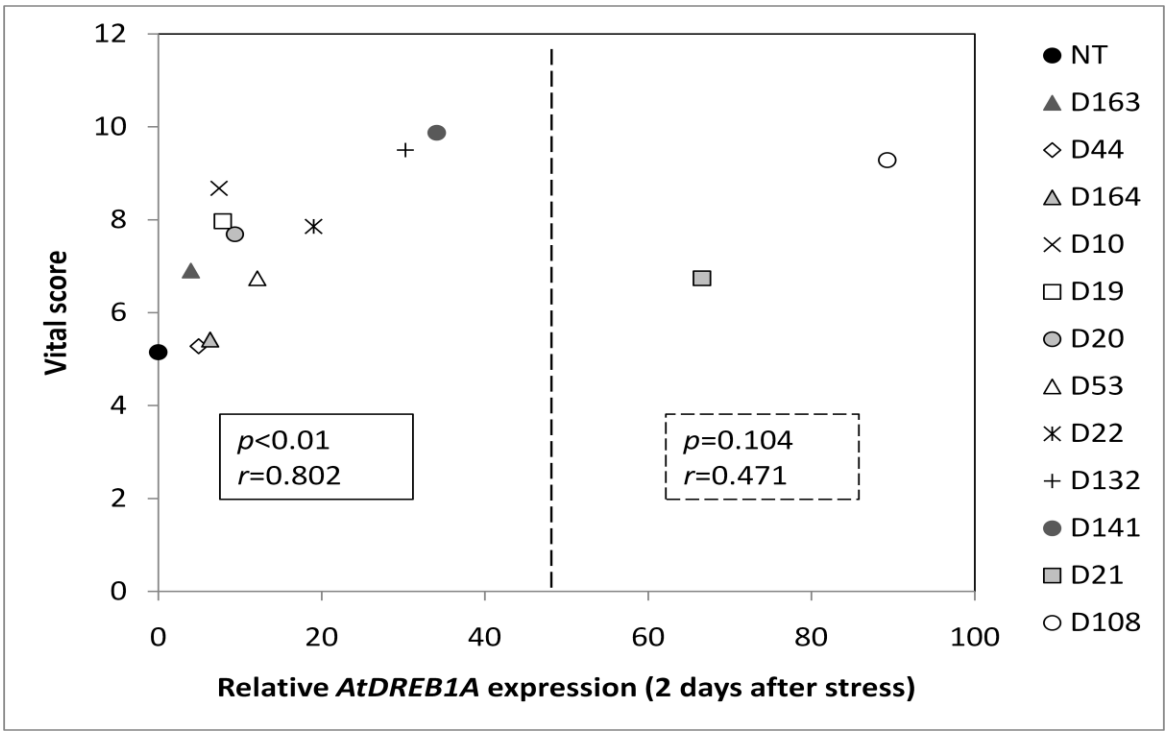


Figure 2.7 Relationships between drought tolerance and *AtDREB1A* expression after 2 days of stress treatment, in differently analyzed pools. The upper part represents the non-transgenic potato line and twelve *rd29A::AtDREB1A* transgenic potato lines; the lower part represents the non-transgenic potato line and 10 transgenic potato lines (after removal of D21 and D108). Each point represents the mean of 3 experimental replications.

CHAPTER 3

Growth room evaluation of drought tolerance in *rd29A::AtDREB1A* transgenic potatoes

3.1 Introduction

In the previous chapter, the preliminary assessment for drought tolerance in 12 *rd29A::AtDREB1A* transgenic potato lines under *in vitro* conditions was performed. In comparison with the non-transgenic line, seven of the transgenic lines – D10, D19, D20, D22, D108, D132, and D141 – showed enhanced drought tolerance. In addition, the enhanced drought tolerance in all transgenic potato lines (except D21 and D108) was indicated highly correlate to the expression of *AtDREB1A* expression under stress. However, growth retardation was observed in some transgenic lines due to high level of transgene expression under non-stress conditions. Considering the balancing between the drought tolerance and growth retardation of the transgenic plants, four transgenic lines D10, D19, D20, and D22 were selected as good candidates for practical application.

The *in vitro* conditions are highly controlled and different from the variable field conditions which need for practical application. Moreover, in order to select some suitable transgenic lines for practical application, further evaluation under unstable environmental conditions is required. On the other hand, to accomplish a

practical use for transgenic plants, evaluation under confined conditions (growth room) is required before further evaluations in semi-confined conditions (special netted-house) and subsequent field conditions (Kikuchi et al. 2008; Hilbeck et al. 2011). Therefore, after *in vitro* evaluation, evaluating drought tolerance of these transgenic potato lines under growth room is necessary.

In this section, drought tolerance of twelve *rd29A::AtDREB1A* transgenic potato lines was evaluated under growth room by using periodical treating PEG to soil-pot plant. Based on this method, the drought tolerant levels of transgenic lines were indicated, and the contribution of *AtDREB1A* expression to drought tolerance under growth room conditions was also investigated.

3.2 Materials and Methods

3.2.1 Plant material

Transgenic potato (*Solanum tuberosum* L. cv. Desiree) lines with introduced *rd29A*::*AtDREB1A* (Kasuga *et al.*, 2004) were generated by *Agrobacterium*-mediated transformation (Celepi-Toprak et al., 2005). In this study, 12 transgenic potato lines (D10, D19, D20, D21, D22, D44, D53, D103, D132, D141, D163 and D164) obtaining different *in vitro* drought tolerant level (chapter 2) were continuously evaluated for drought tolerance under growth room condition.

3.2.2 Growth room acclimation

Pre-cultured transgenic and non-transgenic line potato plants on solid MS medium (Murashige and Skoog 1967) for around one month were acclimated in the growth room for 2 weeks. Shoot cuttings were performed from these plants with root inducer (Rootone; Sumitomo Chemical Inc., Osaka, Japan), and were cultivated for to regenerate root system for 2 week. The grown plantlets were transplanted into quadrangular pyramid pots (4.2 cm x 4.2 cm x 12 cm) 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 perlite (Fuyou perlite Inc., Nagano, Japan). After 10 days pre-cultivation, the pot-plants obtained 7-9 leaves. These were used for further drought tolerance evaluation. The conditions in the growth room were 12-h light/12-h dark photoperiod, 150 µmol m^{-2} s^{-1} light intensity, and at 25±1^{o}C temperature.

3.2.3 Drought tolerance evaluation

The osmotic potential of Hoagland solution (Hoagland and Arnon 1950) plus PEG were fixed on the basis of the osmotic potential calculation for PEG 8000 (Micheal B. E. 1983). The drought stress imposing to experimental plants was performed by soaking the plant pots into PEG solution for 1 hour, and periodically

treated every two days. Before evaluating drought tolerance in transgenic potato lines, stress strength and durations of the drought and recovery treatments were determined by investigating the response of non-transgenic plants to various levels of osmotic stress (-1.0 MPa, -1.2 MPa, -1.4 MPa, -1.6 MPa, -1.8 MPa, -2.2 MPa, and -2.6 MPa). At the end of 3 trials, I fixed the stress strength at -1.8 MPa osmotic potential, and the durations of drought and recovery treatments at 21 days and 9 days, respectively.

The vital score of each leaf was determined as the scoring of *in vitro* drought tolerance evaluation (0, leaf fully wilted or dead; 1, more than half of leaf wilted; 2, small part of leaf wilted; 3, leaf not wilted). The youngest (1st) leaf was often not fully expanded, and the oldest (7-9th) leaf began to be senescence. For this reason, the whole-plant condition was evaluated by the vital score that was calculated as the sum of the score from second to sixth leaf. In this section, I used whole plant score to assess drought response and tolerance of transgenic potato lines. Vital score is represented for whole plant score. For each potato line, the drought and recovery treatment procedure was replicated 3 times, by using 5 plants/line. The temperature was maintained at $25\pm1^{o}C$ and the evaluation was performed at two different relative humidity conditions, 75% and 55%.

3.2.4 RT-PCR and real time PCR

Total RNA was extracted from the leaf sample by using RNAqueous® Kit (Ambion, Austin, Texas, USA) and cDNA was synthesized using PrimeScript RT reagent Kit with gDNA Eraser (Takara Bio Inc., Shiga, Japan).

The procedure from collecting sample to assessing quality and quality expression *AtDREB1A* expression level were performed according to the ones used in the *in vitro* evaluation section, Also, the same sequences of *AtDREB1A* and *ubiquitin* primers that used in the *in vitro* evaluation section were applied in this section.

3.2.5 Statistical analysis

To analyze and evaluate drought tolerance among transgenic potato lines, the same statistical analysis methods and software used in the *in vitro* evaluation section were applied.

3.3 Results

3.3.1 Relative growth of transgenic potato lines

For growth room evaluation, all of the potato plants were pre-cultivated without stress for 10 days. All material showed no abnormal phenotype (Figures 3.1A and B). In this time, the height of all plant was measured. In comparison to non-

transgenic line, all of the transgenic lines showed dwarfing phenotype under both relative humidity (RH) 75% and 55% condition (Figures 3.2A and B). Under RH 75%, the height of non-transgenic line reached a height of higher than 10 cm while of transgenic lines reached a height of less than 5 cm. Under RH 55%, non-transgenic line reached a height of higher than 8 cm while all transgenic lines reached a height of less than 5.5 cm. The growth difference among potato lines was evaluated by using one-way ANOVA, lines were ranked into 3 groups (a-c) by Tukey-Kramer test at $p < 0.05$ (Figures 3.2A and B). For both RH conditions, the results showed that only the non-transgenic line was categorized into a group while the transgenic lines were significantly smaller and categorized into b or c groups. Among transgenic lines, D141, D132, and D108 showed smaller (but non-significant difference) in comparison with the other transgenic lines under both RH 75% and 55% conditions.

3.3.2 Drought stress response of *rd29A::AtDREB1A* transgenic potato lines

To evaluate drought stress response, all of the non-transgenic and transgenic potato plants were imposed to -1.8 MPa PEG solutions every 2 days for 21 days, and then, were subjected to recovery treatment for 9 days. The drought and recovery treatment were performed under two different RH conditions (75% or 55%). The feather of these plants under drought stress and recovery was observed.

All potato lines showed leaf wilting under drought stress and partially recovered after drought stress releasing (Figures 3.1A and B). The drought response profiles of potato lines grown in both RH conditions were also investigated by measuring leaf vital score. During first two days of drought stress, the vital score did not change in all potato lines, after that the vital score gradually decreased until the end (21st day) drought stress. After releasing the drought stress, the vital score gradually increased in all of the non-transgenic and transgenic lines, except D44 line. Although all transgenic lines showed similar profile in vital score during the drought stress, the vital scores were different in each line during recovery. The score became flat after 6 days. It was found that the vital scores of potato lines under RH 75% (< 6) are higher these under RH 55% (< 4) at the end of the drought stress. However, the vital scores of lines between two RH conditions are not different at the end of the recovery.

On the basis of the vital score on the sixth day after recovery treatment (Figures 3.3A and B), D44 and non-transgenic lines showed weaker than the other transgenic lines (Figures 3.4A and B). Drought tolerance of potato lines grown under 75% and 55% RH conditions were analyzed separately by using one-way ANOVA. Under high relative humidity (75%) condition, there was a significant difference in drought tolerance among potato lines, and therefore drought tolerance of these lines could be ranked into 2 groups (a-b) according to Tukey-Kramer test

at $p < 0.05$ (Figure 3.4A). Accordingly, transgenic lines D44 (b), D132 (ab), D141 (ab), D108 (ab), D21 (ab) and D22 (ab) were classified into "b" group, same as non-transgenic line. In contrast, the transgenic lines D163 (a), D53 (a), D20 (a), D19 (a), D10 (a) and D164 (a) were regarded as tolerant lines. On the other hand, under low relative humidity (55%) condition, due to high variation between replications, no significant difference in drought tolerance among potato lines was detected (Figure 3.4B). However, high differences in vital score means among lines were observed, in which non-transgenic and D44 lines showed low scores (< 2) while the D20, D10, and D53 lines showed very high scores (> 8).

3.3.3 *AtDREB1A* expression profiles

For assessing *AtDREB1A* expression, another set of potato plants was treated drought stress, and then collected for gene expression analysis under relative humidity (RH) 75%.

On the basis of the previous *AtDREB1A* expression profile under *in vitro* conditions, four transgenic lines (D164, D163, D132 and D141) were selected for qualitative expression analysis. The results of RT-PCR showed that *AtDREB1A* was expressed in all four lines before the stress treatment (0d). While weak expression was observed in D163 and D164 lines, strong expression was detected in D132 and D141 lines (Figure 3.5A). During drought stress (2 days, 4 days and

15 days) and recovery treatment (2 days), all transgenic lines showed some expression of *AtDREB1A* (Figure 3.5A), however D163 and D164 lines showed lower expression compare to D132 and D141 line. In D163 and D164 lines, the expression of *AtDREB1A* was up at 2 days and remained unchanged at 4 days drought stress. The expression level decreased at 15 days drought stress, and not increased at 2 days recovery. On contrary, in D132 and D141 lines, *AtDREB1A* was highly expressed before drought stress and kept this expression at 2 days and 4 days drought stress. The expression was a little decreased at 15 days drought stress but increased again at 2 day recovery.

The expression profiles indicate that although some difference in expression pattern between these 4 transgenic lines, the *AtDREB1A* expression level is stable in the middle stress (2 days and 4 days). Therefore, the before and 2 days after drought stress treatment were selected to clarify the quantitative expression of *AtDREB1A* in each transgenic potato line (Figure 3.5B). The results showed that the induction of *AtDREB1A* under drought stress was observed in all transgenic lines. However, heterogeneity in gene expression was also observed among transgenic lines. Most of transgenic lines showed low *AtDREB1A* expression before the stress (< 2-fold) while D21, D44, D108, D132, and D141 showed higher expression levels (> 3.5-fold). This expression pattern under growth room conditions is similar to the expression pattern under *in vitro* conditions previously.

3.4 Discussions

3.4.1 Growth room evaluating drought tolerance of transgenic potato lines by periodically imposing PEG to potted-plants

For experiments on drought tolerance, the onset and intensity of the stress need to be defined clearly and controlled precisely during experiment. Besides, the other environmental conditions such as light, temperature, relative humidity, etc., also need to be controlled or managed. These stress and other environmental conditions are needed for a conduct of repeatable and interpretable experiments.

Growth room evaluation is an intermediate step between *in vitro* and greenhouse/special netted-house evaluations. Growth room evaluation allows very precise control of main environmental factors – temperature, air humidity, light, etc., – that can affect on plants growth and development. For drought tolerance evaluation under growth room conditions, soil water depletion /soil drying method is usually used to apply drought stress to the pot plants (Verslues et al. 2006; Tuberosa 2012). In this method, drought stress imposing to each plant depends on the water using of the plant and/or leaf water loss rate. As a result, different size-plants sense different drought stress levels and therefore drought tolerance is a dependent factor of plant size. On the other hand, variations in plant size within or among populations assigned to evaluated lines/genotypes due to variability in growth are unavoidable (Poorter et al. 2012). In addition, plants from different

genotypes possess different plant characters usually lead to variation in water using (Parent et al. 2010). In this study, a non-transgenic and twelve transgenic lines showed heterogeneous in plant size (Figures 3.1A and B), although grown in the same growth room conditions. Therefore, to homologize drought stress treatment to potato lines that had different plant sizes, a new drought treating method to soil pot plants, periodically PEG treating, was applied.

In this study, 10-day-old potato plants which grown in soil-pots were used at the beginning of drought treating. Based on the precisely controlling osmotic potential of PEG solution that has been used in previous *in vitro* drought tolerance evaluation, a new PEG solution treating method for the growth room evaluation was developed. In this method, PEG solution was applied periodically (every two days) at the designed drought stress level (-1.8 MPa) to all soil pot plants. Different potato lines (non-transgenic and transgenic lines) can be imposed at the same drought stress level and then evaluated drought tolerance comparably due to the independence of drought treatment to the plant size. Moreover, by periodically imposing PEG solution to soil pot plants, the hypoxia effect of long-time PEG treating can be reduced due to the air diffusion to the treated soil pot can be maintained during drought treatment.

When drought stress was applied to experimental plants, all potato lines showed leaf wilting after 2 days (Figures 3.3A and B) and almost plant leaves wilted at the

end (21st day) of the drought stress. These wilting symptoms of plants that were periodically treated with PEG solution are similar to the symptoms of plants treated by soil drying (data not shown). On the basis of the leaf wilting scoring in previous *in vitro* evaluation, the vital score (0-3) for each leaf according to its wilting level was determined. Consequently, drought response and tolerance of each potato line were evaluated by using whole plant score as a total vital score of second to sixth leaves (Figures 3.3A and B, and Figure 3.4A and B). Under two different relative humidity (RH) conditions, 75% and 55%, the response profiles of a non-transgenic and 12 transgenic lines under drought stress and recovery showed similar pattern (Figures 3.3A and B). No difference among transgenic lines in response to drought stress while variation in recovery among these lines was observed. Under the recovery, the vital scores were different among potato line, and the recovery became stable after 6 days drought stress releasing. From these results, drought tolerance level of transgenic lines was determined according to the vital score at 6th day recovery (Figures 3.4A and B). No difference in drought tolerance was observed among potato lines at RH 55% due to the high variation between experimental replications, while at RH 75%, with low variation between experimental replications, the drought tolerance among potato lines was classified. This result showed that when the environmental factors become more extreme, the variation in plant responses tend to increase. However, a significant positive

correlation ($p < 0.001$, $r = 0.97$) was recognized between drought tolerance these two relative humidity conditions (Figure 3.10B). This result indicated that the drought tolerance order among the transgenic potato lines was not affected by the changing of relative humidity conditions.

In summary, by using periodically imposing PEG solution to soil pot plants, the same drought strength was applied to all potato lines with different plant sizes. From this method, the drought tolerance levels of twelve *rd29A::AtDREB1A* transgenic potato lines were indicated.

3.4.2 Plant growth and leaky expression of *AtDREB1A* gene

Before the drought stress, all transgenic potato lines showed reduced plant height (< 5 cm) in comparison with the non-transgenic line (> 10 cm) (Figure 3.2A). Otherwise, all transgenic lines showed leaky expression of *AtDREB1A* before the stress treatment (Figures 3.5A and B). Moreover, among transgenic potato lines, a result of a significant negative correlation ($p < 0.01$, $r = -0.84$) between plant height and *AtDREB1A* expression was observed (Figure 3.6).These results indicate that the leaky expression of *AtDREB1A* before the stress considerably affects on growing of transgenic potato plants, and highly corresponds with the reduced growth among them.

DREB gene-induced plant growth retardation was observed in many transgenic plants including *Arabidopsis*, tobacco, rice, etc. (Nakashima et al. 2009; Agarwal

and Jha 2010; Mizoi et al. 2012). Moreover, in *Arabidopsis* (Magome et al. 2004, Achard et al. 2008), tobacco (Cong et al. 2008), tomato (Li et al. 2012), and soybean (Suo et al. 2012), the growth retardations by over-expression of *DREB* genes were indicated in relation to the acceleration of DELLA proteins accumulation and/or gibberellins biodegradation. These results suggest that the growth inhibition in transgenic potato may relate to the increased accumulation of DELLA proteins or decreased gibberellins by the leaky expression of *AtDREB1A* during non-stress conditions before the drought treatment.

Under growth room conditions, my transgenic potato lines showed growth reduction due to the leaky expression of *AtDREB1A* under non-stress condition although this gene was driven by an abiotic-stress-inducible *rd29A* promoter. The leaky expression in these transgenic potatoes may occur because of a native action of *Arabidopsis rd29A* promoter and/or a heterogeneous promoter, as mentioned in the *in vitro* evaluation. On the other hand, this leaky expression may result from the sensing of *rd29A* promoter to mild drought stress during pre-stress treatment. In this study, potato plants were grown in small soil pots with two days irrigation interval. The small soil pot contains low water content and water demand of potato plant over soil pot water is high. Demands for water increase strongly with the size of the plants, so the water availability may become limiting at the later developmental stages when the plant is bigger (Poorter et al. 2012). Therefore, the

soil pots could be temporarily dried between two irrigation time by water loss due to evaporation of soil pot water and transpiration of plant.

3.4.3 Relationship between drought tolerance and *AtDREB1A* expression

In comparison to the non-transgenic line, all of the *rd29A::AtDREB1A* transgenic potato lines (except D44) showed reduced damage at the end of the drought stress and enhanced recovery during the stress releasing (Figure 3.3A). In addition, based on vital score, all transgenic lines (except D44) showed high drought tolerance (> 5.5) in comparison with the non-transgenic line (< 4) (Figure 4A). On the other hand, under drought stress, the expression of *AtDREB1A* was high in all transgenic lines (Figures 3.5A and B). These results indicate the conferring drought tolerance of *AtDREB1A* in these transgenic lines, and this indication is consistent with the previous indication under *in vitro* conditions.

To assess the contribution of *AtDREB1A* expression to drought tolerance of transgenic potato lines, the association between drought tolerance (vital score on the sixth day of recovery) and the relative expression of *AtDREB1A* after 2 days of drought stress was investigated. However, there was no correlation ($p = 0.447$, $r = 0.23$; Figure 3.7A) between the drought tolerance and *AtDREB1A* expression. This result indicated that the contribution of *AtDREB1A* on drought tolerance was

heterogeneous under growth room conditions, and not consisted with the high positive contribution of *in vitro* result.

In transgenic plant production, although the same gene is introduced into plants, transformed event/lines are usually not homologous in performance. Each line is independent from the others. In addition, the effect of transgene is not simply to be an effect of fixed magnitude (De Wolf et al. 2008), and it can depend on the insertion copy number, insertion position, and the interaction of transgene and background genes (Sinclair et al. 2004). Therefore, heterogeneous contribution of *AtDREB1A* on drought tolerance in my transgenic potato lines can be result from the heterogeneous among transgenic lines.

Although the contribution of *AtDREB1A* on drought tolerance was heterogeneous, a significant positive correlation ($p < 0.05$, $r = 0.66$; Figure 3.7B) was observed between drought tolerance and promotion level of *AtDREB1A* expression. Promotion level of *AtDREB1A* expression is defined as promotion expression of *AtDREB1A* under drought stress compare to before stress. This result indicated that the higher induction of *AtDREB1A* expression (from non-stress to stress conditions) conferred higher drought tolerance. However, it was found that low tolerant lines D108, D132, and D141 showed high relative expression of *AtDREB1A* under drought stress while high tolerant lines D10, D19, and D20 showed the lower expression (Figures 3.4A and 3.5B). Additionally, it was also

found that a higher expression of *AtDREB1A* before the stress was also recorded in D108, D132, and D141 lines (Figure 3.5B) in comparison with the transgenic lines D10, D19, and D20. From these results, it was suggested that the contribution of *AtDREB1A* expression under stress on drought tolerance in transgenic potato lines can be affected by the leaky expression of *AtDREB1A* under non-stress conditions.

In transgenic *Arabidopsis*, the over-expression of *AtDREB* correlated to the enhanced accumulation of some inhibitor proteins as DELLA and/or the reduced bioactive gibberellins content (Magome et al. 2004; Achard et al. 2008). DELLA proteins and gibberellins have important roles in plant growth and development, beside the roles in abiotic stress tolerance (Harberd et al. 2009). On the other hand, in transgenic plants, a too high expression of one functional gene can break the intrinsic balance in the plant due to gene-to-gene interactions, consequently result in un-appropriate function in plant (Wilson et al. 2006). In this study, beside the considerably negative effects on plant growth of leaky *AtDREB1A* expression, a significant positive correlation ($p < 0.05$, $r = 0.624$; Figure 3.8) was identified between plant height and drought tolerance in transgenic potato lines. These results suggest negative effects of high leaky *AtDREB1A* expression on the drought tolerance may result from breaking the integration balance of gene-to-gene interaction. The higher abiotic stress tolerance of a stress inducible *AtDREB1A* expression (*rd29A::AtDREB1A*) line in comparison with a constitutive expression

(*35S::AtDREB1A*) line was observed in *Arabidopsis* (Kasuga et al. 1999). Both transgenic lines showed strong *AtDREB1A* expression under stress conditions (drought, salinity, and cold) while the expression of *AtDREB1A* in *rd29A::AtDREB1A* plants was much less than in *35S::AtDREB1A* under non-stress conditions.

Plant recovers from drought stress depend on the activation of genes relate to growth and development and on the repression of genes relate to growth inhibition. Accordingly, many of rehydration-upregulated genes were dehydration-downregulated genes, and conversely, many of rehydration-downregulated genes were dehydration-upregulated genes. In *Arabidopsis*, when the plants were released from drought stress, many drought-stress-inducible genes and also *AtDREB1A* target genes, such as *RD29A*, *cor15A*, *kin1*, *kin2*, *RD17*, *ERD13*, *RD28*, *ERD4*, *RD20*, *ERD9*, *ERD7*, and *RD22* were included in rehydration-downregulated genes (Oono et al. 2003). This result showed that dehydration-inducible genes were repressed in the rehydration process. On the other hand, it is known that gibberellins have important role in plant recovery. Gibberellins induced recovery from water stress in *Brassica campestris* (Banyal and Rai 1983). Besides, gibberellins can stimulate many cell activities such as cell proliferation, differentiation and expansion (Claeys et al. 2012) that are important for plant recovery. In this study, the high expression of *AtDREB1A* after recovery in some

transgenic lines as D132, and D141 was recorded (Figure 3.5A). The high expression of *AtDREB1A* can enhance the expression of many stress inducible gene (Seki et al. 2001; Flower and Thomashow 2002; Maruyama et al. 2004) and reduce the bioactive gibberellins content (Magome et al. 2004; Achard et al. 2008), so then inhibit plant growth and recovery. Otherwise, the expression of *AtDREB1A* under recovery in *rd29A::AtDREB1A* transgenic potato lines is considered similar to the expression before stress treatment or under non-stress conditions due to the same conditions applied. Therefore, the negative effects of leaky *AtDREB1A* expression on drought tolerance in transgenic potato lines result from the inhibition of leaky *AtDREB1A* expression on the recovery process.

In summary, these results indicated the conferring drought tolerance of *AtDREB1A* in *rd29A::AtDREB1A* transgenic potatoes under growth room conditions. A heterogeneous contribution of *AtDREB1A* expression on drought tolerance was indentified in transgenic potato lines. The heterogeneous contribution might be due to gene-to-gene interaction and high relation of *AtDREB1A* to plant growth, development, and also recovery processes under the effect of environment.

3.5 Proposed practical applications

Under growth room conditions, although *rd29A::AtDREB1A* transgenic potato plants grown in soil-pot showed variation in plant size (Figures 3.1A and B), by using periodically PEG treating, the same drought stress level was applied to all experimented plants. In comparison to non-transgenic line, all transgenic lines (except D44) showed reduced damages/injuries at the end of the drought stress and enhanced recovery (Figures 3.3A and B, and Figures 3.4A and B). On the basis of this method, drought tolerance of transgenic potato lines was indentified at RH 75% (Figure 3.4A). Under RH 55%, even though no significant difference in drought tolerance among potato lines was detected (Figure 4B), these potato lines showed similarity in drought response (Figure 3B) and drought tolerance (Figure 4B and Figure 10B) in comparison with that of grown under RH 75%. In the transgenic potatoes, the leaf size of almost transgenic lines (except D108, D132, and D141) was not reduced and the leaf number was also higher (transgenic lines, 8-9 leaves; non-transgenic line, 7-8 leaves) compare to non-transgenic line (Figures 3.1A and B) although all transgenic lines showed shorter than non-transgenic line (Figures 3.2A and B). In considerations of effects of *AtDREB1A* expression on enhanced drought tolerance, plant growth, six of the transgenic lines – D10, D19, D20, D53, D163 and D164 – showed high drought tolerance (Figures

3.4A and B), with low *AtDREB1A* expression under non-stress conditions (Figure 3.5B) may represent good candidates for practical application.

3.6 Figures

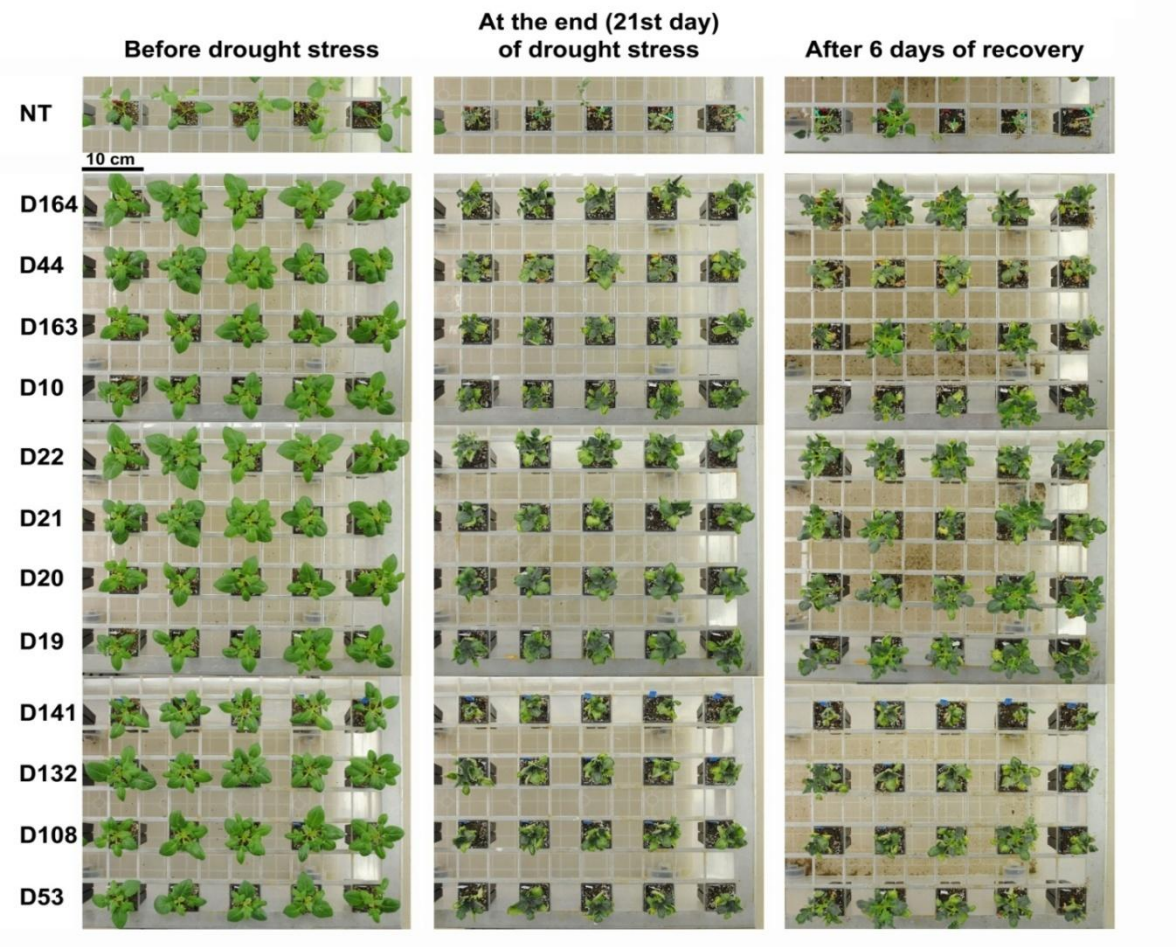


Figure 3.1A Phenotypic responses of growth room non-transgenic (NT) and *rd29A::AtDREB1A* transgenic (D) potato lines of drought stress and recovery at RH 75%. The plants were grown in soil pots (4.2 cm x 4.2 cm x 12 cm). Stress and recovery was carried out with or without PEG solution (-1.8 MPa). The scale bar represents 10 cm.

Figure 3.1B Phenotypic responses of growth room non-transgenic (NT) and *rd29A::AtDREB1A* transgenic (D) potato lines of drought stress and recovery at RH 55%. The plants were grown in soil pots (4.2 cm x 4.2 cm x 12 cm). Stress and recovery was carried out with or without PEG solution (-1.8 MPa). The scale bar represents 10 cm.

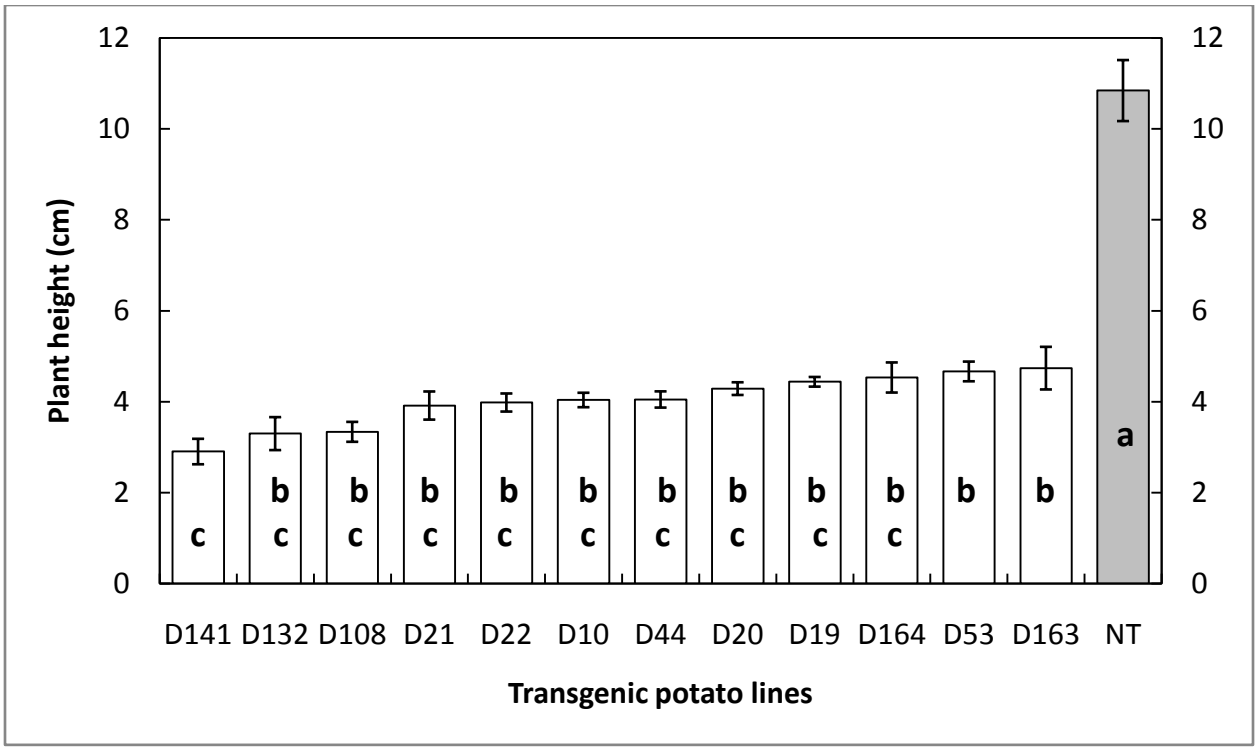
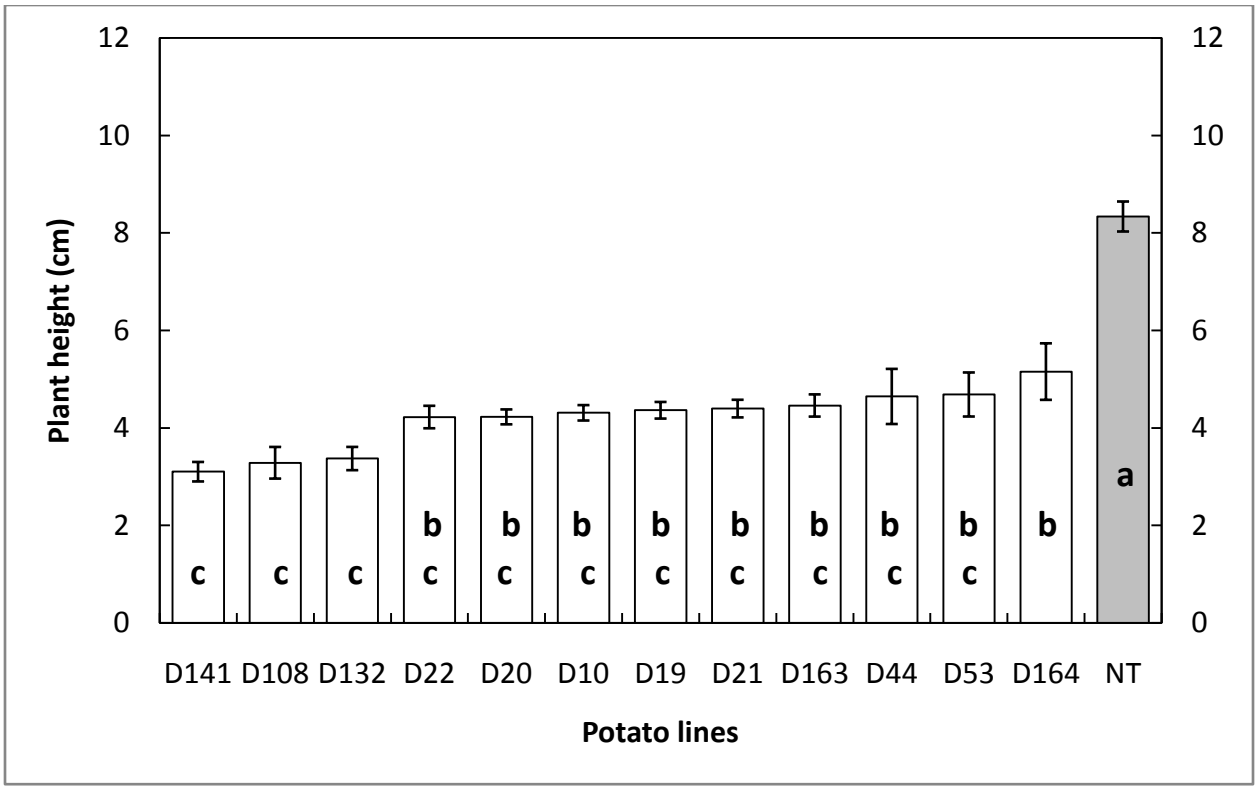


Figure 3.2A Plant growths of potato lines under growth room condition at RH 75%. A non-transgenic and twelve *rd29A::AtDREB1A* transgenic potato lines were cultured for 10 days without stress treatment at 25^{o}C. The plant height was measured immediately before the stress treatment. Each bar represents mean ± standard error.



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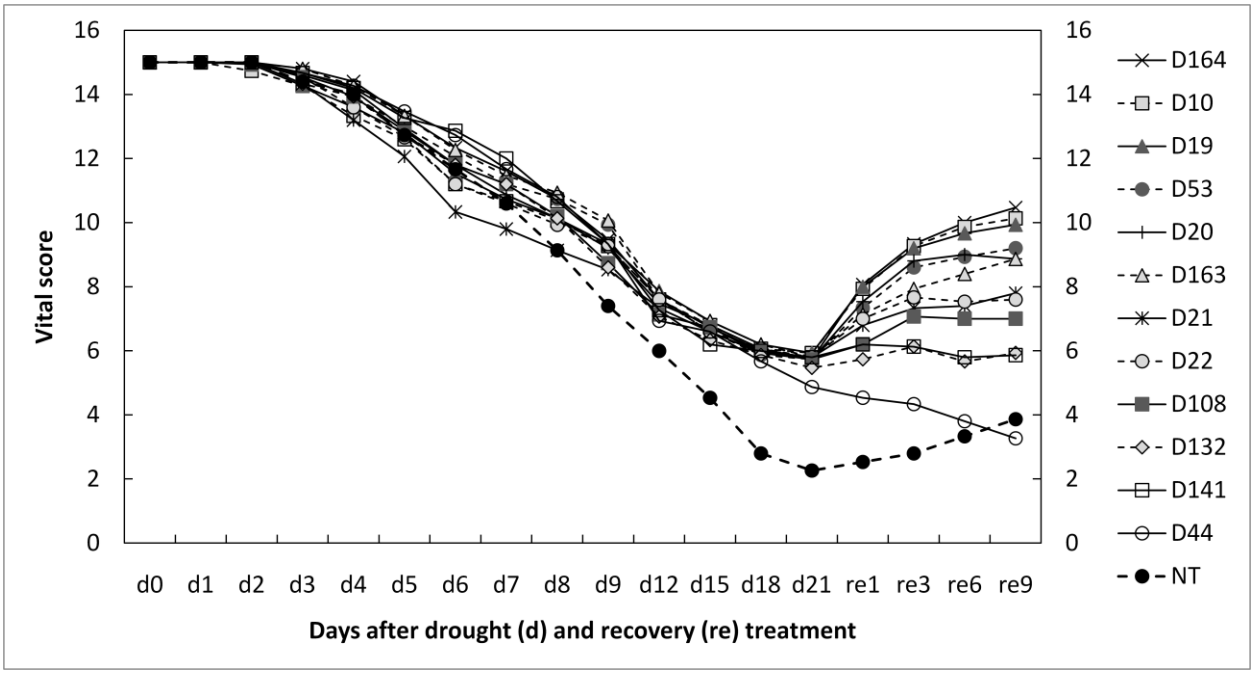

Figure 3.3A Drought stress and recovery responses of potato plants under growth room condition at RH 75%. A non-transgenic line and twelve *rd29A::AtDREB1A* transgenic potato lines were treated with -1.8 MPa PEG solution and recovered by normal culture medium. Each point represents means of whole-plant leaf-resistance scores derived from 3 experimental replications. Whole-plant leaf-resistance score = total leaf wilting resistance score of 5 leaves (second to sixth leaf). Each experimental replication included 5 plants.

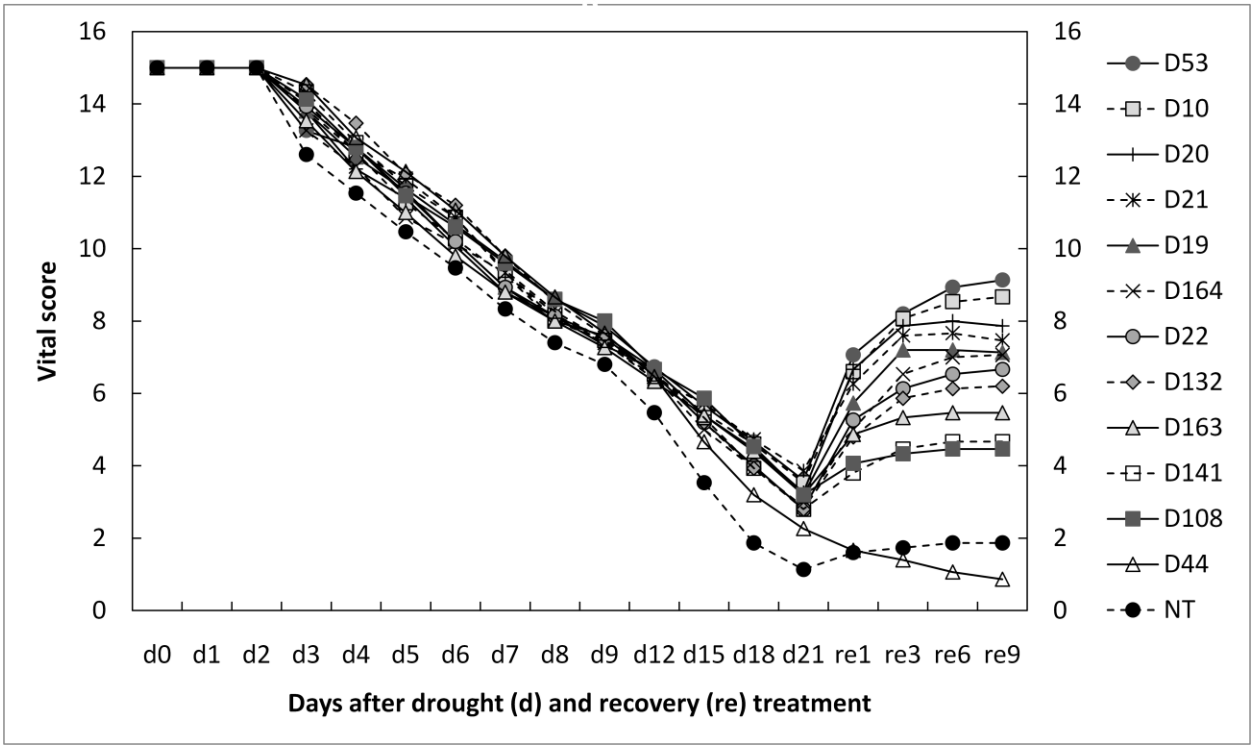

Figure 3.3B Drought stress and recovery responses of potato plants under growth room condition at RH 55%. A non-transgenic line and twelve *rd29A::AtDREB1A* transgenic potato lines were treated with -1.8 MPa PEG solution and recovered by normal culture medium. Each point represents means of whole-plant leaf-resistance scores derived from 3 experimental replications. Whole-plant leaf-resistance score = total leaf wilting resistance score of 5 leaves (second to sixth leaf). Each experimental replication included 5 plants.

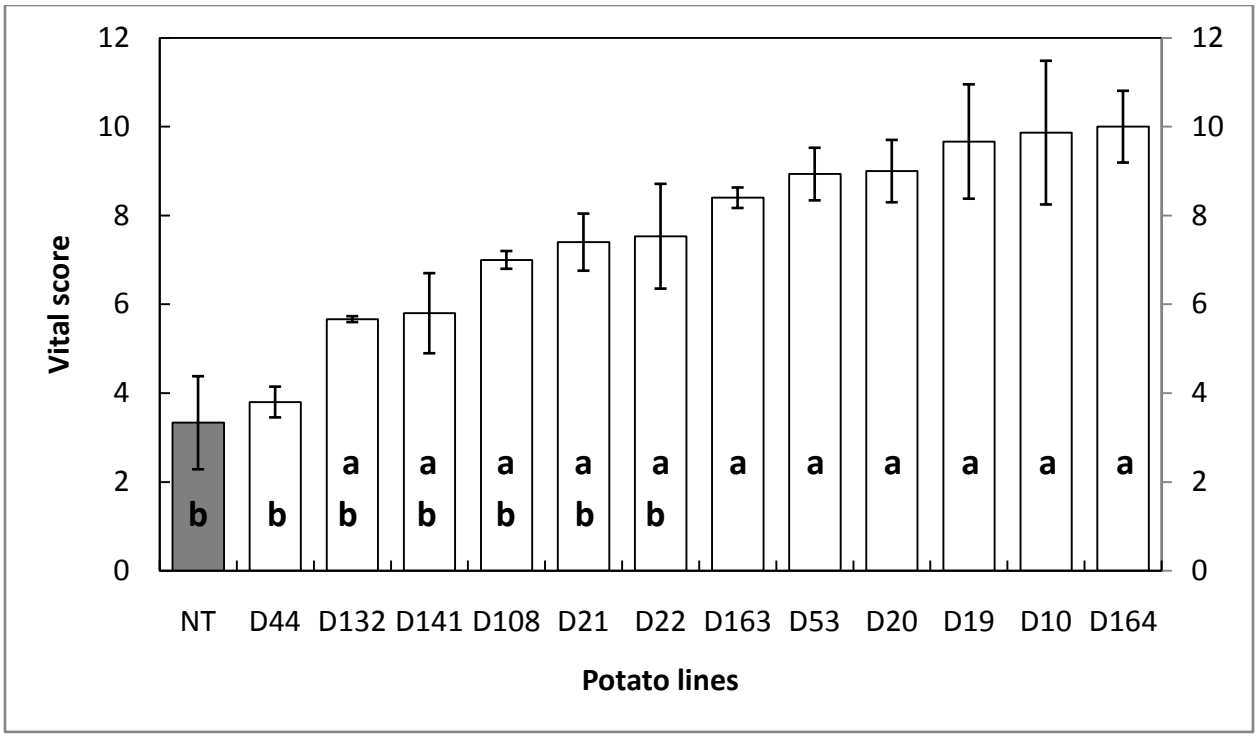

Figure 3.4A Drought tolerances of non-transgenic (NT) and *rd29A::AtDREB1A* transgenic (D) potato lines grown under growth room condition at RH 75%. Drought tolerance was represented by the mean vital score after 6 days of recovery. Difference between means were analyzed by using one-way ANOVA, and ranked according to the Tukey-Kramer test ($p < 0.05$). Lines not sharing the same letter differ significantly. No letter is presented if the difference between means is not significant. Each bar represents mean ± standard error.

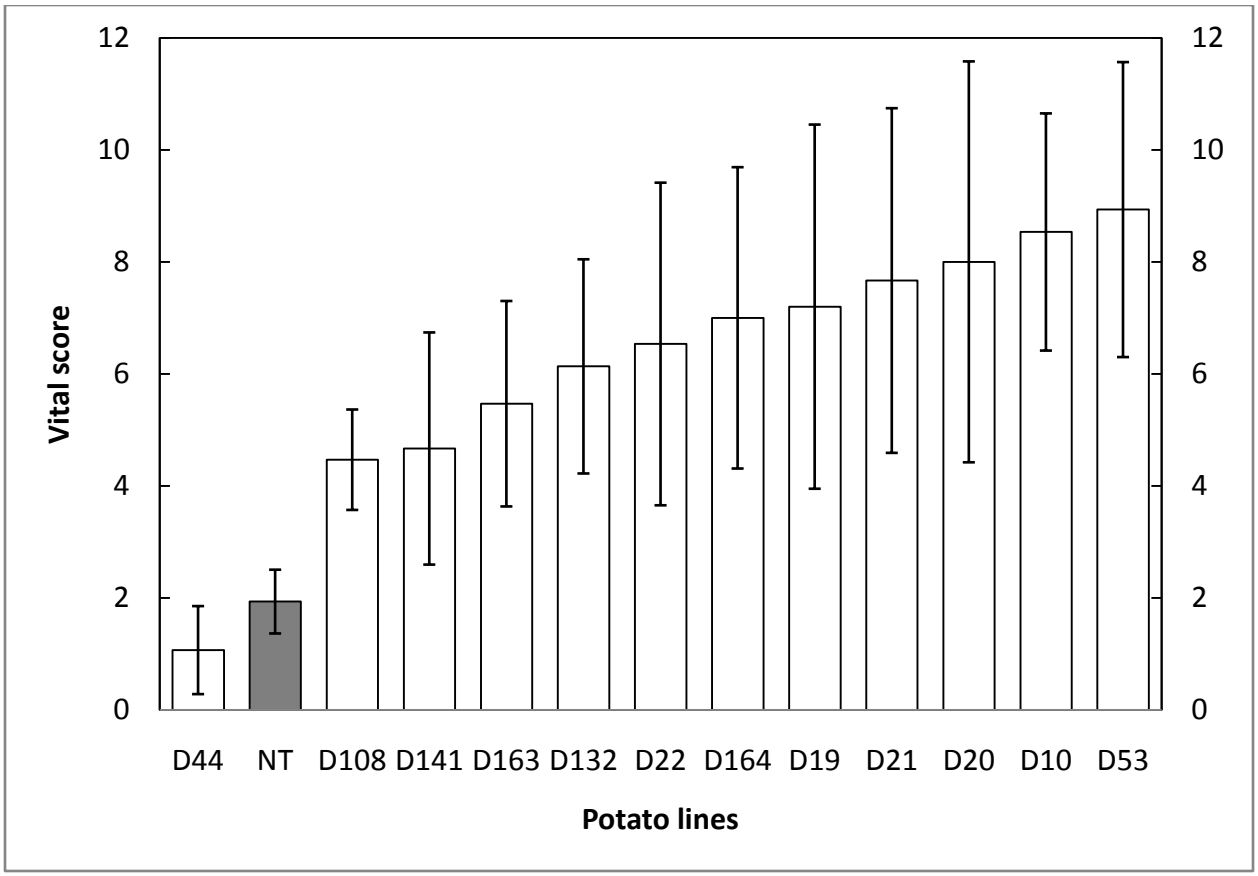

Figure 3.4B Drought tolerances of non-transgenic (NT) and *rd29A::AtDREB1A* transgenic (D) potato lines grown under growth room condition at RH 55%. Drought tolerance was represented by the mean vital score after 6 days of recovery. Difference between means were analyzed by using one-way ANOVA, and ranked according to the Tukey-Kramer test ($p < 0.05$). Lines not sharing the same letter differ significantly. No letter is presented if the difference between means is not significant. Each bar represents mean ± standard error.

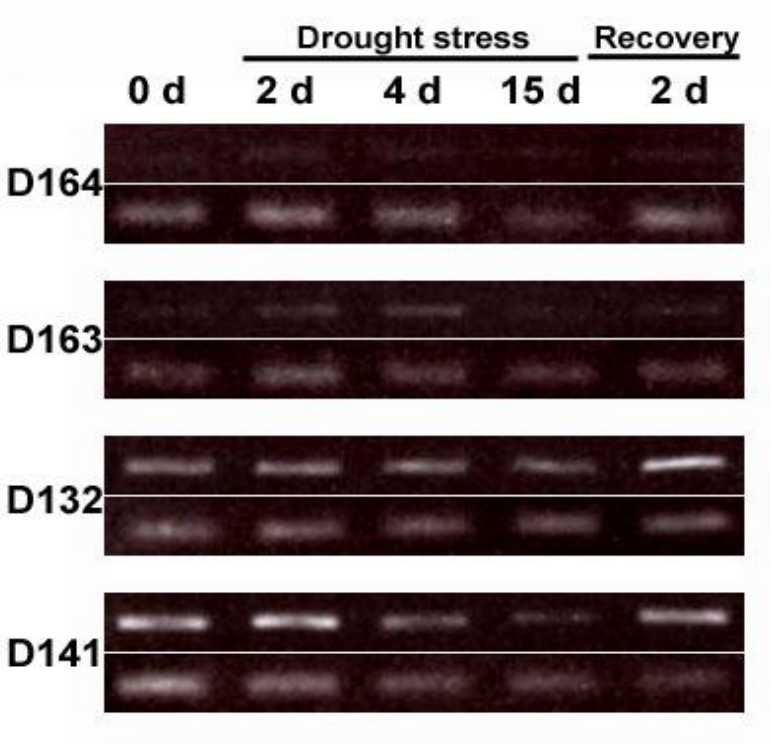

Figure 3.5A RT-PCR analysis of *AtDREB1A* expression in *rd29A::AtDREB1A* transgenic potato lines. For each transgenic line, the upper row represented the expression of *AtDREB1A* gene, and the lower row represented the expression of reference *ubiquitin* gene.

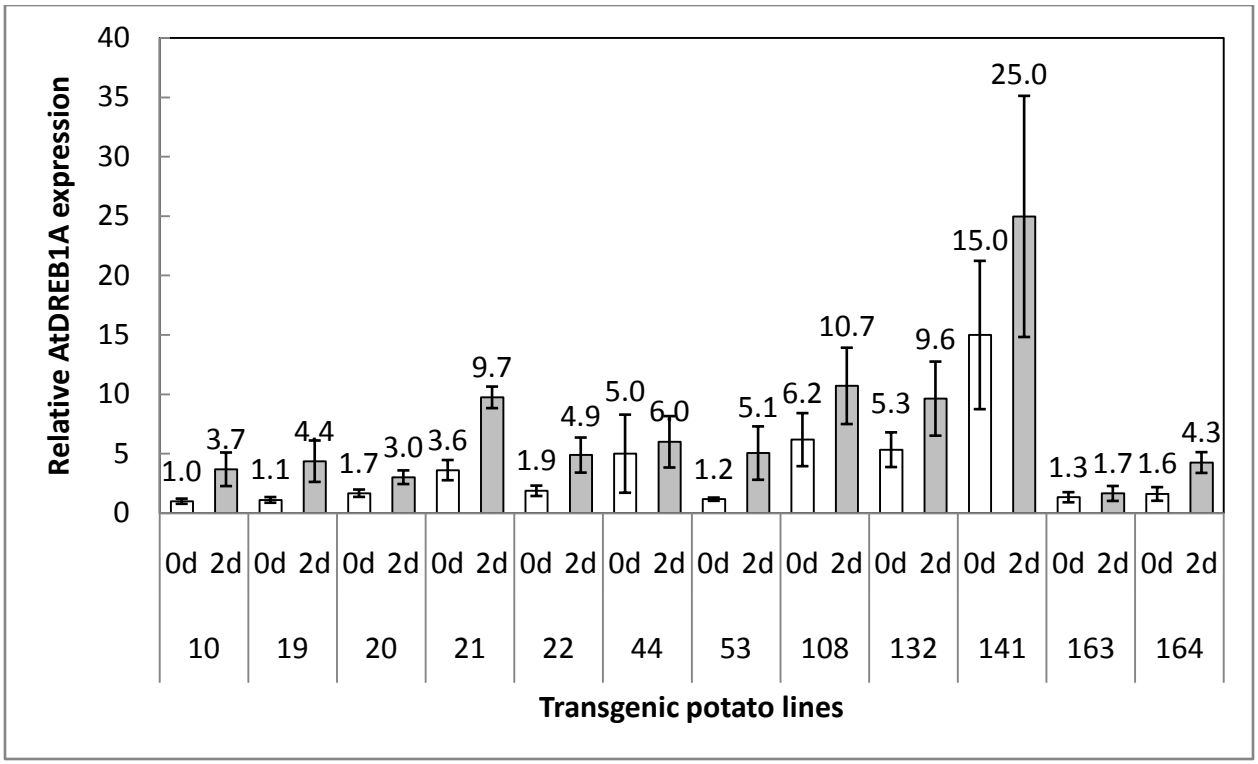

Figure 3.5B Quantitative *AtDREB1A* expression in *rd29A::AtDREB1A* transgenic potato lines before (0 d) and after 2 days (2 d) of drought stress. The relative *AtDREB1A* expression of each transgenic line was derived from 3 experimental replications, and normalized against constitutive expression of *ubiquitin*. The *AtDREB1A* expression level of line D10 before the drought stress was set at 1. Each bar represents mean ± SE of three independent experiments. The plants grown at RH 75% were sampled for these analyses.

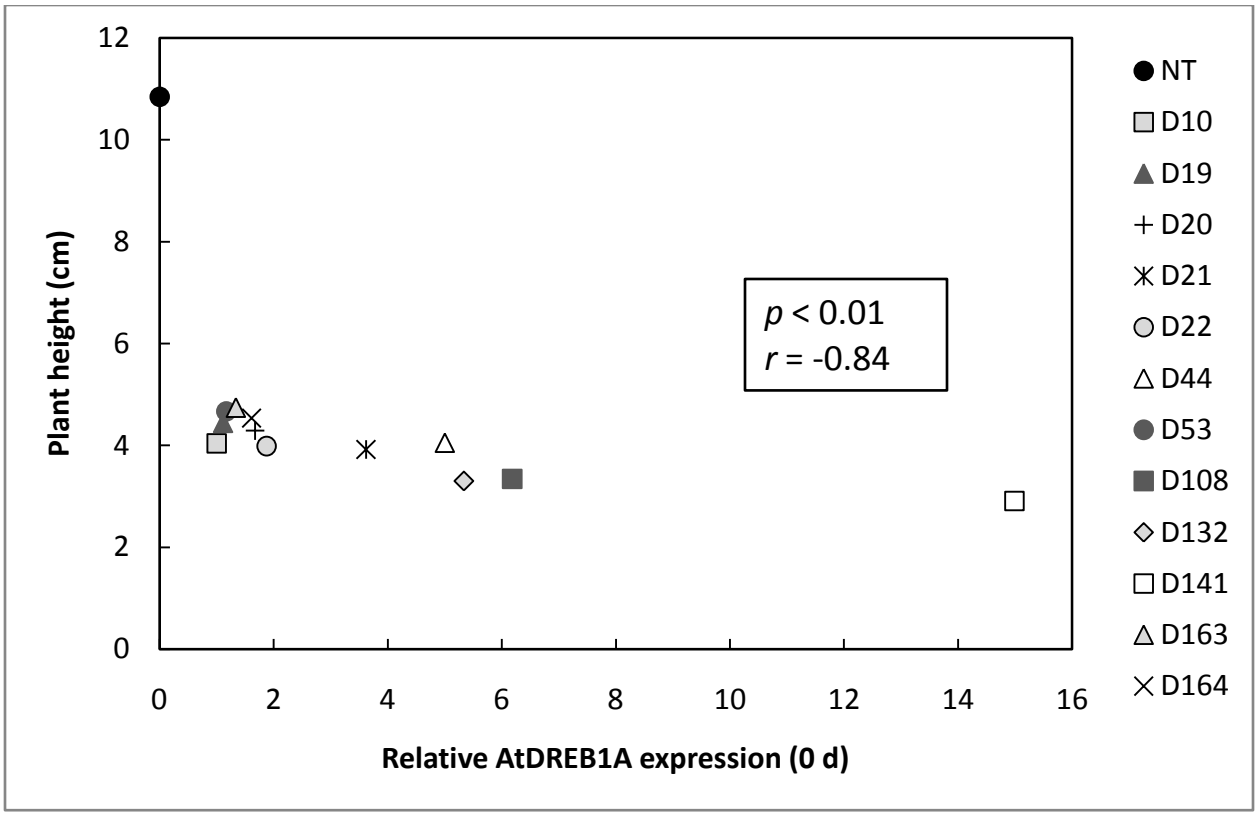

Figure 3.6 Relationships between *AtDREB1A* expression before stress treatment (0 d) and plant height in twelve *rd29A::AtDREB1A* transgenic lines (excluding non-transgenic line). The plants grown at RH 75% were used in this analysis. Each point represents the mean of 3 experimental replications.

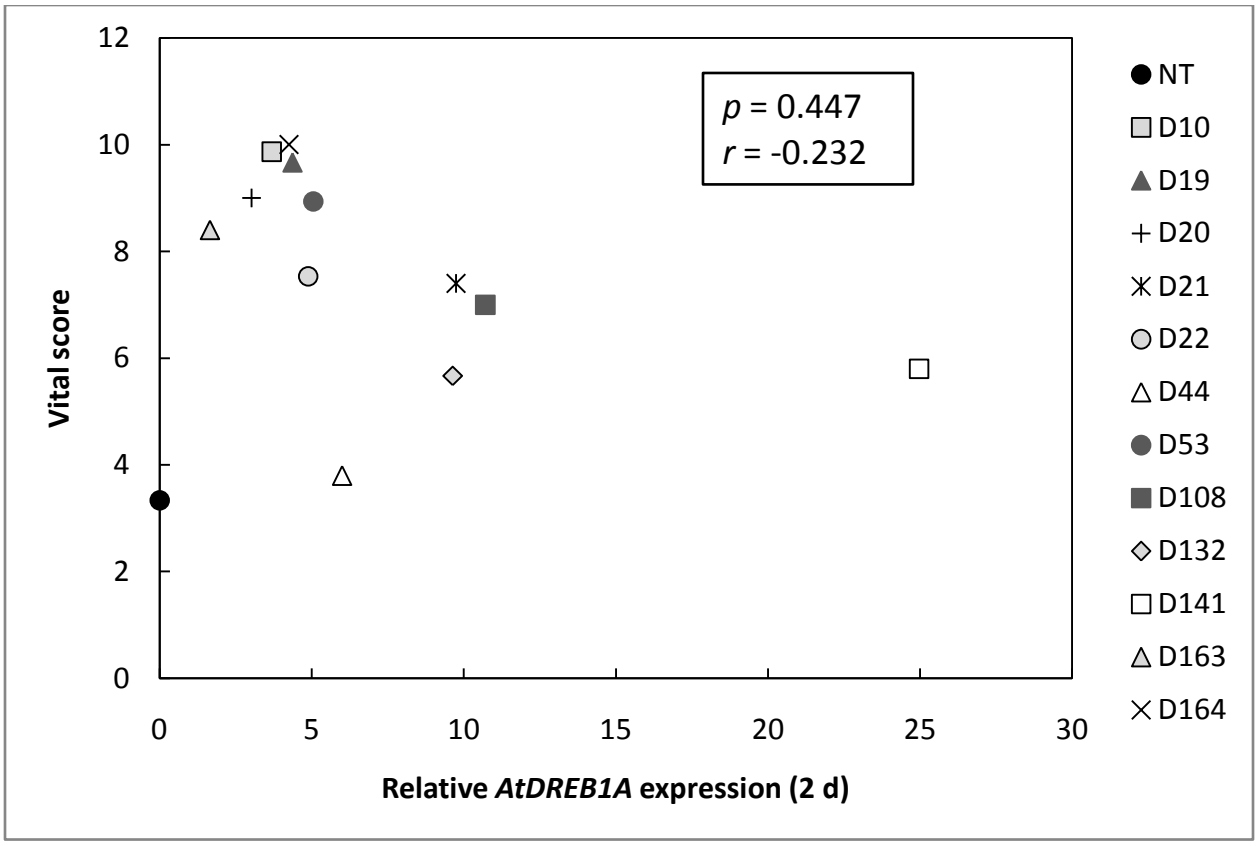

Figure 3.7A Relationships between drought tolerance and *AtDREB1A* expressions after 2 days stress treatment (2 d) in a non-transgenic (NT) and twelve *rd29A::AtDREB1A* transgenic (D) potato lines. The plants grown at RH 75% were used in this analysis. Each point represents the mean of 3 experimental replications.

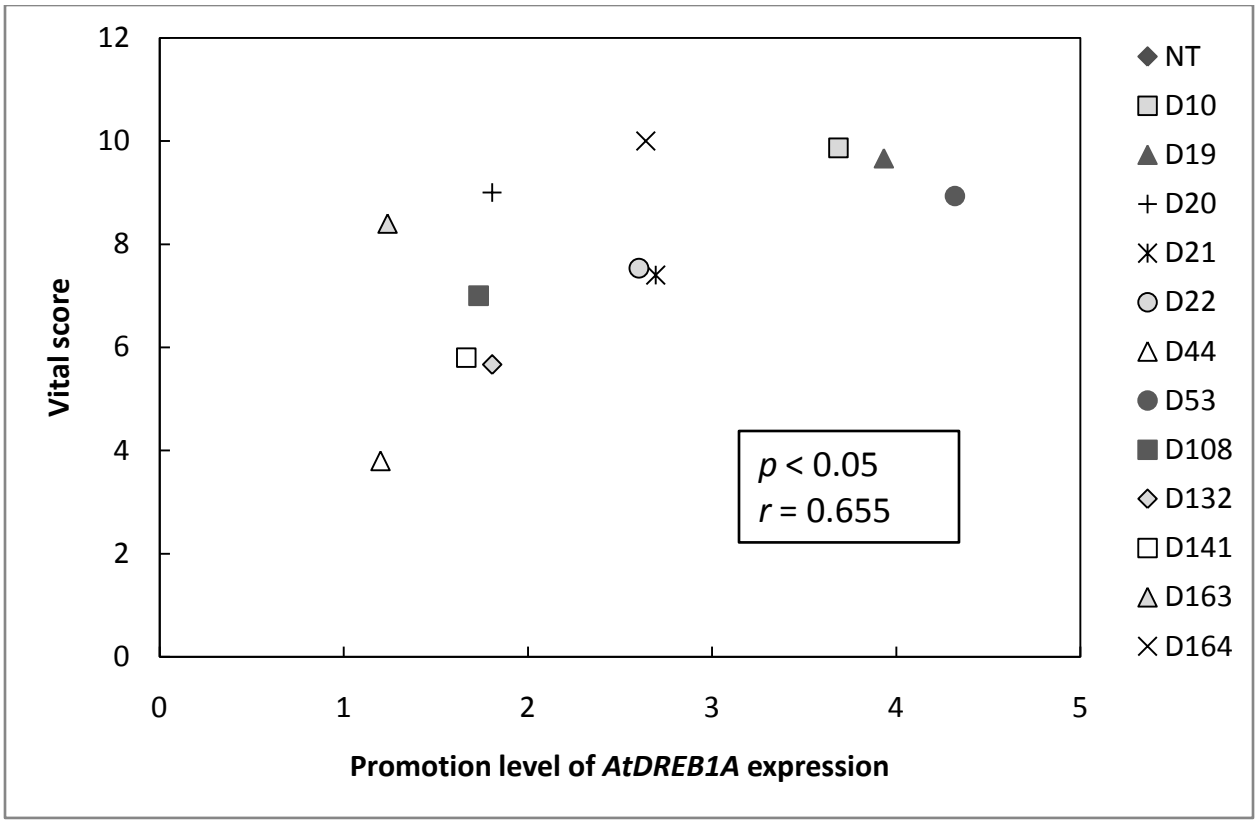

Figure 3.7B Relationships between drought tolerance and promotion level of *AtDREB1A* expression in a non-transgenic (NT) and twelve *rd29A::AtDREB1A* transgenic (D) potato lines. The plants grown at RH 75% were used in this analysis. Each point represents the mean of 3 experimental replications.

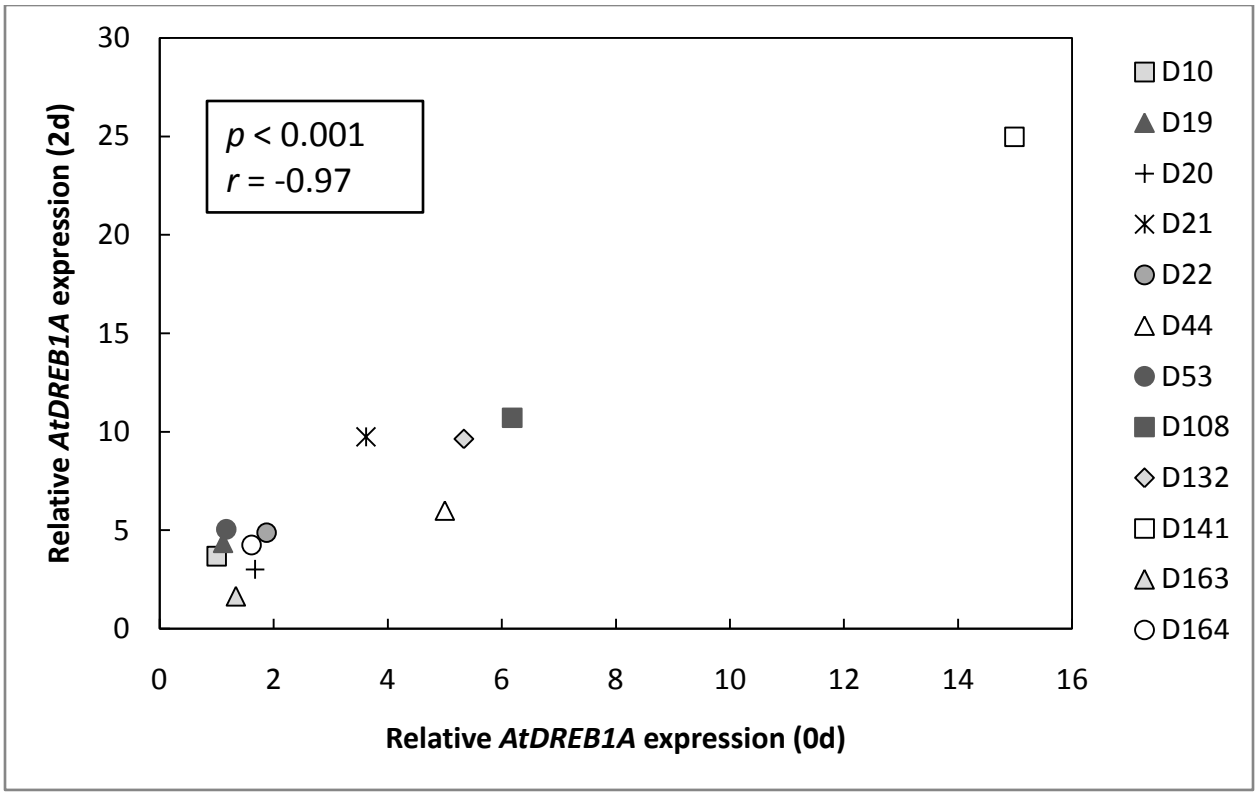

Figure 3.8 Correlation between *AtDREB1A* expression before (0 d) and after 2 days (2 d) stress treatment in twelve *rd29A::AtDREB1A* transgenic (D) potato lines. The plants grown at RH 75% were used in this analysis. Each point represents the mean of 3 experimental replications.

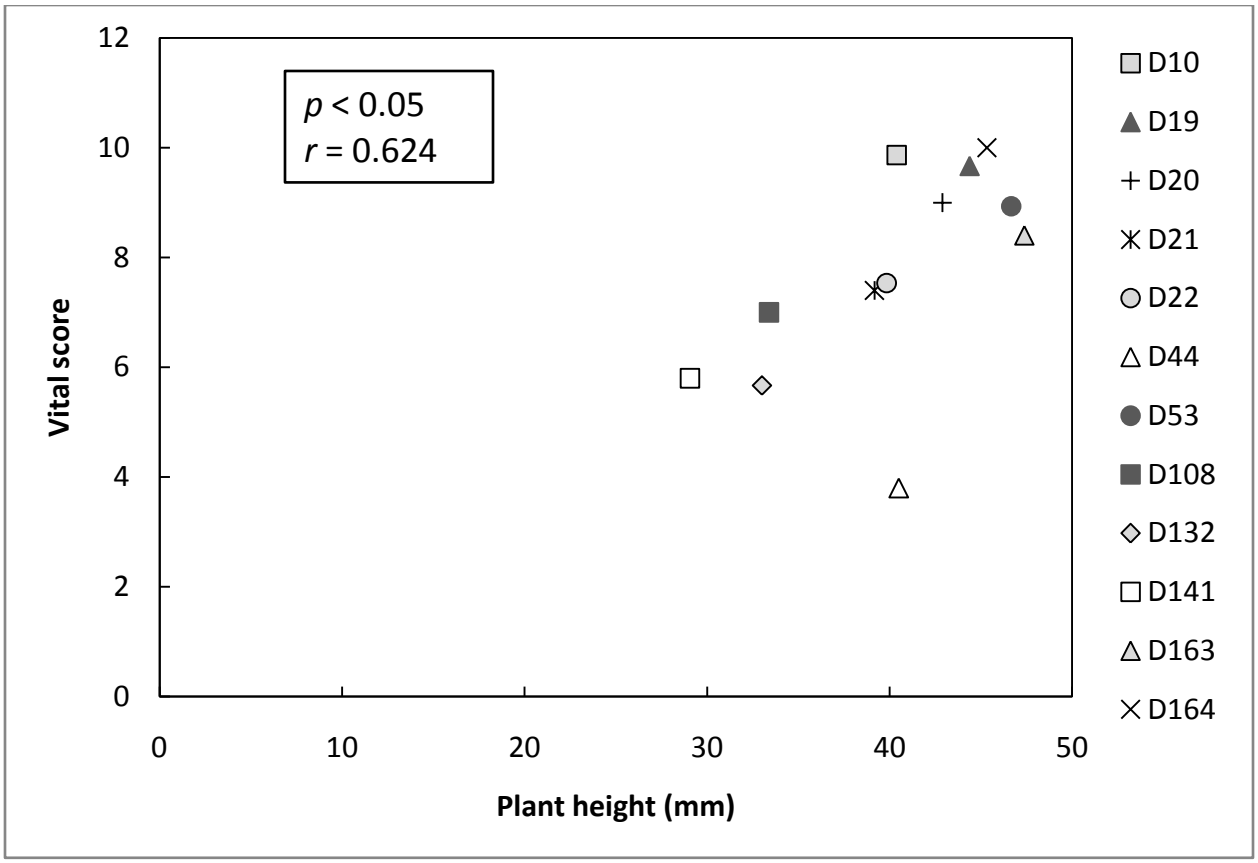

Figure 3.9 Correlation between drought tolerance and plant height in twelve *rd29A::AtDREB1A* transgenic (D) potato lines. The plants grown at RH 75% were used in this analysis. Each point represents the mean of 3 experimental replications.

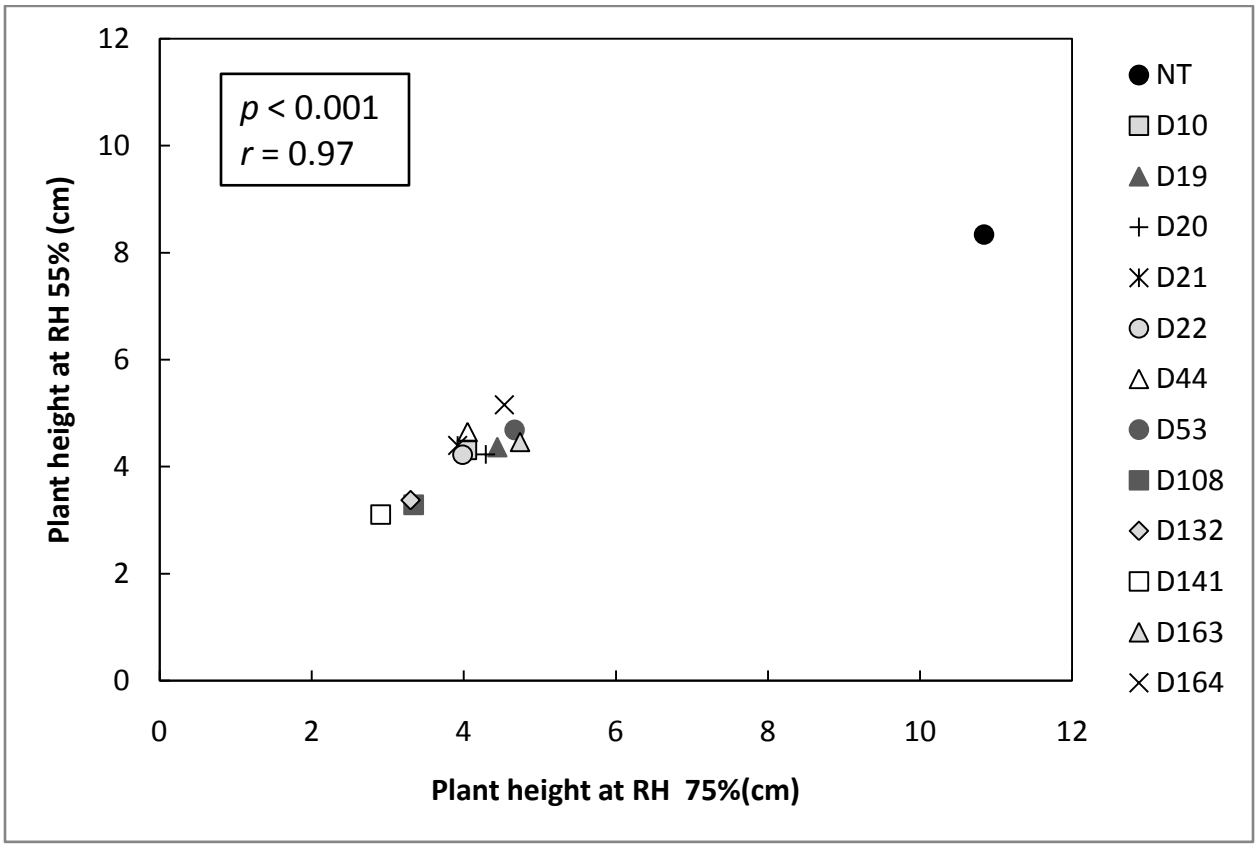

Figure 3.10A Plant height correlation between potato lines grown under growth room at different relative humidity conditions (RH 75% and RH 55%). Each point represents the mean of 3 experimental replications.

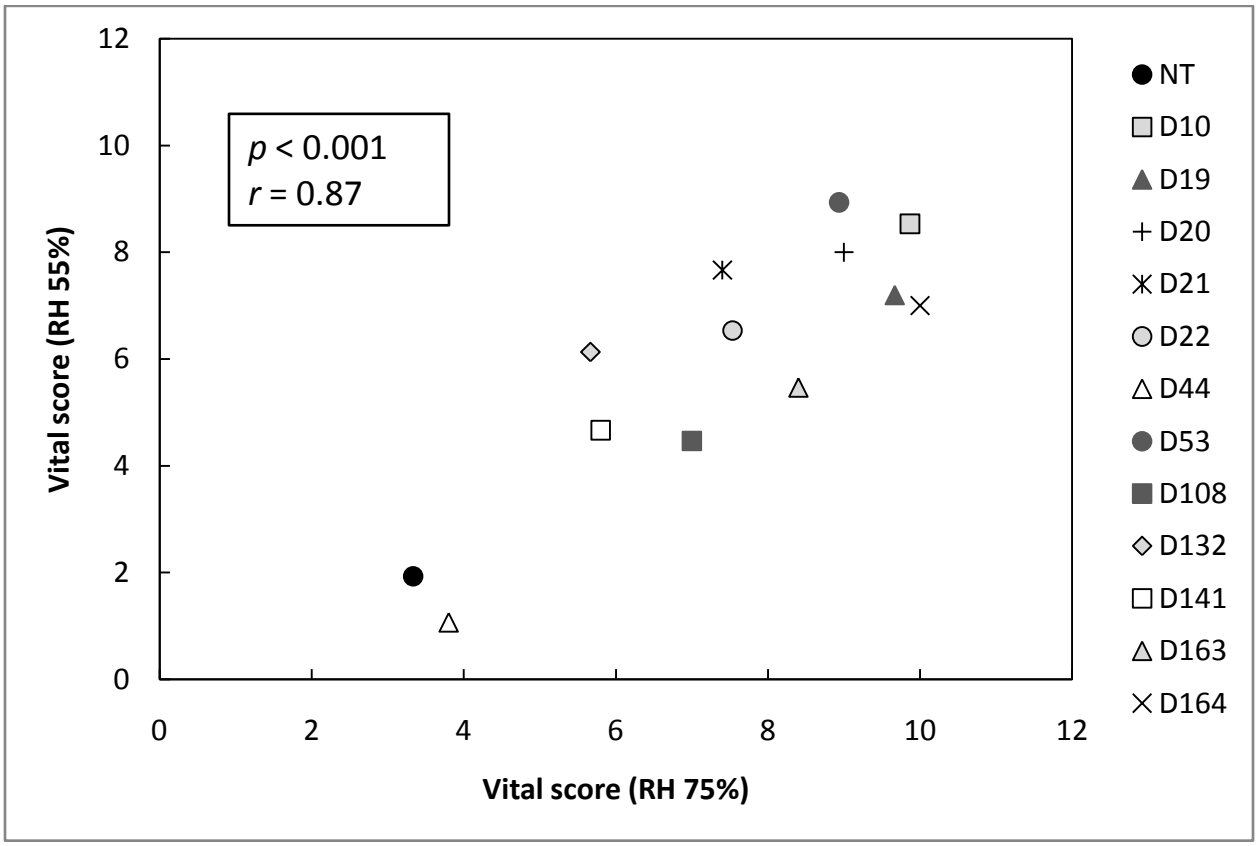

Figure 3.10B Drought tolerance correlation between potato lines grown under growth room at different relative humidity conditions (RH 75% and RH 55%). Each point represents the mean of 3 experimental replications.

CHAPTER 4

General discussions

4.1 Role of *in vitro* and growth room evaluations on assessing drought tolerance in *rd29A::AtDREB1A* transgenic potatoes

Considering practical uses of transgenic plants, they should be evaluated in practical fields. Final screening of drought tolerant transgenic lines for practical uses needs to be evaluated under field conditions. However, only a few of elite drought tolerant lines can be performed under these large-scale evaluations due to the cost of performance. In contrary, small-scale evaluations such as *in vitro* and growth room with low cost performance allow rapid screening a large number of transgenic lines in controlled environmental conditions. Therefore, in this study, twelve *rd29A::AtDREB1A* transgenic potato lines were evaluated drought tolerance under *in vitro* and growth room conditions to select a few suitable lines for further evaluations in greenhouse and field conditions. Furthermore, effect of transgene also should be clarified. To elucidate association between transgene expression and performance of each transformant, controlled condition is essential for evaluation since *rd29A* promoter is stress inducible property (Kasuga et al. 1999; Liu et al. 1998) and strength of drought stress is apt to be affected by atmospheric conditions.

In this study, the applying drought stress strength to each transgenic line was precisely controlled. Rotary liquid culture combined with PEG was applied for *in vitro* evaluation, and soaking the soil pot with plant to PEG solution periodically was applied for growth room evaluation under controlled relative humidity. During both evaluations, other environmental conditions such as light and temperature were controlled and managed. By using these methods, the same drought stress strength was applied to all potato lines. Therefore, classification of drought tolerance in each *rd29A::AtDREB1A* potato line and contribution of *AtDREB1A* on drought tolerance and plant growth were able to be carried out by those established methods in this study (Chapters 2 and 3).

4.2 Difference between *in vitro* and growth room drought tolerance in *rd29A::AtDREB1A* transgenic potato lines

In this study, on *in vitro* evaluation, seven of the transgenic lines—D10, D19, D20, D22, D108, D132, and D141—showed enhanced drought tolerance in comparison to non-transgenic line (Figure 2.4). On growth room evaluation (at RH 75%), it was indicated that six of the transgenic lines – D10, D19, D20, D53, D163 and D164 – showed more drought tolerant than the non-transgenic line (Figure 3.4A). In addition, no correlation was observed between *in vitro* and growth room drought tolerance in transgenic lines (p = 0.78, r = 0.09; Figure 4.1). Thus, each

rd29A::AtDREB1A transgenic potato line showed different performance in different evaluating conditions.

In this study, relative humidity was stable at 100% under *in vitro* condition while was lower (55% and 75%) and relatively fluctuated under growth room conditions. Growth room potato plants grown in soil pots had to synthesize energy and carbohydrate by themselves while *in vitro* potato plants grown in sucrose-contained media could use directly energy and carbohydrate resources. Although the same drought strength (-1.8 MPa) was applied to materials in both evaluations, the applying method of drought was quite different (materials and methods in Chapters 2 and 3). In transgenic maize, differences in number and expression level of metabolic genes were observed between *in vitro* and greenhouse transgenic plants due to different in growing condition (Coll et al. 2009; Barros et al. 2010). Difference of activated genes was observed when various drought stress applying methods were applied to *Arabidopsis* plants (Bray 2004). Therefore, various kinds of native genes were influenced by growth conditions or stress applying methods. Furthermore, the variations in transgene expression levels were also observed in transgenic *Populus* under different growing conditions (Strauss et al. 2004). In this study, difference of expression level in transgene was also observed in two different conditions (Figures 2.5B, 3.5B, and 4.2). These variations of gene

expression might cause the difference in tolerance level of each transgenic line between *in vitro* and growth room evaluations in this study.

4.3 Expression profile of *AtDREB1A* and its effects in *rd29A::AtDREB1A* transgenic potato lines

The expression of transgene depends on inserted position and structure of the locus in transgenic plants (Kohli et al. 2010). In addition, it is known that different transgenic lines often vary in levels, patterns or stability of transgene expression (Schubert et al. 2004). In this study, different expression levels of *AtDREB1A* before and after stress treatment were identified among *rd29A::AtDREB1A* transgenic potato lines under both *in vitro* and growth room conditions (Figures 2.5A and B, Figures 3.5A and B). The promotion ratios of *AtDREB1A* expression before and after stress treatment were also different in each transgenic line (Figure 4.2). Therefore, expression patterns are independent in all transgenic lines because each transgenic line is derived from an individual transformation event.

Environment can influence to the expression of transgene in transgenic plants (Strauss 2003). In this study, the result of promotion ratio of *AtDREB1A* expression showed that transgenic lines D10, D19, D21, D22, D53, and D164 showed high promotion ratio (>2.5) while D20, D44, D108, D132, D141 and D163 showed low promotion ratio (<2.0) under both evaluated conditions (Figure 4.2).

However, there was no correlation of promotion ratios of *AtDREB1A* expression between *in vitro* and growth room conditions ($p = 0.49$, $r = 0.11$; Figure 4.2). This result reflected that differences between *in vitro* and growth room conditions might influence on the promotion ratio of *AtDREB1A* expression in these transgenic lines.

The influence of *AtDREB1A* on growth retardation has previously reported in many transgenic plants such as *Arabidopsis* (Glimour et al. 2000; Kasuga et al. 1999; Liu et al. 1998), tobacco (Kasuga et al. 2004; Cong et al. 2008), tomato (Hsieh et al. 2002), soybean (Suo et al. 2012), rice (Ito et al. 2006; Oh et al. 2005), and wheat (Pellegrineschi et al. 2004). In this study, strong negative correlations between the leak expression level of *AtDREB1A* and growth retardation in transgenic lines were also observed under both *in vitro* ($p < 0.01$, $r = -0.92$; Figure 2.6) and growth room ($p < 0.01$, $r = -0.84$; Figure 3.6) conditions. However, due to the strongly expression of *AtDREB1A* under non-stress conditions (Figures 2.5B and 3.5B), the severe growth retardants were observed in D108, D132, and D141 whose phenotypes were small leaf size (Figures 2.1 and 3.1) and low plant height (Figures 2.2 and 3.2) in comparison to the other transgenic lines. The other transgenic lines showed lower expression of *AtDREB1A* under non-stress conditions (Figures 2.5B and 3.5B), and resulted in low plant height (Figures 2.2 and 3.2) but no leaf size reduction. These result reflected that the negative effects

of *AtDREB1A* caused severe growth retardation in *rd29A::AtDREB1A* transgenic when its expression reached a threshold level as in D108, D132, and D141 transgenic lines.

The effect of a transgene is known not to be a fixed magnitude, and different desired traits can be occurred due to different interactions between transgenic plants and environment (Sinclair 2004; De Wolf et al. 2008). On *in vitro* evaluation, a highly positive correlation between *AtDREB1A* expression and drought tolerance was recognized ($p < 0.01$, $r = 0.8$; Figure 2.7) while there was no correlation between promotion ratio of *AtDREB1A* expression and drought tolerance ($p = 0.45$, $r = -0.23$). In contrast, on growth room evaluation, there was no correlation between *AtDREB1A* expression and drought tolerance ($p = 0.45$, $r = -0.23$; Figure 3.7) while a positive correlation was identified between promotion ratio of *AtDREB1A* expression and drought tolerance ($p < 0.05$, $r = 0.66$; Figure 3.7B). On the other hand, different environmental conditions between *in vitro* and growth room evaluation were identified above (Section 4.2). Therefore, the differences between *in vitro* and growth room drought tolerance may be caused by the different effects of *AtDREB1A* on drought tolerance in *rd29A::AtDREB1A* transgenic potato lines. In transgenic wheat, under differences between greenhouse and field conditions, transgene had different effects on desired trait (Zeller et al. 2010).

4.4 Selecting drought tolerant candidates from *rd29A::AtDREB1A* transgenic potato lines

The drought tolerance in *rd29A::AtDREB1A* transgenic potatoes with expression of *AtDREB1A* was identified under both *in vitro* and growth room conditions (Chapters 2 and 3). On the other hand, the leaky expression of *AtDREB1A* was also observed in all transgenic lines before stress treatment and suppressed the plant growth according to its expression level (Figures 2.6 and 3.6). However, only lines D108, D132, and D141 showed severe growth reduction compare to the other transgenic lines (Figures 2.2 and 3.2). On the basic of the *in vitro* and growth room assessments, it was indicated that three of the transgenic lines – D10, D19, and D20 – showed enhanced drought tolerance without growth retardation under both *in vitro* and growth room conditions. Stable tolerance to different drought stress conditions and evaluation stages is one of the strategies in selecting elite drought tolerant candidate (Saint Pierre et al. 2012). Therefore, with this strategy, stable tolerance of the three D10, D19, and D20 *rd29A::AtDREB1A* transgenic lines under both *in vitro* and growth room conditions is also expected on other drought stress conditions.

It is known that drought tolerance is complex, and may change according to plant stage and environment. Therefore, one genotype may tolerate to drought under this drought condition but may not in the other drought conditions. On the

other hand, plants may change kinds of response types depending on developmental stage and drought environment (Chaves et al. 2003, Mullet 2009). In this study, transgenic lines D22, D53 and D164 showed high values of promotion ratio of *AtDREB1A* expression under *in vitro* and growth room conditions. In addition, transgenic line D22 showed tolerance to *in vitro* drought stress, and lines D53 and D164 showed high tolerance to growth room drought stress. These transgenic lines also showed no growth retardation. Therefore, in considering on the dependence of drought tolerance on plant developmental stage and the promising tolerance of the *rd29A::AtDREB1A* transgenic lines obtaining high promotion ratio of *AtDREB1A* expression, transgenic lines D22, D53 and D164 are expected tolerance to some other drought stress conditions.

4.5 For future practical application

In this study, *in vitro* and growth room evaluations were performed under precisely controlled conditions of drought 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 drought tolerance profiles are manifested differently under various conditions of drought (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) (Kikuchi et al. 2006; Hilbeck et al. 2011), are required. Japanese regulations regarding the practical application of transgenic plants specify the environmental biosafety assessments in special netted-houses prior to field trials. Moreover, in my evaluations, the drought tolerance evaluations were performed only at vegetative-stage of potato plants. For practical used, these transgenic potato lines need to be evaluated yield performance under drought conditions. Thus, further screening of the drought-tolerant transgenic potato lines by using pot cultivation under special netted-houses and isolated field, combined with environmental biosafety assessment, is required. In future studies, I aim to perform field trials with these transgenic potato lines by using step-by-step evaluations.

4.6 Figures

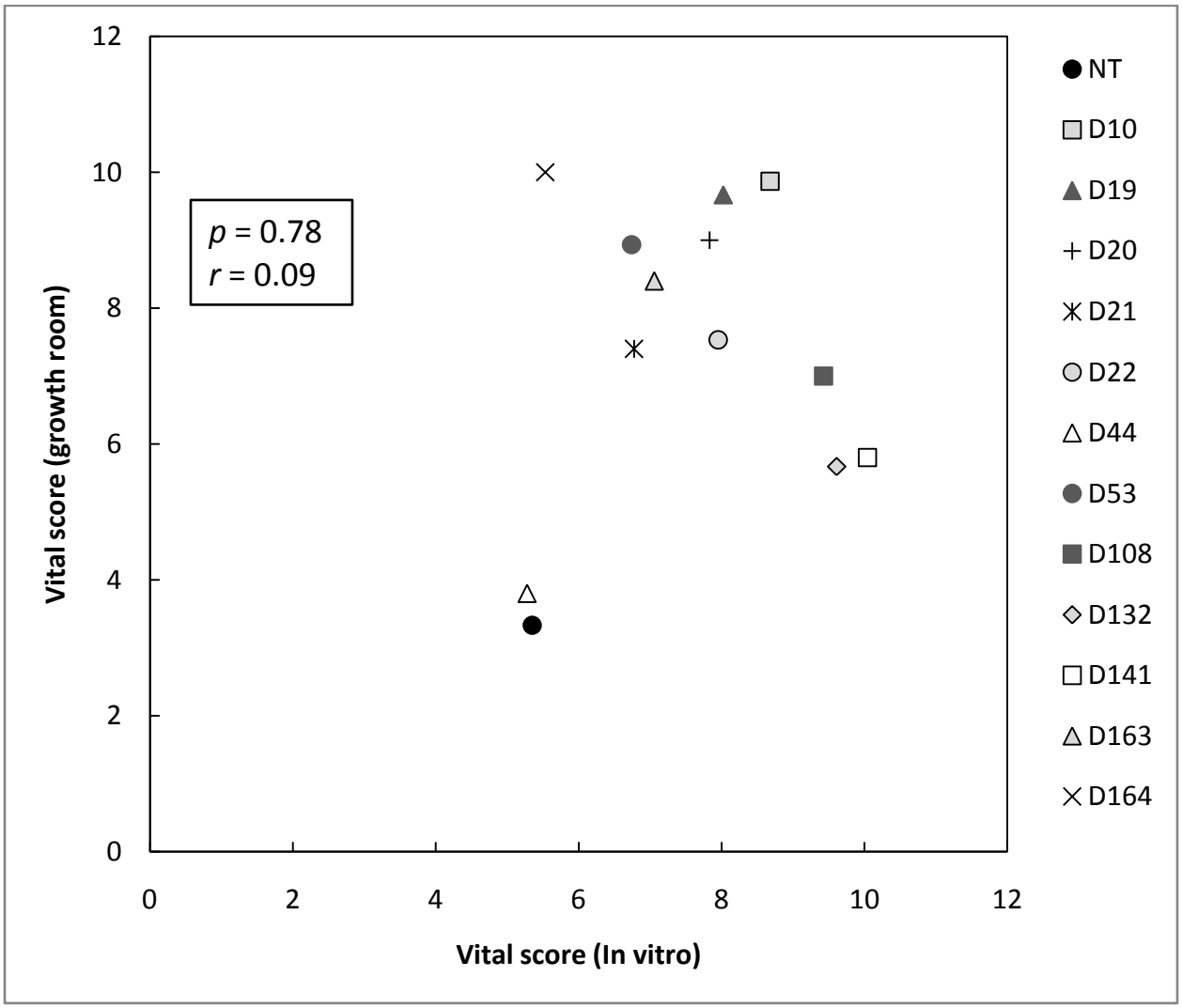

Figure 4.1 Correlation between *in vitro* and growth room drought tolerance of twelve *rd29A::AtDREB1A* transgenic potato lines potato lines. The plants grown under growth room conditions at RH 75% were used for this analysis. Each point represents the mean of 3 experimental replications.

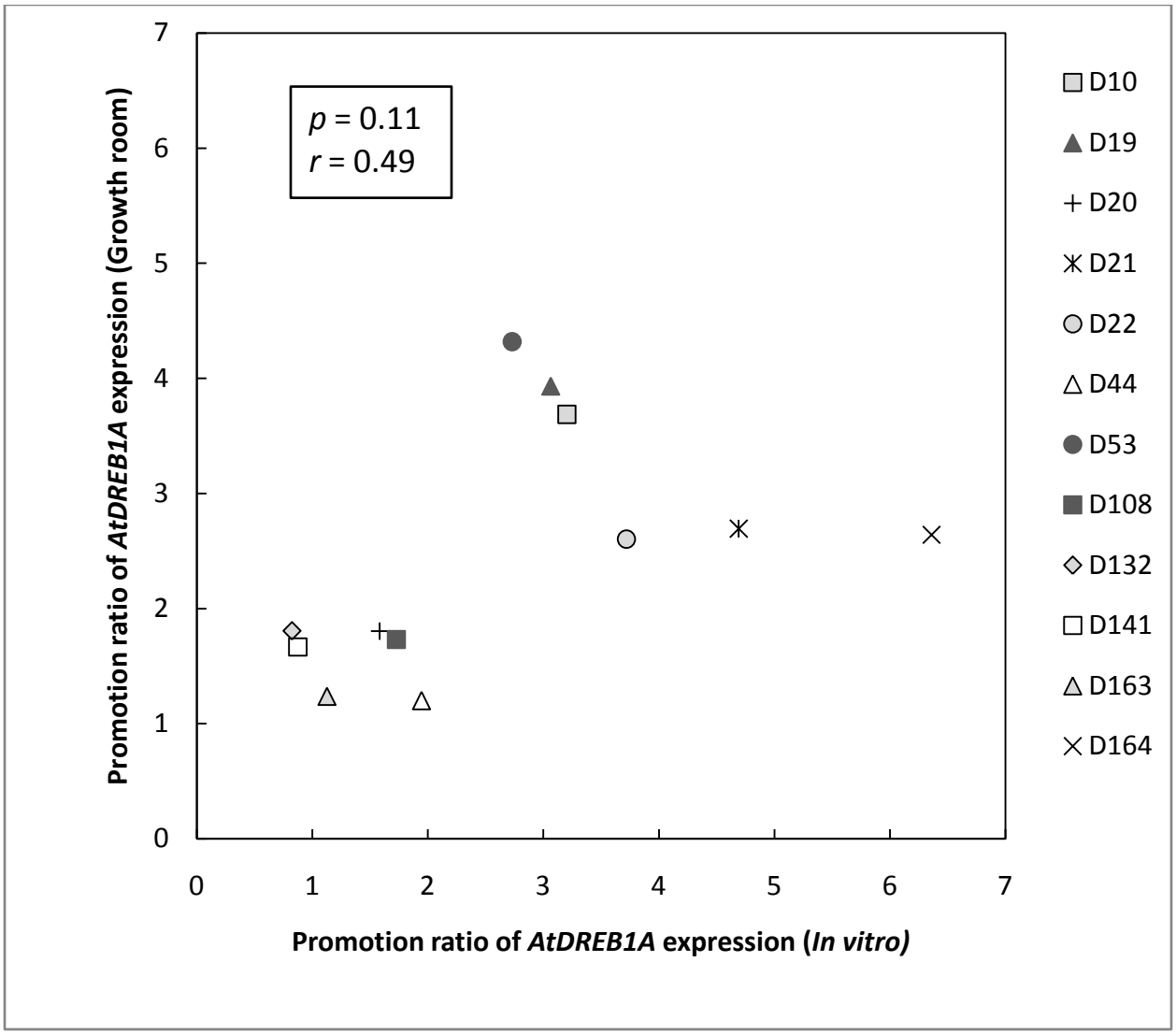

Figure 4.2 Correlation between *in vitro* and growth room promotion ratio of *AtDREB1A* expression of twelve *rd29A::AtDREB1A* transgenic potato lines. The plants grown under growth room conditions at RH 75% were used for this analysis. Each point represents the mean of 3 experimental replications.

LIST OF PUBLICATIONS AND PRESENTATIONS

Publications

Huu Duc Huynh, Akira Kikuchi, Takayoshi Shimazaki, Mie Kasuga, Kazuko Yamaguchi-Shinozaki, Kazuo N. Watanabe (2014) *In vitro* evaluation of dehydration tolerance in *AtDREB1A* transgenic potatoes. Plant Biotechnology (accepted December 8th 2013).

Presentations

Huynh Huu Duc, Akira Kikuchi, Taichi Oguchi, Takayoshi Shimazaki, Kazuo N. Watanabe (2010) An *in vitro* Screening for Drought Tolerance in Transgenic Potatoes. The 4th AG-BIO/PERDO Graduate Conference on Agriculture biotechnology and UT-KU Joint Seminar at Kasetsart University (Nokhon Pathom, Thailand).

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