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Enhanced salinity tolerance in transgenic maize plants expressing a *BADH* gene from *Atriplex micrantha*

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Abstract Salinity is an adverse environmental stress that limits the yield and quality of maize. As one of the most important osmolytes present in higher plants, glycinebetaine helps stabilize metabolism in plant cells and protects the constituents of cells from damage. In this study, a gene from *Atriplex micrantha* that encodes betaine aldehyde dehydrogenase was introduced by *Agrobacterium*-mediated transformation into maize inbred lines Zheng58 and Qi319 under the control of the maize ubiquitin promoter. Putative transgenic plants were confirmed by PCR and Southern blotting analysis. The transgenic maize plants expressed higher amounts of betaine aldehyde dehydrogenase activity and also grew better than the WT plants under NaCl stress. Compared with the wild type, the transgenic plants had increased fresh weight, lower malondialdehyde content, lower relative electrical conductivity, higher chlorophyll content, taller plant height, and higher grain yield under salt stress, which indicated that the expression of *BADH* gene in maize seedlings enhanced the salt tolerance of these plants.

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Introduction

Maize (*Zea mays* L.) is one of the important crops in the world, and serves as food for humans, feed for livestock and poultry, and as a raw material for agriculture-based industries. Meeting human demand will require cereal production to increase to over 400 million tons by 2050, which represents an increase in yield of 37 % from current values (Tester and Langridge 2010). Salinity is an adverse environmental stress that limits the yield and quality of maize. Estimates indicate that about 6 % of the world's total land area, including 20 % of the world's irrigated area, is affected by salinity (UNESCO Water Portal 2007). Because demand for maize production is increasing and arable land becomes more limited every year, it is critical to improve the salt tolerance of maize germplasm via efficient breeding technologies. With the application of the transgenic technology, researchers can accelerate the breeding process by introducing genes that encode proteins involved in abiotic stress-protective mechanisms. One common mechanism plants use for self-protection is the accumulation of osmoprotectants that stabilize metabolism and protect cellular components from damage due to abiotic stress (Wood et al. 1996).

Glycinebetaine (GB), a common osmoprotectant in many organisms and in higher plants, raises osmolarity within cells in response to different stresses and

stabilizes biological macromolecules (Rhodes and Hanson 1993; Holmstrom et al. 2000). GB is synthesized from choline in eukaryotes by a two-step process with betaine aldehyde as an intermediate compound. The choline is oxidised to betaine aldehyde by choline monooxygenase in the first step, followed by another oxidation reaction catalysed by betaine aldehyde dehydrogenase (BADH) that generates glycinebetaine. GB significantly increases the cellular osmotic levels of many salt-tolerant plants (Rhodes and Hanson 1993). Many studies have shown that overexpression of the *BADH* gene in transgenic plants could enhance tolerance to a wide range of abiotic stresses including salt, heat, drought, and chilling (Park et al. 2004; Quan et al. 2004; Su et al. 2006; Fan et al. 2012). In previous studies, heterologous overexpression of the *BADH* gene in transgenic plants resulted in the accumulation of GB, which subsequently improved the plant tolerance to salt, oxidative, and cold stresses (Fan et al. 2012; Ahmad et al. 2013; Tang et al. 2014). Researchers have proposed that such improvements in stress tolerance are mediated by induction or activation of ROS scavenging; increased proline accumulation and improved membrane integrity under stress have also been observed. GB has also been shown to counteract the inhibitory effects of salt stress-mediated photoinhibition on the degradation and synthesis of the D1 protein in *Synechococcus* (Ohnishi and Murata 2006).

The complexity of the maize genome, its relatively long life cycle, and the low transformation efficiency of most maize genotypes make generating transgenic maize difficult. In this study, we used established *Agrobacterium*-mediated methods to introduce a binary plasmid containing a betaine aldehyde dehydrogenase gene, *BADH*, from salt-tolerant *Atriplex micrantha* into maize inbred lines, and subsequently evaluated the stress tolerance of the transgenic plants. Here, we aimed to understand the function of BADH in the tolerance of maize plants to salinity stress, with the ultimate goal of creating new abiotic stress-tolerant maize germplasm.

Materials and methods

Agrobacterium tumefaciens strain and plasmid construct

Agrobacterium tumefaciens strain LBA4404 harboring pCAMBIA3300-*Ubi-BADH* was used to overexpress the *BADH* gene in the present study (Fig. 1). The 1503-bp coding sequence of the *BADH* gene (GenBank ID EF208902, and Supplementary Fig. 1) was originally cloned from *Atriplex micrantha*, a very salt-tolerant plant that grows in salt marshes (Zhu et al. 2007). The *BADH* gene was then cloned into vector pCAMBIA3300 (Zhu et al. 2007) after appropriate digestion following standard protocols (Sambrook et al. 2000). The recombinant plasmid was transformed into *A. tumefaciens* strain LBA4404 using the freeze-thaw method.

Plant materials and maize transformation

The elite maize inbred lines Zheng58 and Qi319 were used as the genetic materials in this study. The maize transformation procedure was performed according to the method published by Li et al. (2008). Seeds of Zheng58 and Qi319 were surface sterilized and were incubated on autoclaved solid MS medium at 28 °C in darkness until the etiolated seedlings had grown to 3.0–4.0 cm in length. Maize shoot tips with exposed meristems were immersed in the suspension of transformed *Agrobacterium tumefaciens* that had been grown to log phase then diluted to OD600 = 0.8 for 10 min under 0.05 MPa pressure, and were then co-cultivated on modified solid MS medium in the dark at 24 °C for 3 days. The plantlets were then transplanted into pots and cultivated in the greenhouse. The seedlings that survived spraying with the herbicide Basta at a concentration of 0.1 % were assayed for the presence of the transgene by PCR, and seeds were harvested from the positive T0 plants. T1 plants were grown from the harvested seed and self-pollinated to produce the T2 generation, followed by repeated herbicide screening and PCR assays until the T4 generation. Southern blotting and RT-PCR were carried out on PCR-positive plants in the T4 generation to confirm the integration and stability of the *BADH* gene, as described below. Experiments were conducted at the Molecular Breeding Experimental Base of Northeast Agricultural University located in Harbin City, Heilongjiang Province, China. Seeds for these plants were stored in a secure room and the plants were incinerated when the experiments were finished, in compliance with laws related to the handling and disposal of transgenic plants in China.
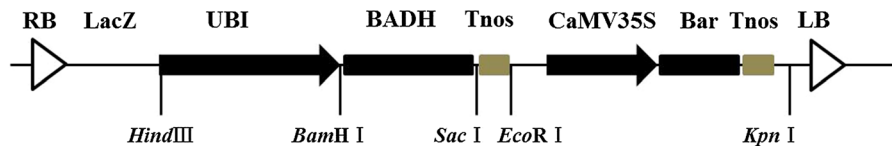
Fig. 1 Schematic diagram of the expression vector p3300-Ubi-BADH. *RB*, right border; *LB*, left border; *UBI*, *Ubiquitin* promoter, *Tnos*, *Nopaline synthase* terminator; *BADH*, betaine aldehyde dehydrogenase gene; *Bar*, *Bialaphos resistance* selectable marker gene; *CaMV35S*, *Cauliflower mosaic virus* 35S promoter. *Hind*III, *Bam*HI, *Sac*I, *Eco*RI, and *Kpn*I are restriction endonuclease recognition sites

PCR analysis and Southern blotting

Genomic DNA was extracted from the two youngest leaves of each plant using the CTAB protocol (Murray and Thompson 1980). PCR assays were performed using the following *BADH* gene-specific primers: F, 5′-TCCAAATCCACTACGC-3′; and R, 5′-TTCTGC TGCCCAACTA-3′. Southern blotting was performed with a digoxigenin (DIG)-labelled probe consisting of the gene fragment amplified using the *BADH* cDNA as template, as described in the DIG System Manual (Roche, Inc., Basel, Switzerland). Genomic DNA from each of the PCR-positive maize plants was digested with *Bam*HI, a restriction enzyme that cuts a single site in the T-DNA and cuts no sites within the *BADH* sequence. Restriction enzyme digestion was followed by electrophoresis of 20 μg of digested genomic DNA on 0.8 % agarose gels and subsequent transfer onto a positively charged nylon membrane (Roche, USA). After drying at 80 °C for 2 h, the membrane was hybridised to digoxigenin (DIG)-labelled probe, as described in the DIG System Manual.

RNA isolation and RT-PCR

Total RNA was extracted using Trizol reagent (Tiangen Biotech, Beijing, China) from 100 mg of young maize seedling leaves or roots, and 500 ng DNase-treated RNA was reverse transcribed using the RT Reagent Kit (Transgene, Beijing, China). The DNase-treated RNA samples were diluted fivefold to serve as templates for the subsequent PCR. The gene-specific primers from the conserved region of the target gene were used to amplify a 661-bp *BADH* fragment. Seedling leaves subjected to salt stress as described below were collected for RNA extraction and semi-quantitative RT-PCR. The β-Actin gene was used as an internal control for RT-PCR, and three replications were performed for each of the RT-PCR experiments in this study.

Salt stress treatment during seedling growth

T4-generation transgenic seeds and WT seeds were germinated and planted in pots containing vermiculite in the greenhouse (22 °C, humidity 40–50 %, with 16-h light and 8-h darkness). The plants were grown under well-watered conditions with 0.5× Hoagland's nutrient solution applied once per day until the three-leaf stage. Plants for the salt treatment were then irrigated with 0.5× Hoagland's nutrient solution containing 300 mmol NaCl once per day for a 7-days period. Control plants were watered at the same time with 0.5× Hoagland's solution containing no NaCl. All plants in the experiment, both salt-treated and control, were then irrigated with 0.5× Hoagland's, every 3 days to prevent the accumulation of excess NaCl in the vermiculite.

Detection of physiological and biochemical parameters and phenotypic changes related to salt tolerance

Plant height, fresh weight, BADH activity, relative electrical conductivity, malondialdehyde (MDA) content, chlorophyll content, phenotypic changes, and physiological parameters were measured for the transgenic and WT seedlings that had been stressed for 3 days. Each measurement was repeated three times. Data were analyzed using the statistical analysis system (SAS).

Plants heights from the first basal leaf to the next leaf immediately above were measured. Seedling fresh weight was scored immediately after removal from vermiculite. Fresh leaves of the transgenic and WT seedlings that had been stressed for 3 days were collected for biochemical parameter assays.

BADH enzyme activity was measured according to previously published procedures (Liu et al. 1997). Relative electrical conductivity was detected using a conductivity meter (DDS-11A) by comparing the electrical conductivity of exudates from the transgenic plant leaves (L1) to those from control leaves after boiling leaf samples (L2) (Li 2000).

Glycinebetaine was extracted according to a published method (Moghaieb et al. 2004). Malondialdehyde (MDA) and chlorophyll contents were measured according to the methods of Bates et al. (1973) and Arnon et al. (1974), respectively.

Maize field trials

Seeds of T4 transgenic lines and their receptor lines were planted in three-row plots in a simulated saline-alkaline field containing soil taken from natural saline-alkaline land in Heilongjiang Province, China. The principle composition of the soil is described in Table 1. The trial plots were arranged in a random complete block design with three replications. Each plot was 2.0 m in length, and 0.60 m wide, with an interval of 0.20 m between plants. The plants were thinned at the five-leaf stage, resulting in 10 plants per plot. Plant and ear heights were measured after fluorescence. Mature ears were harvested, dried, and threshed to determine hundred-grain weight, the number of rows per ear, and the number of grains per row from ten randomly selected ears for each plot.

Results

Molecular characterization of transgenic maize plants

Transgenic maize plants overexpressing the *BADH* gene from *Atriplex micrantha*, a plant that is extremely resistant to salt, were successfully generated using *Agrobacterium*-mediated transformation of binary vector pCAMBIA3300-*Ubi-BADH* (Fig. 1) into the inbred lines Qi319 and Zheng58. More than 30 T0 independent transgenic plant lines were established and propagated in the greenhouse. A 661-bp PCR product amplified from these lines with *BADH* gene-specific primers is shown in Fig. 2. Four transgenic maize lines (BZ-136, BQ-2, BQ-12, and BQ-20) with single *BADH* integration events were verified *via* Southern blot analysis by hybridising *Bam*HI-digested genomic DNA samples with a *BADH* probe. The probe did not produce a signal in wild-type plants. Two copies of *BADH* were detected in the line BQ-16 (Fig. 3). The lines BQ-2, BQ-12, BQ-16, and BQ-20 are transformants of Qi319, and line BZ-136 originated from transformed Zheng58.

The expression of the *BADH* gene in T4 plants leaves was monitored by reverse transcriptase-polymerase chain reaction (RT-PCR), and the results are shown in Fig. 4. The RT-PCR did not produce a product in wild-type plant samples.

BADH activities and GB contents of transgenic plants

BADH enzyme activity measured in the transgenic lines was 5.7-fold higher than that in the WT plants after 3 days of 300 mM NaCl treatment. BADH activities in the transgenic lines were between 10.92 and 12.68 U, and varied slightly among the different transgenic plant lines. Line BQ-2 showed the highest BADH activity (Table 2).

The lines WT1 and WT2 accumulated 1.02 and 1.25 mg GB per g fresh weight, whereas the *BADH*-transgenic maize lines accumulated 15- to 20-fold more GB in their leaves than did wild-type plants under the 300 mM NaCl treatment. Analyses of variance indicated significant differences between transgenic and wild-type plants in terms of GB accumulation (Table 2).

Table 1 Composition of saline-alkali soil planted the transgenic lines

pH value	SAR value	Electrical conductivity (mS/cm)	Available nitrogen (mg/kg)	Available phosphorus (mg/kg)	Available potassium (mg/kg)	Organic content (g/kg)
8.88 ± 0.35	18.74 ± 0.24	1.59 ± 0.08	155.6 ± 3.24	113.6 ± 2.56	385.1 ± 3.87	30.1 ± 1.68

SAR value means sodium adsorption ratio, a measure of the sodicity of soil, as determined from analysis of water extracted from the soil. The formula for calculating sodium adsorption ratio is: $S.A.R = \frac{Na^+}{\sqrt{\frac{1}{2}(Ca^{2+}+Mg^{2+})}}$
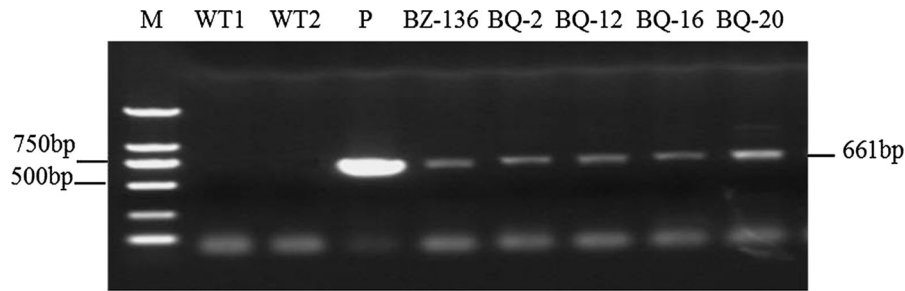
Fig. 2 PCR analysis of transformed plants using *BADH* primers. *M*, DNA marker DL2000 (TaKaRa); *WT1*, wild-type plants of Zheng58; *WT2*, wild-type plants of Qi319; *P*, pCAMBIA1301-BADH plasmid; *BZ-136* derived from Zheng58; *BQ-2*, *BQ-12*, *BQ-16*, and *BQ-20* are *transgenic lines* derived from Qi319

Fig. 3 Southern blot analysis of transgenic plants and wild-type plants. Total plant DNA was digested with *Bam*HI. *M*, DNA marker DL15000 (TaKaRa); *WT1*, wild-type plants of Zheng58; *WT2*, wild-type plants of Qi319; *P*, pCAMBIA1301-BADH plasmid; *BZ-136*, *transgenic line* derived from Zheng58; *BQ-2*, *BQ-12*, *BQ-16*, and *BQ-20*, *transgenic lines* derived from Qi319 transformed with *BADH*
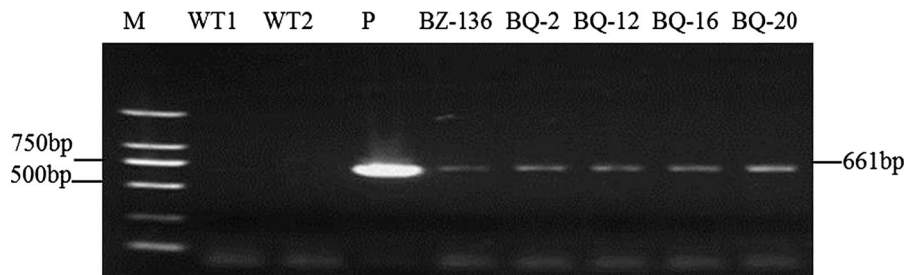

Fig. 4 RT-PCR analysis of transformed samples using *BADH* primers. *M*, DNA marker DL2000 (TaKaRa); *WT1*, wild-type plants of Zheng58; *WT2*, wild-type plants of Qi319; *P*, pCAMBIA1301-BADH plasmid; *BZ-136*, *transgenic line* derived from Zheng58; *BQ-2*, *BQ-12*, *BQ-16*, and *BQ-20*, *transgenic lines* derived from Qi319 transformed with *BADH*
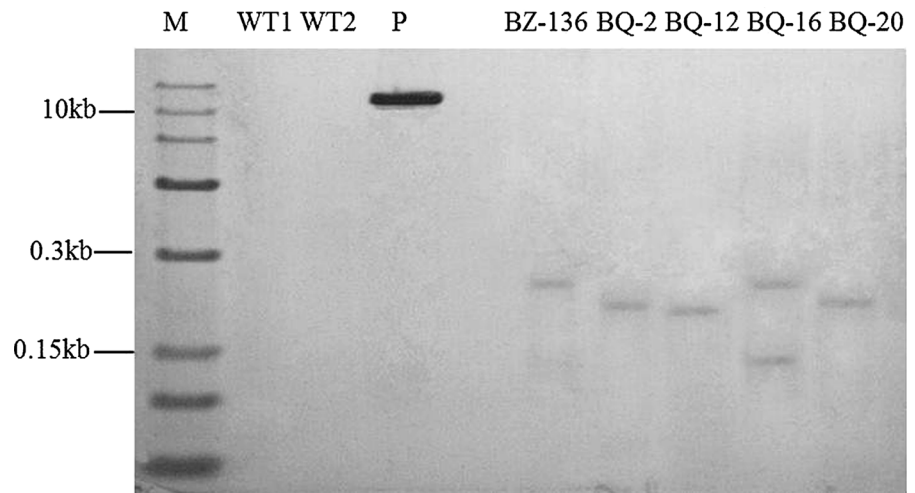
Over-expressing the *BADH* gene enhanced the tolerance of maize seedlings to salt stress

Seedlings that had reached the three-leaf stage were treated with a control $0.5\times$ Hoagland's solution or 300 mM NaCl solution for 7 days, then growth of the transgenic and wild-type lines were compared under control and salt-stressed conditions. Growth of the transgenic maize plants was better than that of the WT plants under NaCl stress. The transgenic plants were 4.2–5.3 cm taller and 9.6–15.8 % heavier in terms of fresh weight per plant compared with WT plants

Table 2 BADH activity, relative electrical conductivity, height, and fresh weight of maize WT and *BADH*-transgenic progeny plants under 300 mM NaCl stress

Lines	GB (mg g^{-1} FW)	BADH activity (U mg^{-1})	Relative electrical conductivity (REC)	Height (cm)	Fresh weight per plant (g)
WT1	1.02 ± 0.75b	2.12 ± 0.22b	0.26 ± 0.02a	22.27 ± 0.81b	3.32 ± 0.52b
BZ-136	20.05 ± 1.21a	12.30 ± 0.18a	0.15 ± 0.03b	27.58 ± 2.46a	4.28 ± 0.41a
WT2	1.25 ± 0.54b	2.08 ± 0.28b	0.28 ± 0.04a	22.20 ± 1.35b	3.55 ± 0.37b
BQ-2	20.42 ± 1.30a	12.68 ± 0.42a	0.14 ± 0.03b	27.47 ± 1.36a	4.32 ± 0.65a
BQ-12	18.43 ± 0.96a	10.92 ± 0.36a	0.17 ± 0.03b	26.40 ± 1.21a	4.08 ± 0.32a
BQ-16	19.85 ± 1.35a	11.37 ± 0.26a	0.15 ± 0.04b	26.90 ± 0.44a	4.26 ± 0.55a
BQ-20	19.65 ± 1.42a	11.06 ± 0.42a	0.16 ± 0.03b	26.57 ± 1.19b	4.12 ± 0.40a

WT1 non-transformed wild-type maize line Zheng58, *WT2* non-transformed wild-type maize line Qi319, values with the same letter are not significantly different at $P \leq 0.05$, by LSD test

(Table 2; Fig. 5). The seedlings of wild-type lines turned almost entirely yellow after 7 days of salt stress treatment. All transgenic plants were still growing well after the 3rd and 4th days of the NaCl stress treatment; however, the leaves had become paler by the 7th day of salt stress and the lower leaves had turned yellow. The line BZ-136 performed best among all of the transgenic lines (Fig. 5).

During the salt-stress treatment, the membrane integrity of the plants was also measured (Table 2; Fig. 6). MDA contents and the relative electrical conductivities (REC) in all maize plants analysed increased due to salt stress. However, the MDA contents and the REC values of the transgenic plants were significantly lower than those of WT under the 300 mM NaCl treatment, which indicated that the membranes of the transgenic plants suffered less salt-induced damage than those of the WT.

Salt stress often results in degradation of photosynthetic pigments, resulting in reduced of photosynthetic capacity. Measurements indicated that the chlorophyll contents of all lines decreased under salt stress. All of the transgenic plants had higher chlorophyll content than the wild type (Fig. 6).

All of the above results indicate that the expression of the *BADH* gene in maize seedlings enhanced the salt tolerance of these transgenic plants, and the lines BQ-2 and BZ-136 appeared most tolerant, which was consistent with results of the BADH enzyme activity assays.

Expression of *BADH* increased grain yield

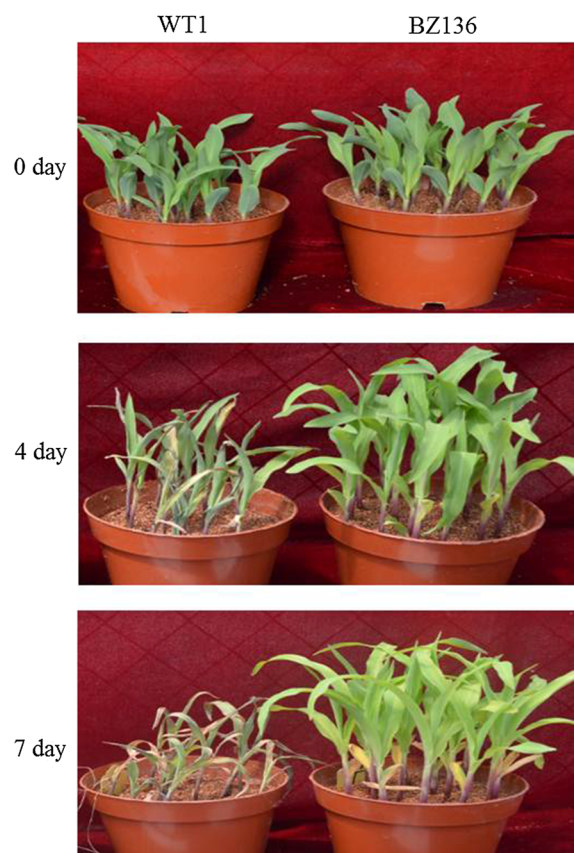The transgenic maize lines were planted in natural soda saline-alkaline soil in order to investigate the

Fig. 5 Enhanced salt tolerance of *transgenic line* compared to wild-type seedlings under 300 mM NaCl. *WT1*, wild-type plants of line Zheng58; *BZ-136*, Zheng58 plants transformed with BADH. Inbred line Zheng 58 was derived from recycled line hybrid 3268, and was classified as belonging to the Reid group

saline-alkali tolerance. The result showed that the characteristics of rows per ear and grains per row were not significantly different from the WT lines. The

Fig. 6 Effects of NaCl stress on REC, MDA, and chlorophyll content of wild-type and the transgenic maize plants. Wild-type (Zheng58 or Qi310) or transgenic maize plants were treated with 300 mM NaCl or 0.5× Hoagland's (0 mM NaCl) for 4 days, then REC, MDA, and chlorophyll contents were measured. **a** REC content **b** MDA content, and **c** Chlorophyll content. *WT1*, wild-type plants of Zheng58; *WT2*, wild-type plants of Qi319; *BZ-136*, Zheng58 plant transformed with BADH; *BQ-2*, *BQ-12*, *BQ-16*, and *BQ-20*, *transgenic lines* from Qi319 plants transformed with BADH. Values shown are mean ± SE of three replicates per plant. *Indicates significantly different at $P \leq 0.05$ according to the LSD test
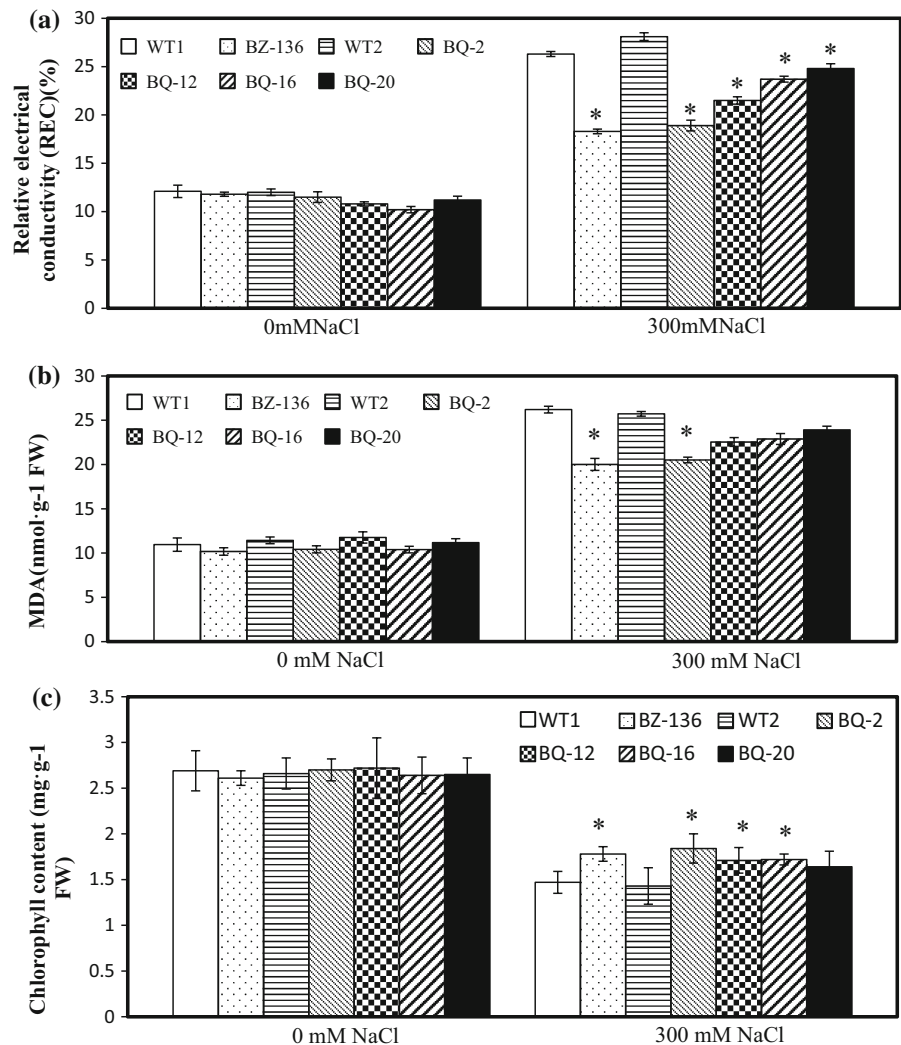
plant height and ear height of transgenic lines was significantly higher than WT lines except for BQ-16 and BQ-20. The hundred-grain weight of lines BQ-2 and BZ-136 was 34.7 and 25.7 % higher than that of the WT plants, respectively. These results indicated that expression of *BADH* increased the tolerance of maize plants to salt stress (Table 3).

Discussion

GB has been detected as an osmoprotectant in many organisms and has been a target of stress-resistance engineering for many years. Heterologous expression of a bacterial choline dehydrogenase (*betA*) or choline oxidase (*codA*), or a plant-derived *BADH* gene, results in increased accumulation of endogenous GB that can enhance stress tolerance (Sakamoto and Murata 2000; Chen and Murata 2008). Interestingly, GB can confer increased tolerance to cold, freezing, heat, drought, and salt stress in *BADH* transgenic plants even at low levels (Sakamoto and Murata 2002; Chen and Murata 2008). In another study, genetic evidence showed that a GB-deficient mutation reduced the salt tolerance of maize (Saneoka et al. 1995).

GB has proven very effective for enhancing salt-stress tolerance and increasing the productivity of various crop species including tomato (Zhou et al.

Table 3 Effect of saline-alkaline stress on the agronomic traits of transgenic maize plants

Lines	Plant height (cm)	Ear height (cm)	Rows per ear	Grains per row	100-grains weight (g)
WT1	161.00 ± 3.73b	43.67 ± 3.79b	12a	24.67 ± 1.53a	27.90 ± 1.31b
BZ-136	171.33 ± 1.33a	52.67 ± 2.52a	12a	22.17 ± 0.58a	35.08 ± 1.28a
WT2	184.67 ± 7.51b	70.67 ± 6.03b	12a	22.00 ± 1.00a	17.53 ± 1.40b
BQ-2	219.67 ± 6.11a	88.67 ± 4.04a	12a	26.67 ± 1.53a	23.62 ± 0.51a
BQ-12	215.00 ± 5.00a	87.33 ± 4.04a	12a	25.67 ± 1.53a	20.31 ± 0.97b
BQ-16	199.00 ± 7.55b	80.33 ± 1.53b	12a	25.00 ± 2.65a	20.34 ± 0.68b
BQ-20	217.00 ± 5.00a	81.67 ± 2.65b	12a	25.67 ± 1.53a	22.71 ± 1.50b

WT1 non-transformed wild-type maize line Zheng58, *WT2* non-transformed wild-type maize line Qi319, values with the same letter are not significantly different at $P \leq 0.05$, by LSD test

2007), sweet potato (Fan et al. 2012), wheat (He et al. 2010), cotton (Zhang et al. 2009), rice (Mohanty et al. 2002), and potato (Zhang et al. 2011).

In the present study, injured shoot tips from sterile germinated maize seeds were used for *Agrobacterium*-mediated transformation to reduce the likelihood of chimeric transformants. This method has previously been used to generate many transgenic maize lines (Li et al. 2008; He et al. 2013). The effectiveness of this transformation method is demonstrated by the molecular characterization of the transgenic plants recovered in this study (Figs. 2, 3, and 4).

The *BADH*-transgenic maize plants synthesised and accumulated more GB than did the WT under salt-stressed conditions, as shown in Table 1, and had 5.7-fold higher BADH activity than did WT under salt stress. Our results indicated that the *BADH* transgene likely enhanced the tolerance of transgenic maize plants to salinity stress through membrane stabilization, as the transgenic plants showed lower relative electrical conductivities and MDA content than did the WT under salt stress (Fig. 6a, b). The *BADH* transgene also enhanced plant height and fresh weight of transgenic seedlings, and increased grain production parameters of transgenic plants under salt-stressed conditions compared to the WT plants (Tables 1, 3).

In other experiments, expressing the *BADH* gene together with other stress-protective genes in transgenic plants has shown additive enhanced tolerance against salt and drought stress (Duan et al. 2009; Zhou et al. 2008; Ahmad et al. 2010; Wei et al. 2011). Therefore, increasing GB levels *via* the technology applied in the present study should be very useful for engineering crop plants with enhanced tolerance to multiple environmental stresses.

In conclusion, these results suggest that the *BADH* gene can be used to improve the salinity tolerance of maize through genetic engineering. Further research will investigate the effects of introducing the *BADH* gene into maize on yield and other agronomic characteristics.

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