Constitutive expression of *Arabidopsis DREB1B* in transgenic potato enhances drought and freezing tolerance

S. MOVAHEDI[1], B.E. SAYED TABATABAEI[1]*, H. ALIZADE[2], C. GHOBADI[1], A. YAMCHI[3] and G. KHAKSAR[1]

Department of Agricultural Biotechnology, College of Agriculture, Isfahan University of Technology, Isfahan, 84156-83111, Iran[1]
Department of Agricultural Biotechnology, College of Agriculture, University of Tehran, Tehran, Iran[2]
Department of Plant Breeding and Biotechnology, Gorgan University of Agricultural Science and Natural Resources, Gorgan, Iran[3]

Abstract

DNA cassette consisting of an *Arabidopsis* dehydration-responsive element binding factor 1 (*DREB1B*) cDNA, driven by a cauliflower mosaic virus 35S promoter, was introduced into potato plants (*Solanum tuberosum* L.) through *Agrobacterium tumefaciens*-mediated gene transfer. The presence and expression of the gene in transgenic plants were confirmed by the PCR and RT-PCR techniques, respectively. Northern hybridization using a *DREB1B* cDNA probe revealed high levels of *DREB1B* expression among the most transgenic lines. Overexpression of *DREB1B* imparted a significant freezing and drought tolerance gain in the transgenic potato lines. In comparison with the wild-type plants, the transgenic potatoes contained higher proline content under drought and freezing conditions, and maintained their relative water content higher under water stress. The enhancement of tolerance in transgenic potato highlights the presence of genes responding to the transcription factor DREB1B in this plant.

Additional key words: abiotic stress, dehydration-responsive element, overexpression, *Solanum tuberosum*, transcription factor.

Introduction

Drought, high salinity and freezing are prevalent environmental stresses that strongly influence the survival and potential yields of crops. Plants display a range of biochemical, physiological and developmental responses to these stresses that allow them to survive (Xiong *et al*. 2002, Nakashima *et al*. 2006). Transcriptome analysis has revealed that genes induced by stress conditions could be classified into two groups according to the functions of their products. The first group consists of proteins function in stress conditions and the second group contains protein factors involved in further regulation of signal transduction and gene expression (Xiong *et al*. 2002, Hu *et al.* 2010). Among several strategies followed to enhance stress tolerance in plants, direct introduction of genes by genetic engineering is thought to be more useful (Agarwal *et al*. 2006, Yang *et al*. 2010). The genes selected for transformation were those involved in encoding stress proteins or the enzymes of the biosynthetic pathways associated with the stress responses (Richards *et al*. 2002, Zhang *et al*. 2004). However, it is well known that stress tolerance is a complex trait, requiring the coordinated regulation of a network of genes that act synergistically and additively (Nakashima *et al*. 2006). Recent studies have shown that it is possible for a single transcription factor to act as a "regulon" by controlling the expression of many down-stream stress-induced genes through the specific binding of the transcription factor to DNA motifs in gene promoters (Umezawa *et al*. 2006). Analysis of expression of dehydration-inducible genes in the model plant *Arabidopsis thaliana* has identified several important transcription factors such as dehydration-responsive element-binding protein (DREB) that could be dichotomized as DREB1/CBF (C-repeat binding factor)

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Abbreviations: bp - base pair; DREB - dehydration responsive element binding protein; PCR - polymerase chain reaction; SR - survival rate; WC - water content per dry mass unit.
* Corresponding author; fax: (+98) 311 3912254, e-mail: sayedt@cc.iut.ac.ir

and DREB2 (Yamaguchi-Shinozaki and Shinozaki 1994). The role of DREB1/CBF regulon in the enhancement of abiotic stress tolerance was indicated by the results of *CBF* overexpression. Constitutive expression of *DREB1A*, *DREB1B* and *DREB1C* genes in transgenic *Arabidopsis* plants activated the expression of stress-responsive genes and increased the drought, salinity and freezing tolerance (Gilmour *et al*. 2004). The development of cold tolerance was also observed in transgenic canola overexpressing the *CBF1, CBF2* and *CBF3* genes (Jaglo *et al*. 2001), and transgenic tomato and rice with overexpression of *CBF1* gene (Hsieh *et al*. 2002, Lee *et al*. 2004).

The potato plant which is widely distributed in the world is not only a major source of food, but also an important target crop for biotechnological applications, as well as a valuable model system for studying signaling processes (Banerjee *et al*. 2006, Rensink *et al*. 2005). Restricted culturing of many potato cultivars because of low resistance to wide range of environmental stresses has led to trying for engineering of new stress-tolerant cultivars (Jeong *et al*. 2001, Celebi-Toprak *et al*. 2005). Therefore the present investigation was performed to determine whether overexpression of *DREB1B* gene in potato enhances tolerance to drought and freezing stresses as it does in *Arabidopsis* and other plants.

Materials and methods

The *Arabidopsis DREB1B* cDNA in the cloning vector *pBluescript* was obtained from *RIKEN Bioresource Center*, Tsukuba, Japan. The 929-bp fragment from the *DREB1B* clone was inserted into the transformation vector pBI121 (Jefferson *et al*. 1987) under the control of the CaMV35S promoter. This DNA cassette also contained the *neomycin phosphotransferase* II (*npt*II) gene as selectable marker (conferring resistance to kanamycin). The prepared construct carrying the *DREB1B* coding sequence, was introduced into *Agrobacterium tumefaciens*.

For transformation, stem segments of vigorous *in vitro* grown potato (*Solanum tuberosum* cv. Desiree) plants were inoculated with *A. tumefaciens* strain LBA4404 containing the plasmid *35S:DREB1B* on modified Linsmaier and Skoog (LS) medium (MS salts and vitamins, 14 mg dm^{-3} adenine sulfate, 1 mg dm^{-3} zeatin riboside, 0.5 mg dm^{-3} benzylaminopurine, 0.1 mg dm^{-3} naphthalene acetic acid) and incubated at room temperature. In the next step, the explants were transferred to modified LS medium containing 500 mg dm^{-3} cefotaxime and 100 mg dm^{-3} kanamycin. On the basis of kanamycin resistance, the rooted transgenic plantlets were selected and further screened by polymerase chain reaction (PCR) for the *DREB1B* and *nptII* genes.

For reverse transcription (RT)-PCR analysis for *DREB1B* gene, leaf materials of PCR positive potato plants were homogenized under liquid nitrogen and subsequently mRNA was extracted and reverse-transcripted into cDNA using mRNA capture kit (*Roche*, Germany). Duplex PCR was performed for amplification of the 780 bp fragment of the *DREB1B* gene.

Twenty three representative transgenic and control *in vitro* grown (4-week-old) potato lines from the T_0 family were transferred to pots containing a mixture of peat moss, sand and soil (1:1:1) and watered daily for a period of 21 d. To impose a drought treatment, water was withheld for 0, 14, 21 and 28 d followed by rewatering. Survival rate (SR) of water stress treatment was defined as percentage of healthy plants to total plants in each line. The water content (WC) was determined as WC = (f.m. - d.m.)/d.m.

Tolerance to freezing stress was analyzed using 35S:*AtDREB1B* potato lines grown in pots as described previously. The plants were exposed to a temperature of -6 °C for 0, 15, 30 and 45 h and returned to 24 °C. The number of surviving plants in each pot was recorded and survival rate (SR) of freezing treatment was determined.

Proline content was determined according to Bates (1973). Leaves detached from transgenic lines and wild-types were extracted using 3-sulfosalicylic acid and the supernatant collected using centrifugation. Ninhydrin and acetic acid were added to the supernatant and incubated at 100 °C for 60 min. It was snap chilled on ice to terminate the reaction, and after adding toluene, the absorbance at 520 nm was measured using spectrophotometer (Beckman DU 500, USA).

For Northern hybridization, total RNA from wild-type plants, tolerant and sensitive transgenic lines to drought and freezing stresses were extracted by *Biozol* RNA extraction reagent (*BioFlux*, Japan) according to the manufacturer's recommendations. Total RNA samples were probed using RNA probe labeled with *DIG-11-UTP* using the *DIG* Northern starter kit (*Roche*).

Three independent Northern hybridization experiments were performed for each line (using the same RNA preparation), and the average of the results was reported.

Results

The introduction of *DREB1B* cassette into *A. tumefaciens* strain LBA4404 and co-cultivation of the stem explants of potato plant with *A. tumefaciens* were successfully carried in the modified LS medium. Supplementation of

the culture media with kanamycin fully suppressed the regeneration of non-transgenic plants and reduced the number of plantlets for the PCR test using the *DREB1B* and *nptII* primers. As a result, one hundred (83 %) transgenic potato lines, the T_0 family, were identified as transgenic. The regenerated wild types appeared to be phenotypically normal, while the shoots containing 35S:*DREB1B* showed growth retardation which has known as a natural effect of constitutive expression of the *DREB1* genes.

RT-PCR studies of cDNA from the putative 35S: *DREB1B* transgenic plants showed positive amplification of the 780 bp *DREB1B* gene fragment using designed primer pair BF1 and BR1 in the selected transgenic lines

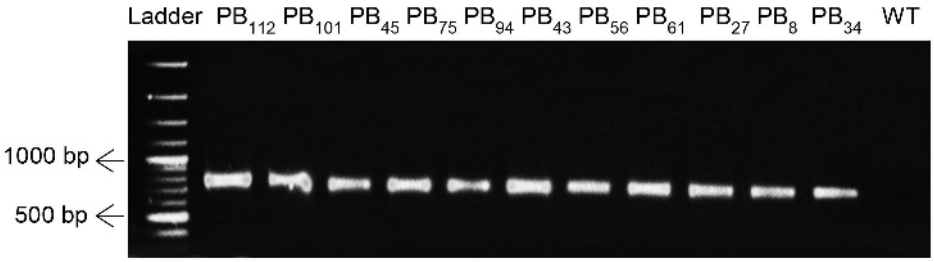

Fig. 1. RT-PCR analysis of *DREB1B* (780 bp) gene expression in non-stress transgenic and wild-type potato plants.

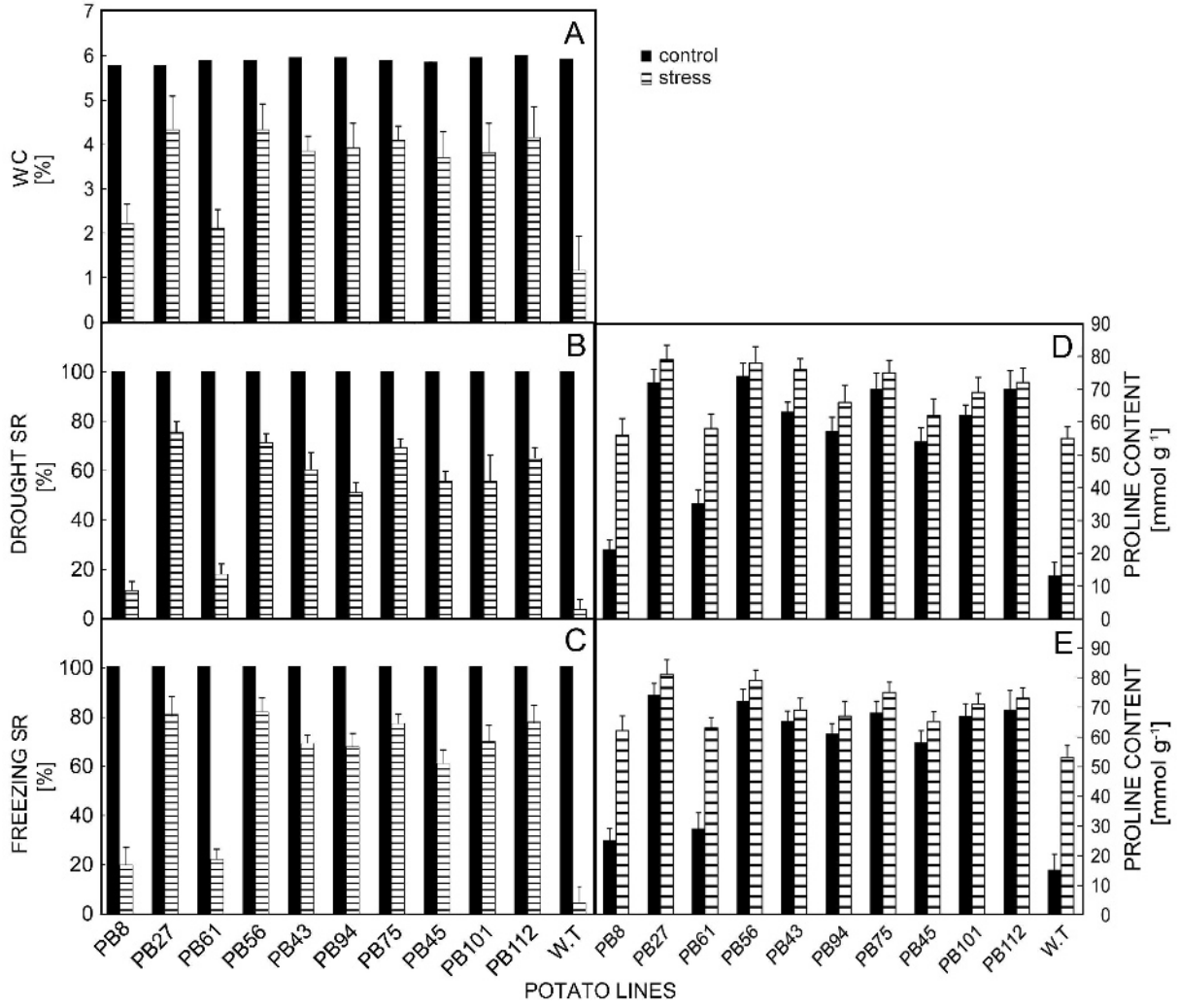

Fig. 2. Water content (WC) of transgenic potato and wild type plants during 0 and 28 d of drought treatment (*A*), survival rate (SR) of potato lines under 0 and 28 d of drought stress (*B*), SR of potato lines under freezing treatment of 0 and 45 h at the temperature of -6 °C (*C*), proline content of potato lines in control and drought treatment (*D*) and freezing treatment (*E*). Means ± SD, $n = 15$.

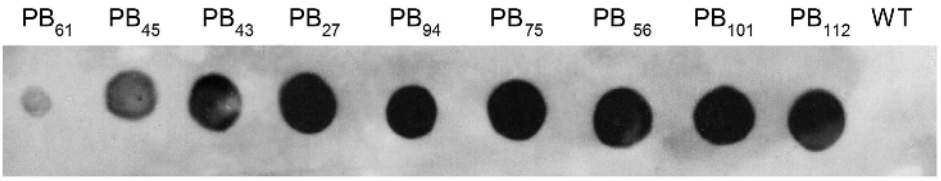

Fig. 3. Northern hybridization of transgenic and wild-type potato plants. Ten micrograms of total RNA was dotted on nylon membranes and hybridized with an RNA probe labeled with DIG-11-UTP.

under non-stress conditions (Fig. 1) indicated the integration of *DREB1B* gene and accumulation of its transcripts in the potato transgenic lines.

During the water stress treatment it was observed that the leaves of wild-type plants became wilted and their stems did not stand upright after 14 d of cessation of watering, whereas the 35S:*DREB1B* transgenic plants were apparently more resistant. By extending the water stress to 28 d, 98 % of the wild-type plants were completely wilted and did not recover during 7-day period after rewatering, while 75.3, 71 and 69 % of the transgenic lines PB27, PB56, and PB75 survived, and continued to grow after rewatering (Fig. 2).

The measurement of WC in leaves of drought-stressed transgenic and wild-type potato plants at various stress times was performed to compare their ability of maintaining water in tissue. It was observed that transgenic plants could preserve their WC during water stress treatments, while a great reduction of WC occurred in the wild-type plants (Fig. 2). This result in combination with the SR under dehydration, suggested that overexpression of *DREB1B* could significantly improve drought tolerance in transgenic potato.

The possible effect of *DREB1B* overexpression on tolerance to freezing in 23 transgenic lines and wild-type plants were tested at -6 °C. The freezing injury on wild-type plants was visible more clear than on the transgenic lines. After 15 h of freezing treatment, 59 and 91 % of wild-type and transgenic plants survived, respectively. Less than 4.6 % of wild-type plants endured a freezing treatment of 45 h, but none of them was able to resume growth when transferred to normal growth conditions and subsequently died, whereas most of the transgenic lines overexpressing *DREB1B* could survive after 45 h freezing treatment (survival rate of 61 - 81 %), and grew after recovery at 22 °C (Fig. 2). This result let us to propose that constitutive expression of *DREB1B* has led to strong resistance of transgenic plants to frost treatment.

The proline content was higher in transgenic lines comparing with the wild-type plants in both normal and stress conditions (Fig. 2). These results suggested that overexpression of the *DREB1B* gene could bring about a great stimulation of the expression of genes responsible for proline accumulation in transgenic potato even without stress signal.

The expression of *DREB1B* inserts under the control of 35S promoter was determined using Northern hybridization for the following transgenic lines. The *DREB1B* transcripts were accumulated only in transgenic potato plants. The line PB61 which posed low tolerance during drought and freezing experiment (SR of 17.6 and 22.3 % in drought and freezing treatment, respectively), did not show a high expression of *DREB1B*; which is probably due to post-transcriptional silencing of *DREB1B* gene expression. On the contrary, transgenic lines BP27, PB56, PB75, PB101 and PB112, exhibited high resistance in both drought and freezing conditions, showed significant expression of *DREB1B*.

Discussion

Although transformation with individual genes has been shown to confer some degree of tolerance in transgenic plants, overexpression of transcription factors can lead to a multiple stress tolerance in plants. In this investigation, transgenic potato lines expressing the *DREB1B* of *Arabidopsis* driven by CaMV35S promoter were successfully generated using *Agrobacterium*-mediated transformation. High percentage of transformants verified by PCR and RT-PCR analysis of the *DREB1B* and the *nptII* genes, revealed the success of transformation procedure in integration and constitutive expression of *DREB1B* in transgenic potato plants. The results of studying the WC and SR showed that overexpression of *DREB1B* in transgenic potato plants can lead to a significant increase in their tolerance to both drought and freezing stresses. The dwarfism of transgenic potato plants led to lower transpiration rate during water stress, which can be the cause of higher WC and SR of transgenic potatoes comparing with the wild-type plants. However, the increased proline content, revealed that *DREB1B* overexpression might result in biochemical changes associated with stress response in transgenic potato plants even without stimulating by stress signals. Similar results were observed in transgenic *Arabidopsis* and transgenic tomato overexpressing *DREB1A* and *DREB1B*, respectively (Gilmour *et al*. 2000, Hsieh *et al*. 2002a). These observations led us to propose that *DREB1B* could enhance stress tolerance due to activation

of the stress responsive genes such as those involved in proline biosynthesis in transgenic potato plants.

However, reduction of growth in transgenic potatoes was a negative consequence of constitutive expression of the *DREB1B*. Similar effect was found in transgenic *Arabidopsis* and transgenic tomato with overexpression of *DREB1B* (Gilmour *et al*. 2000, Hsieh *et al*. 2002a), and transgenic *Arabidopsis* overexpressing of two chrysanthemum *DgDREB1* genes (Tong *et al*. 2009); suggesting that dwarfism is a result of *DREB1* genes expression. It seems that DREB1 proteins have a multiple impact on developmental process in transgenic plants (Hsieh *et al*. 2002b, Achard *et al*. 2008). To overcome this problem, stress inducible promoters such as RD29A that have low background expression under non-stress conditions have been used in conjunction with *DREB1* genes to strengthen stress tolerance without growth reduction in transgenic plants (Kasuga *et al*. 2004, Pellegrineschi 2004, Bhatnagar-Mathur *et al*. 2007, Jin *et al*. 2009).

In this experiment, despite the fact that stress treatments were conducted on juvenile 7-week-old plants and multiple stresses might be combined, 78 % of the *DREB1B* transgenic potato lines showed relatively a strong tolerance against drought and freezing. The lines PB27, PB56, PB75 and PB112 showed the highest SR (Fig. 2), and even some cutting did not experience any damage due to the exposure to stress conditions. The results of Northern analysis indicated a high expression of *DREB1B* transcripts in transgenic lines which were more resistant to drought and freezing conditions. This result besides other similar studies (Hsieh *et al*. 2002b, Celebi-Toprak *et al*. 2005, James *et al*. 2008) strongly suggests the existence of a direct relation between the expression level of *DREB1B* mRNA and tolerance level in transgenic plants. The variation among the lines associated with *DREB1B* expression could be presumably due to the position effect of the *DREB1* gene insertion and gene silencing on transcriptional and post-transcriptional level (Angell and Baulcombe. 1997, Celebi-Toprak *et al*. 2005).

As a whole, the results presented lead us to propose that *DREB1B* can integrate the activation of multiple genes in response to drought and freezing stresses in transgenic potato. In similar approaches, expression of *DREB1A* in transgenic potato led to a high resistance to salinity and freezing stresses (Celebi-Topark *et al*. 2005, Behnam *et al*. 2007). These results suggest that DREB mechanism in potato is similar to *Arabidopsis* and other plant species; since there was no DRE or DRE-related motif sequences in the promoter region of stress-responsive genes, no variation in stress tolerance would be observed in this transgenic plant. However, farther studies for identification and functional analysis of the stress tolerance mechanisms are required in transgenic potato and other plants besides the application of stress-induced promoters. Until now, the orthologous genes of *DREB1* have been found in many plants such as canola, rice, wheat, tomato, alfalfa, barley and maize (Zhang *et al*. 2004). This indicates that DREB regulon system is ubiquitous within higher plants, and "DREB technology" with controlling the expression of DREB regulons is expected to improve abiotic stress tolerance in crop plants.

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