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ORIGINAL ARTICLE

Enhanced drought and salinity tolerance in transgenic potato plants with a BADH gene from spinach

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Abstract Drought and salinity are the most important abiotic stresses that affect the normal growth and development of plants. Glycine betaine is one of the most important osmolytes present in higher plants that enable them to cope with environmental stresses through osmotic adjustment. In this study, a betaine aldehyde dehydrogenase (BADH) gene from spinach under the control of the stress-induced promoter *rd29A* from *Arabidopsis thaliana* was introduced into potato cultivar Gannongshu 2 by the *Agrobacterium tumefaciens* system. Putative transgenic plants were confirmed by Southern blot analysis. Northern hybridization analysis demonstrated that expression of *BADH* gene was induced by drought and NaCl stress in the transgenic potato plants. The BADH activity in the transgenic potato plants was between 10.8 and 11.7 U. There was a negative relationship ($y = -2.2083x + 43.329$, $r = 0.9495$) between BADH activity and the relative electrical conductivity of the transgenic potato plant leaves. Plant height increased by 0.4–0.9 cm and fresh weight per plant increased by 17–29% for the transgenic potato plants under NaCl and polyethylene glycol stresses compared with the control potato plants. These results indicated that the ability of transgenic plants to tolerate drought and salt was increased when their BADH activity was increased.

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Abbreviations

BADH	Betaine aldehyde dehydrogenase
BCIP	5-Bromo-4-chloro-3-indolyl phosphate
CMO	Choline monooxygenase
CTAB	Cetyl-trimethyl-ammonium bromide
DTT	Dithiothreitol
GB	Glycine betaine
GUS	β-Glucuronidase
MS	Murashige and Skoog medium
NBT	Nitro blue tetrazolium
NptII	Neomycin phosphotransferase
PEG	Polyethylene glycol
SSC	Sodium chloride/sodium citrate buffer

Introduction

Drought and salinity are the most important abiotic stresses that limit agricultural production globally. Many important crop plants, such as rice, potato and tobacco, are sensitive to drought and salinity. One of the fundamental physiological mechanisms that enable higher plants to cope with environmental stresses is osmotic adjustment, and glycine betaine (GB) is one of the most important osmolytes (Chen and Murata 2008).

Many plant species can accumulate GB in response to drought and salinity (Rhodes and Hanson 1993). In higher plants, GB is synthesized by converting choline to GB through a two-step oxidation via the intermediate betaine aldehyde (Hanson and Scoff 1980). The relevant enzymes

are choline monooxygenase (CMO) and betaine aldehyde dehydrogenase (BADH) (Sakamoto and Murata 2000). Genes or cDNAs that encode the enzymes involved in the biosynthesis of GB have been cloned. They include genes for *CMO* from spinach (Rathinasabapathi et al. 1997), and *BADH* from spinach (Weretilnyk and Hanson 1990), sugar beet (Mccue and Hanson 1992), barley (Ishitani et al. 1995) and sorghum (Wood et al. 1996). Transgenic plants of various species have been produced, including tobacco (Nuccio et al. 1998), wheat (Guo et al. 2000), rice (Kishitani et al. 2000) and so on, that express CMO or BADH transformed with the corresponding genes. These transgenic plants accumulate GB at various levels and exhibit enhanced tolerance of drought and salinity (Sakamoto and Murata 2000).

The fact that many important crops—such as rice, potato and tomato—are betaine deficient has inevitably led to the proposal that it might be possible to increase drought and salinity tolerance by genetically engineering GB synthesis (McCue and Hanson 1990). Ahmad et al. (2008) obtained transgenic potato plants with enhanced tolerance of oxidation, salt, and drought stresses by synthesizing GB via the introduction of the bacterial choline oxidase (*codA*) gene. In this study, we introduced the *BADH* gene from spinach, which is responsible for GB synthesis, into potato by genetic transformation. The resulting transgenic plants acquired the ability to tolerate drought and salinity.

Materials and methods

Plant materials and growth conditions

Experiments were carried out with the potato (*Solanum tuberosum* L.) tetraploid cultivar Gannongshu 2. The potato plants were propagated in vitro by subculturing single-node cuttings on MS medium supplemented with 3% sucrose and 0.6% agar. Plantlets were grown in 150 ml flasks under a 16 h white fluorescent light and 8 h dark cycle at 20°C.

Plasmid construction

The 1,556 bp cDNA of the *BADH* gene (AY156694) (Zhang et al. 2004) isolated from spinach was cloned into the *Sma*I–*Sac*I site of the plasmid pBIrd containing the stress-induced promoter *rd29A* from *Arabidopsis thaliana* (Zhang et al. 2005), such that the β-glucuronidase (GUS) gene was replaced by the *BADH* gene. The recombinant plasmid was named pBIrB, and then introduced into *Agrobacterium tumefaciens* strain LBA4404 using the freeze–thaw method (Hofgen and Willmitzer 1988). The presence of the plasmid was verified by restriction enzyme digestion and PCR amplification.

Potato transformation and PCR analysis

Slices of microtubers of potato cultivar Gannongshu 2 were used as the receptor for *Agrobacterium*-mediated transformation, which was performed as described previously (Si et al. 2003).

Genomic DNA was isolated from the putative transformed and control potato plants using the cetyl-trimethylammonium bromide (CTAB) method. The presence of the transferred *BADH* gene was demonstrated by employing standard PCR techniques using the primers 5′-GTTGA CAAGATTGCCTTTAC-3′ (703–722 bp) and 5′-CTTAA CAA AAACAACACCGT-3′ (1,537–1,556 bp). The PCR product was expected to be a 853 bp fragment. Amplification was performed in a thermal cycler (UNOII, Biometra) programmed for one cycle of 3 min at 94°C followed by 36 cycles of 45 s at 94°C, 45 s at 50°C, and 1 min at 72°C. A final extension step was performed for 5 min at 72°C. The PCR products were electrophoresed on a 0.8% agarose gel.

Southern blot analysis

Genomic DNA was extracted using the CTAB method. Forty micrograms of genomic DNA were digested with the restriction endonuclease *Sac*I and separated on 0.8% agarose gel, denatured, and transferred to a positively charged nylon membrane (Roche) using standard methods. Membranes were hybridized at high stringency in a 50% (v/v) formamide hybridization buffer at 42°C for 16 h. The hybridization buffer was 50% (v/v) formamide, 5× SSC, 2% blocking reagent (w/v), 0.02% SDS (w/v) and 0.1% (w/v) *N*-lauroylsarcosine. Membranes were washed twice in 2× SSC, 0.1% (v/v) SDS at room temperature for 10 min each, and twice in 0.5× SSC, 0.1% (v/v) SDS at 65°C for 15 min. The gene-specific probe of the *BADH* gene was labeled using a DIG High Prime DNA Labeling and Detection Starter Kit I (Roche). The hybridization signal generated using NBT/BCIP color substrate was detected after 8–24 h. Hybridization and detection were carried out according to the manufacturer's instructions.

Northern blot analysis

Total RNA was isolated using the guanidine–HCl method (Chomczynski and Sacchi 1987). Thirty micrograms of total RNA per lane were separated by electrophoresis on 1% agarose formaldehyde gels and transferred to a positively charged nylon membrane (Roche) using standard methods. The conditions used in the hybridization and

washing of RNA blotting were the same as those described for Southern blotting hybridization, except that hybridization was performed at 68°C. The RNA probe of the *BADH* gene was labeled using a DIG RNA Labeling Kit (SP6/T7) (Roche). The concentration of hybridization probe was 40 ng ml^{-1}. Hybridization and detection were carried out according to the manufacturer's instructions.

Assay of BADH activity

Fresh leaves (1 g) from the transgenic and control plants were powdered in liquid nitrogen and homogenized in 2 ml of extraction buffer containing 50 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES)-KOH (pH 8.0), 1 mM ethylene diamine tetraacetic acid (EDTA), and 5 mM dithiothreitol (DTT). Plant debris was removed by centrifugation at 10,000×*g* for 10 min at 4°C. The protein concentration of the supernatant was determined using the Bradford method (Bradford 1976). BADH activity was measured according to the procedures of Liu et al. (1997).

Assay of relative electrical conductivity

Fresh leaves (2 g) from the transgenic plants and the control plants taken 3 days after the NaCl and PEG treatments had been completed were used for the assay of relative electrical conductivity using a conductivity meter (DDS-11A) according to the method described by Li (2000). The relative electrical conductivity was obtained by comparing the electrical conductivity of exudates from the transgenic plant leaves (L1) with that of exudates from control leaves killed in boiling water (L2).

Bioassay for salt and drought tolerance

The transgenic and control potato plants were grown in vermiculite in 3 L pots in a greenhouse under natural light at 25°C, and watered and fertilized weekly with a complete nutrient solution. When the plants reached 10 cm in height, they were treated with NaCl and polyethylene glycol (PEG, MW 6,000), respectively. NaCl treatment was begun at a concentration of 50 mM and increased stepwise by 50 mM every day until the final concentration, 500 mM, was reached. PEG treatment was conducted with 15% PEG solution once a day for 10 days. After the treatment had been finished for 3 days, the plants were harvested in order to perform measurements of BADH activity, relative electrical conductivity, height, and fresh weight. Total RNA was isolated for northern blot analysis from plants treated for 5 days and then left another 3 days after the treatment had finished. Data were analyzed using the statistical analysis system (SAS).

Results

Plasmid construction and potato transformation

The plasmid pBIrB containing the *BADH* gene was constructed, and is shown schematically in Fig. 1. Green shoots were produced directly from the surfaces of the transformed microtuber slices after 4 weeks of culture in the selective medium (Fig. 2). Roots were formed in about 10 days when green shoots were transferred to the selective

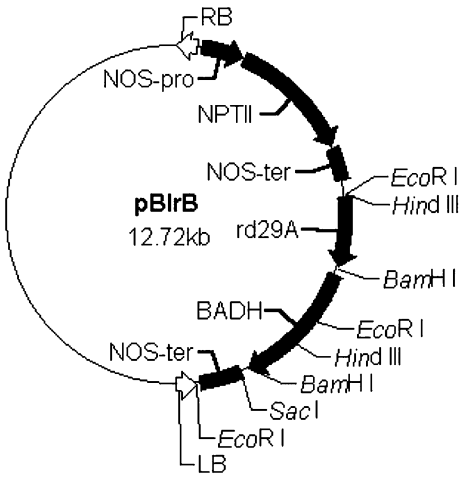

Fig. 1 Schematic diagram of the expression vector pBIrB. *RB* right border, *LB* left border, *NOS-pro* nopaline synthase promoter, *NOS-ter* nopaline synthase terminator, *NPTII* neomycin phosphotransferase II gene, *rd29A Arabidopsis thaliana* stress-induced promoter, *BADH* betaine aldehyde dehydrogenase gene. *Eco*RI, *Hin*dIII, *Bam*HI, and *Sac*I are restriction endonuclease recognition sites

Fig. 2 Formation of shoots directly from transgenic microtuber discs of the potato cultivar Gannongshu 2 after 4 weeks of culture in the selective medium (MS medium containing 1 mg l^{-1} IAA + 0.2 mg l^{-1} GA_3 + 0.5 mg l^{-1} BA + 2 mg l^{-1} zeatin) supplemented with 75 mg l^{-1} kanamycin and 400 mg l^{-1} carbenicillin and incubated under a photoperiod with 16 h light and 8 h dark cycles at 24°C

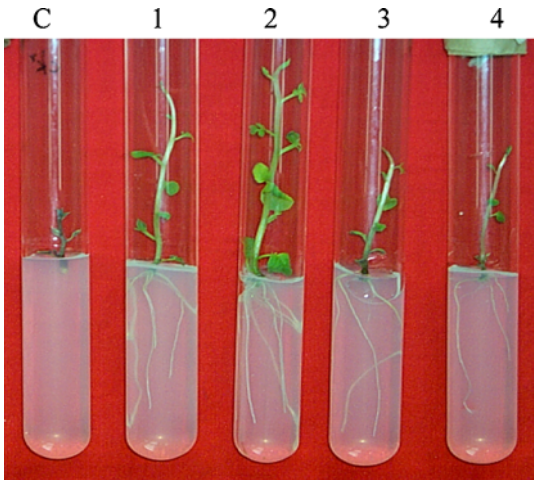

Fig. 3 Roots were formed in about 10 days when green shoots were transferred to the selective rooting medium (MS medium containing 50 mg l^{-1} kanamycin and 200 mg l^{-1} carbenicillin). *C* nontransgenic potato plant, *1–4* transgenic potato plant lines

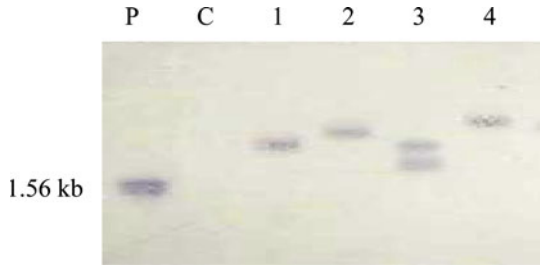

Fig. 4 Southern blot analysis of genomic DNA from PCR-positive transformed potato plant lines. *Lane P* positive control, showing 1.56 kb *BADH* gene insertion obtained from plasmid pBIrB DNA digested with *Bam*HI, *Lane C* genomic DNA from the untransformed control plant, *lanes 1–4* genomic DNA from transformed plant lines. The genomic DNA was digested with *Sac*I and hybridized with a *BADH* probe labeled with digoxigenin

rooting medium (Fig. 3). Plantlets with well-developed roots were propagated for further molecular analysis.

Molecular blotting analysis

PCR analysis using *BADH* gene-specific primers showed that the putatively transformed plants had an amplification product of 853 bp that was missing in the control plant. The PCR amplification results were further conformed by Southern blot analysis, which showed that the *BADH* gene was integrated into the potato genome (Fig. 4). When analyzed using northern blot analysis, the expression profiles for *BADH* mRNA in the leaves from transgenic plant line 1 showed that the *BADH* gene was expressed weakly in the unstressed transgenic plants while there was no transcript in the control plants, and the level of *BADH* mRNA increased steadily with the number of treatment days of NaCl and PEG, respectively (Fig. 5). Transgenic

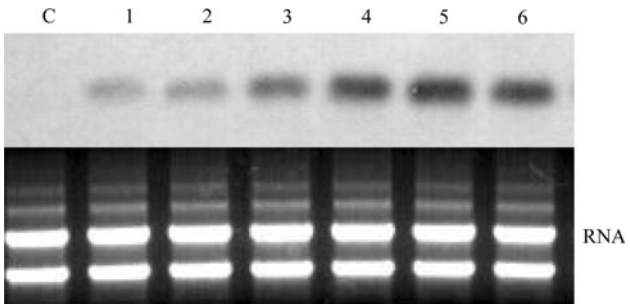

Fig. 5 Northern blot analysis of *BADH* gene expression in the transgenic potato plant line 1. *Lane C* untransformed potato plant; *lanes 1 and 2* untreated transgenic potato plants, *lanes 3 and 4* transgenic potato plants subjected to NaCl and PEG treatments for 5 days, respectively, *lanes 5 and 6* transgenic potato plants 3 days after being subjected to NaCl and PEG treatments for 10 days, respectively. Each lane in the electrophoresis contained a similar 30 μg RNA sample stained with ethidium bromide

Table 1 BADH activities of the transgenic potato plants stressed with NaCl and PEG

Transgenic plant lines	BADH activity (U)		
	Untreated	Treated with NaCl	Treated with PEG
C	0.0 ± 0.00d	0.0 ± 0.00b	0.0 ± 0.00b
1	0.8 ± 0.10b	11.2 ± 0.46a	11.7 ± 0.44a
2	0.3 ± 0.17cd	10.9 ± 0.62a	11.5 ± 0.53a
3	1.2 ± 0.20a	11.4 ± 0.36a	10.8 ± 1.22a
4	0.5 ± 0.26bc	11.6 ± 0.36a	11.3 ± 0.62a

C untransformed potato plant, *1–4* transgenic potato plant lines. Values represent the mean ± standard error (SE) from three replicates. Means within a column followed by different small letters are significantly different at the 0.05 probability level, as tested with Duncan's multiple range test

plant lines 2, 3, and 4 showed similar expression profiles (data not shown).

BADH activities and relative electrical conductivities of the transgenic plants

The BADH activities of the transgenic plants were rather low when they were not stressed, but increased greatly 3 days after the treatment with NaCl and PEG had finished. The BADH activities were between 10.8 and 11.7 U and varied a little among the different transgenic plant lines (Table 1). The results were similar to those observed in the northern blot analysis.

The relative electrical conductivities of the transgenic plants after NaCl and PEG stress were about 17.4–19.6%, less than those of the control plants (45.6%), which showed that the cell membranes of the transgenic plants were less injured than those of the control plants under NaCl and PEG stress. A significant negative linear relationship

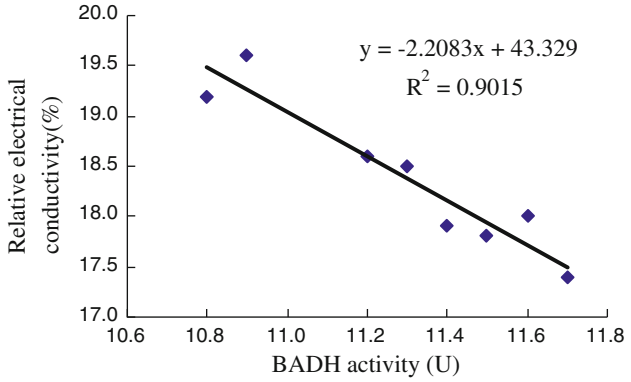

Fig. 6 Relationship between BADH activity and relative electrical conductivity of transgenic potatoes. Values of BADH activity and relative electrical conductivity were obtained from the transgenic plants 3 days after 10 days of NaCl and PEG treatment had been completed, respectively

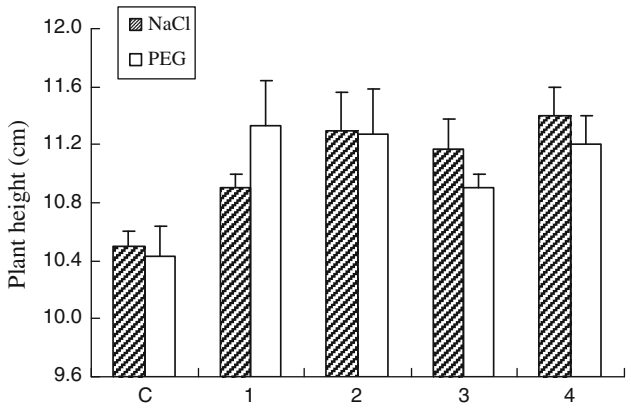

Fig. 7 Height of transgenic potato plants 3 days after 10 days of NaCl and PEG treatment had been completed, respectively. Plants were grown in vermiculite in 3 L pots in a greenhouse under natural light. The data are the mean ± standard error (SE) from three replicates. *C* nontransgenic potato plant, *1–4* transgenic potato plant lines

between the relative electrical conductivity (y) and BADH activity (x) was observed, which could be represented by a function of $y = -2.2083x + 43.329$ ($r = 0.9495$), revealing that BADH activity was positively related to protection of cell membrane permeability (Fig. 6).

Salt and drought stress tolerance of the transgenic potato plants

The growth of the transgenic potato plants was normal and better than the untransformed plants under NaCl and PEG stresses. The transgenic plants were 0.4–0.9 cm higher and 17–29% heavier (fresh weight per plant) compared with the control potato plants (Figs. 7, 8). These results suggested that the *BADH* gene can be used to improve the drought and salinity tolerance of important crops that are betaine deficient through genetic engineering.

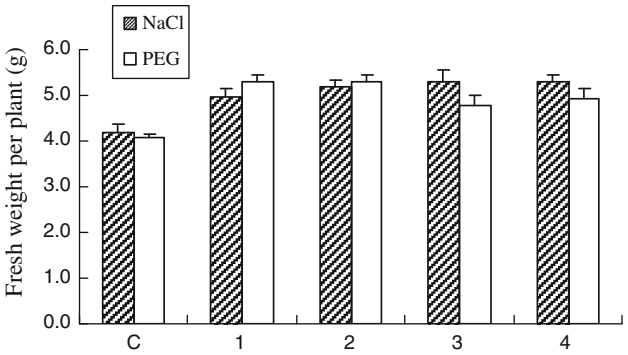

Fig. 8 Fresh weight per transgenic potato plant 3 days after 10 days of NaCl and PEG treatment had been completed, respectively. Plants were grown in vermiculite in 3 L pots in a greenhouse under natural light. The data are the mean ± standard error (SE) from three replicates. *C* nontransgenic potato plant, *1–4* transgenic potato plant lines

Discussion

Potato is regarded as a drought-sensitive crop compared with other crops based on field experiments (Salter and Goode 1967; van Loon 1981). Drought always influences the development and growth of stems, roots and tubers of potato (Ojala et al. 1990), and it also reduces the number of tubers and the yield of potato (Cavagnaro et al. 1971). Genetic engineering has produced potato plants that have drought and salinity tolerance. Goddijn et al. (1997) engineered trehalose biosynthesis in potato (*Solanum tuberosum*) by introducing the *otsA* and *otsB* genes from *Escherichia coli*, which encode trehalose-6-phosphate synthase and trehalose-6-phosphate phosphatase, respectively. Jeong et al. (2001) introduced the glyceraldehyde-3-phosphate dehydrogenase gene into potato by genetic engineering. The transgenic potato plants exhibited some degree of salinity tolerance.

GB is an osmoprotectant found in many organisms, including higher plants. Many plant species that accumulate betaine inhabit arid and saline areas, and accumulate this compound in response to drought and salinity. GB accumulation has long been a target of research aimed at engineering stress resistance (Le Rudulier et al. 1984). The idea that introducing the GB pathway into plants that lack it will enhance their stress tolerance is based on both comparative physiology (Yancey 1994) and genetic evidence from a glycinebetaine-deficient maize mutation line that showed reduced salt and heat tolerance (Saneoka et al. 1995; Yang et al. 1996). Potato plants are incapable of synthesizing betaine. Therefore, it might be possible to increase the drought and salinity tolerance of transgenic potato by introducing the *BADH* gene related to GB synthesis into potato.

In previous experiments, a cDNA encoding BADH was isolated from spinach (*Spinacia oleracea* L.) (Zhang et al.

2004) and the stress-induced promoter *rd29A* was isolated from *Arabidopsis thaliana* (Zhang et al. 2005). In the present study, we constructed a recombinant vector pBIrB containing the *BADH* gene under the control of the promoter *rd29A*, and then introduced it into potato and obtained transgenic plants. To investigate the drought and salinity tolerance of BADH-transgenic potato plants, four independent transgenic potato lines were selected via molecular identification. Both northern blot analysis and BADH activity assay demonstrated that *BADH* gene expression was induced by NaCl and PEG stresses since the *BADH* gene was controlled by the stress-induced promoter *rd29A*. When the plants that were grown in pots were exposed to NaCl and PEG stresses, all transgenic plants showed high drought and salinity tolerance due to the expression of the *BADH* gene compared to the control plants. This was similar to the results of the research described by Ahmad et al. (2008). They obtained transgenic potato plants with enhanced tolerance of NaCl and drought stress at the whole plant level by introducing bacterial choline oxidase (*codA*) gene for GB synthesis under the control of an oxidative stress-inducible *SWPA2* promoter. These results demonstrated that engineering GB synthesis, especially through the use of a stress-inducible promoter such as *SWPA2* and *rd29A*, is an effective way to impart stress tolerance to the potato plant. However, this work was conducted using the *BADH* gene from spinach, which seems to be more favorable to transgenic biosafety than the *codA* gene from bacterium.

GB is an amphoteric compound that is electrically neutral at physiological pH and is a nontoxic, low molecular weight, highly soluble compound. Although the actual roles of GB in plant osmotolerance remain controversial, GB is thought to protect the plant by acting as an osmolyte that maintains the water balance between the plant cell and the environment, and by stabilizing macromolecules, enzyme activities, and membranes under stress conditions (Sakamoto and Murata 2002; Chen and Murata 2008). Our results indicated that the transgenic potato plants gained enhanced tolerance to drought and salinity stress through membrane stabilization, since the transgenic plants showed lower relative electrical conductivities than the control plants under NaCl and PEG stress. The exogenous application of GB and the engineering of transgenic plants that can synthesize GB can both enhance tolerance to drought and salinity (Sakamoto and Murata 2000; Sakamoto and Murata 2002; Ashraf and Foolad 2007; Chen and Murata 2008). There are some reports that demonstrate positive effects of the exogenous application of GB (see Ashraf and Foolad 2007 and references therein) as well as the genetic engineering of GB synthesis (Ahmad et al. 2008) on plant growth and final crop yield under stress. Our transgenic plants also evidenced enhanced plant heights and fresh weights under stress conditions compared to the control plants (Figs. 7, 8). Similarly, transgenic potato plants with the *codA* gene for GB synthesis also presented enhanced fresh and dry weights and tuber yields in pot experiments (Ahmad et al. 2008), and transgenic tomato plants with the *codA* gene showed enlarged flowers and heavier ripe fruits (Park et al. 2007).

In conclusion, these results suggested that the *BADH* gene can be used to improve the drought and salinity tolerance of important crops that are betaine deficient through genetic engineering. Further research will determine the effects of the introduction of the *BADH* gene into potato on its agronomic characteristics and tuber yield.

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