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Transformation of tomato with the *BADH* gene from *Atriplex* improves salt tolerance

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Abstract Glycinebetaine is an important quaternary ammonium compound that is produced in response to salt and other osmotic stresses in many organisms. Its synthesis requires the catalysis of betaine aldehyde dehydrogenase encoded by *BADH* gene that converts betaine aldehyde into glycinebetaine in some halotolerant plants. We transformed the *BADH* gene, cloned from *Atriplex hortensis* and controlled by two 35S promoters of the cauliflower mosaic virus, into a salt-sensitive tomato cultivar, Bailichun, using *Agrobacterium tumefaciens* strain LBA4404 carrying a binary vector pBin438, and using a leaf regeneration system. Polymerase chain reaction and Southern hybridization analyses demonstrated that the *BADH* gene had integrated into the genome of tomato. Transgenic tomato plants showed significantly higher levels of mRNA and BADH enzyme activity than wild-type plants. Observations on rooting development and relative electronic conductivity suggested that the transgenic plants exhibited tolerance to salt stress, with these plants growing normally at salt concentrations up to 120 m*M*.

Keywords Tomato · Glycinebetaine · *Agrobacterium tumefaciens* · Betaine-aldehyde dehydrogenase · Salt tolerance

Abbreviations *BA*: Benzylaminopurine · *IAA*: Indole-3-acetic acid · *REc*: Relative electronic conductivity · *ZT*: Zeatin

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Introduction

Plants are often exposed to various adverse environmental stresses such as drought, salinity, and high and low temperatures. Salinity is one of the major factors that limit the geographical distribution of plants and is responsible for significant reductions in the yield and quality of many important crops (Boyer 1982). Plants utilize a number of protection mechanisms to maintain normal cellular metabolism and prevent damage to cellular components (Wood et al. 1996). One common metabolic adaptation to salinity stress is the accumulation of osmoprotectants. One of these osmoprotectants, glycinebetaine, is a bipolar quaternary ammonium compound accumulated in many plant species (Rhodes and Hanson 1993). Glycinebetaine protects the cell from salt stress by maintaining an osmotic balance with the environment (Robinson and Jones 1986) and by stabilizing the quaternary structure of complex proteins (Bernard et al. 1988; Papageorgiou and Murata 1995). In photosynthetic systems, glycinebetaine stabilizes the oxygen-evolving photosystem II complex (Murata et al. 1992) and Rubisco at elevated salt concentrations.

In plants, glycinebetaine is synthesized by the two-step oxidation of choline in the chloroplast (Hanson et al. 1985). The first step is catalyzed by choline monooxygenase (CMO) (Brouquisse et al. 1989); the second by betaine aldehyde dehydrogenase (BADH) localized in the chloroplasts (Weigel et al. 1986). The *BADH* gene has been cloned from *Spinacia oleracea* (Weretilnyk and Hanson 1990), *Atriplex hortensis* (Xiao et al. 1995), *Beta vulgaris* (McCue and Hanson 1992), *Sorghum bicolor* (Wood et al. 1996), and *Avicennia marina* (Hibino et al. 2001) and is well characterized. However, many test results have demonstrated that some plant species, such as *Arabidopsis thaliana*, tobacco, and tomato, do not accumulate glycinebetaine (Weretilnyk et al. 1989; Rhodes and Hanson 1993; Nuccio et al. 1998). This has led to great interest in the metabolic engineering of the glycinebetaine biosynthesis pathway as an approach for enhancing salt stress resistance in salt-sensitive species (LeRudulier et al. 1984; McCue and Hanson 1990).

Nomura et al. (1995) postulated that the introduction of exogenous genes related to the synthesis of glycinebetaine into salt-sensitive crops would lead to the accumulation of glycinebetaine and an improvement in their tolerance to salt stress. To date, several genes catalyzing the synthesis of glycinebetaine, such as *CMO* from spinach, *CodA* from *Arthrobacter globiformis*, *BADH* from spinach or sugar beet, and *BetA* and *BetB* from *Escherichia coli*, have been introduced into different plants (Rathinasabapathi et al. 1994; Hayashi et al. 1997; Holmstrom 1998; Nuccio et al. 1998; Sakamoto et al. 1998). The transgenic plants produced minimal glycinebetaine but, in some cases, showed small but significant increases in tolerance to salt or other stresses (Nuccio et al. 1999). In a previous investigation, we introduced the *BADH* gene from *Atriplex hortensis* into watercress (Li et al. 2000). The transgenic watercress plants grew normally on medium containing 100 m*M* NaCl and survived on medium containing 160 m*M* NaCl.

Tomato is a vegetable crop grown world wide. Most of its cultivars are moderately sensitive to salt, with yield being seriously limited by the salinity of the soil or irrigating water (Cuartero and Munoz 1999; Foolad 1999). Extensive work has been done on breeding for enhanced tolerance to salinity and identifying quantitative trait loci-associated markers to enhanced tolerance and yield under stress. However, there have been very few reports published on the metabolic engineering of salt tolerance of tomato. As a non-accumulator of glycinebetaine, tomato has no glycinebetaine synthesis pathway (Weretilnyk et al. 1989). In the investigation reported here, we established a regeneration system from leaves of Bailichun, a salt-sensitive tomato cultivar, most of whose plants can not withstand the stress of 90 m*M* salt, and introduced the *BADH* cDNA cloned from *Atriplex hortensis* (Xiao et al. 1995) to allow the biosynthesis of glycinebetaine.

Materials and methods

Plant materials

Seeds of tomato (*Lycopersicon esculentum* Mill cv. Bailichun) obtained from the Chinese Academy of Agricultural Science (CAS) were surface-sterilized for 1.5 min in 70% ethanol, then 15 min in 0.1% $HgCl_2$, followed by three washes in sterile distilled water. They were then germinated on a hormone-free MS (Murashige and Skoog 1962) basic medium with 2.0% sucrose, 0.7% agar, pH 5.8, in 200-ml plastic culture boxes (Jiana Corporation, Shanghai, China) at 25°C and under a 12/12-h (light/dark) photoperiod (light intensity: 150 μmol m^{-2} s^{-1}).

Callus initiation and shoot regeneration

Discs of cv. Bailichun leaves were dissected from 20- to 30-day-old sterile seedlings and cultured on the induction medium (IM) (MS + 0.2 mg/l IAA + 2.0 mg/l BA + 0.1 mg/l ZT) for callus induction and shoot regeneration. They were transferred to fresh IM medium every 20 days until shoot regeneration occurred. Shoots (2–3 cm long) were excised and transferred to root induction medium (RM) (MS + 0.5 mg/l IAA) for rooting. Culture conditions for tissue culture were the same as for germination.

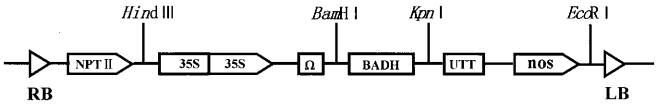


Fig. 1 Construction of the binary vector plasmid vector pBin438 that carries the *BADH* gene. *RB* Right border, *LB* left border, *NPTII* neomycin phosphotransferase, *35S* cauliflower mosaic virus 35S promoter, Ω TMV translation enhancer, *UTT* termination sequence of transcription, *nos* nopaline synthase terminator

Agrobacterium-mediated transformation and selection of transgenic shoots

Agrobacterium tumefaciens strain LBA4404 carrying pBin438 (size: 13 kb) (Tian et al. 1991) with the *BADH* structural gene from *Atriplex hortensis* and selection marker *NPTII* (Fig. 1) was provided generously by Prof. Chen-Shouyi (Institute of Genetics, Chinese Academy of Sciences, CAS).

A. tumefaciens in YEB medium were cultured overnight at 28°C, with shaking at 220 rpm, until the OD_{560} reached 0.5. Acetosyringone was added with continued shaking for 2–3 h. Before transformation, the bacterial suspension was diluted to 1/10 with liquid hormone-free MS medium. Leaf discs were immersed in the bacterial suspension for 5 min immediately after excision and then blotted with sterile filter paper. The inoculated leaf discs were co-cultured on IM medium in darkness for 48 h followed by transfer to selective induction medium (SIM) (IM + 500 mg/l carbenicillin + 50 mg/l kanamycin) for shoot differentiation. Differentiated shoots were transferred to selective rooting medium (SRM) (RM + 200 mg/l carbenicillin + 50 mg/l kanamycin).

DNA extraction and polymerase chain reaction

Nuclear DNA of the wild-type and transgenic plants was prepared by the simplified CTAB method (Murray and Thompson 1980). The PCR procedure was performed as followed. DNA (0.5 μl) was added to a final volume of 25 μl with 0.2 μ*M* of each primer, 0.2 m*M* each of dNTP, and 1 U *Taq* DNA polymerase. The reaction consisted of 35 cycles of 1.0 min at 95°C for denaturation, 1.0 min at 45°C for annealing, and 1.5 min at 72°C for extension. The two primers for *Atriplex hortensis BADH* gene were: 5′-AGAATGGCGTTCCCAATTCCTGCTC-3′ and 5′-TTCAAGGAGACTTGTACCATCCCCA-3′ (Xiao et al. 1995).

Southern and Northern hybridization

The probe used for Southern and Northern hybridizations was the 1.5 kb *Bam*HI-*Kpn*I *BADH* cDNA fragment from the cloning vector plasmid (Fig. 1) labeled with α-[^{32}P]-dCTP via the Random Primer DNA labeling system (Takara Biotechnology. China). Genomic DNA (20 μg) isolated from leaves was digested with restriction endonucleases *Hin*dIII and *Eco*RI, respectively, separated on a 1.0% (w/v) agarose gel by electrophoresis, and transferred onto a Hybond-N^+ nylon membrane (Amersham Pharmacia Biotech, Buckinghamshire, UK). Hybridization was carried out as following standard procedures (Sambrook et al. 1989).

Total RNAs were extracted from young leaves by the Trizol (GibcoBRL, Gaithersburg, Md.) one-step method (following manufacturer's instructions), denatured with formaldehyde and formamide, and subjected to electrophoresis on 1.2% (w/v) agarose gels. The separated RNAs were transferred to a Hybond-N^+ nylon membrane and hybridized with the same probe and following the same procedure as that used in the Southern hybridization.

BADH activity and the REc assay of transgenic plants

Transgenic lines were propagated and rooted on SIM and SRM medium, then transplanted into a mixture of soil and vermiculite (1:1) in pots in the greenhouse, and watered with MS nutritional solution. NaCl at 0 m*M* and 180 m*M* was added into the nutritional solution to stress the plants for 24 h until they grew to the four- to six-leaf stage. The third leaves from the top down were used for the BADH activity and REc assays. BADH activity was measured according to Guo et al. (1997), with one unit of BADH activity defined as consuming 1 nmol/l NAD per minute in the reaction volume (nmol min^{-1} mg^{-1} protein) (Guo et al. 1997). Each measurement was repeated three times.

REc was tested using a HI9033 conductivity meter (HANNA, Italy) according to Leopold and Toenniessen (1984). The wild-type and transgenic lines were stressed with 0 m*M*, 90 m*M*, 180 m*M*, and 270 m*M* NaCl for 24 h.

Results and discussion

Regeneration and selection of transgenic shoots

In earlier investigations, the explants used for tomato regeneration and transformation were usually cotyledons and hypocotyls or surface-sterilized leaf discs (Roekel et al. 1993; Costa et al. 2000). We established a regeneration system for cv. Bailichun using leaf discs from surface-sterilized seedlings. To determine the intrinsic kanamycin resistance, we placed untransformed leaf discs on SIM media; all of these turned yellow within 2 weeks and ultimately died (Fig. 2A). Among the 333 inoculated leaf discs, 77 began to produce callus and shoot primordia from the excision cuts 18 days post-inoculation on SIM medium (Fig. 2B), which then differentiated into green shoots or leaf-like bodies after 40 days (Fig. 2C). When the shoots were 2–3 cm long, they were excised and transplanted to SRM medium for rooting. All of these potentially transgenic shoots survived and rooted normally after 5–8 days, and their roots had reached a length of 2.0 cm or longer 8–10 days later. There were no apparent phenotypic differences between the wild-type and transgenic plants.

Integration and expression of the *BADH* gene in transgenic tomato

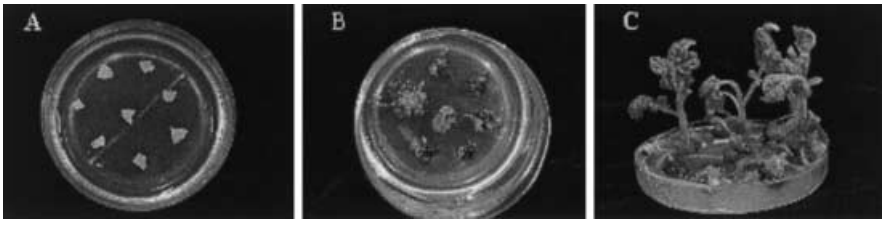
To eliminate the potential for false positives arising from persistent *Agrobacterium*, we surface-sterilized explants of these potentially transgenic shoots and put them on carbenicillin-free SIM medium. No *Agrobacterium* appeared on this medium after 10 days or longer. PCR analysis detected a 1.5-kb band in 6 of the 11 shoots obtained from the SRM medium, while none of the wild-type seedlings showed this band (Fig. 3A). Subsequent Southern analysis (Fig. 3B) with the α-[^{32}P]-dCTP-labeled probe showed excellent consistency with PCR results. The results from these two separate analyses demonstrated that the *BADH* gene had been integrated into the genome of these six transgenic lines.

Levels of BADH transcripts were found to increase almost twofold in leaves of salt-stressed spinach plants and almost three- and fourfold in the taproots and leaves of sugar beet, respectively (Weretilnyk and Hanson 1990; McCue and Hanson 1992). The accumulation of *BADH* mRNA levels from barley plants exposed to salt stress increased up to eightfold in the leaves, and these levels decreased when the stress was attenuated (Ishitani et al. 1995). Moreover, the increases in *BADH* mRNA levels also occurred when the barley plants were subjected to drought and water stresses, a result consistent with previous findings (Arakawa et al. 1992a, b). The results of these studies indicate that betaine accumulation in salt-stressed plants is regulated (at least in part) via changes in the expression of the genes of the betaine biosynthetic pathway and that the BADH gene probably has stress responsive *cis* regulatory elements (Rathinasabapathi et al. 1997). These may be essential to reproduce the natural pattern of stress-induced glycinebetaine accumulation in engineered crops (Rathinasabapathi et al. 1997). In our investigation, the *BADH* gene was introduced with the CaMV 35S promoter that can promote the expression of genes constitutively and a non-salt inducible Ω enhancer. Transcripts of the *BADH* gene were detectable no matter whether salt stress was present or not, but the expression levels were different among different transgenic lines (Fig. 3C). This phenomenon has been generally ascribed to different integration sites of the transgenes into the plant genome in each independent transformant (position effect) (Guo et al. 1997; Liu et al. 1997; Li et al. 2000; van Leeuwen et al. 2001). We did not find any transcript signal in line 2 and supposed that transgene silencing may have occurred (Chandler and Vaucheret 2001).

BADH activity and salt tolerance of the transgenic plants

BADH activity was not detectable in wild-type plants under either the NaCl-free condition or under NaCl

Fig. 2A–C Selection of *BADH* transformed tomato callus and shoots. **A** Untransformed leaf discs that have died on SIM medium, **B** antibiotic-resistant callus initiated on SIM medium, **C** antibiotic-resistant shoots on SIM medium



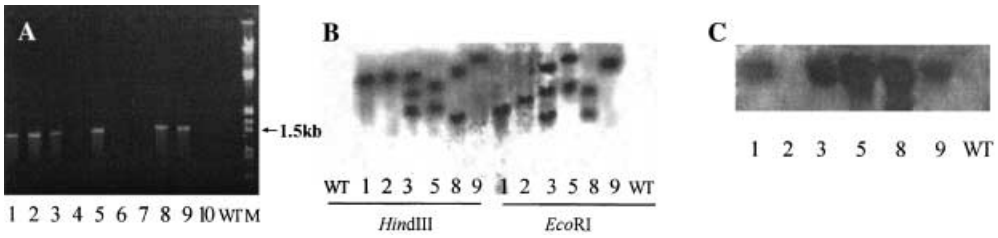


Fig. 3A–C Molecular assay of transformed tomato shoots. **A** PCR analysis. *WT* Wild-type plants, *M* λ-DNA/*Eco*RI+*Hin*dIII marker, *lanes 1–10* ten independent transformant lines. **B** Southern hybridization analysis with the α-[^{32}P]-labeled *BADH* cDNA probe. Nuclear DNA was digested with *Hin*dIII or *Eco*RI restriction enzymes, respectively. **C** Northern hybridization analysis with α-[^{32}P]-labeled *BADH* cDNA probe

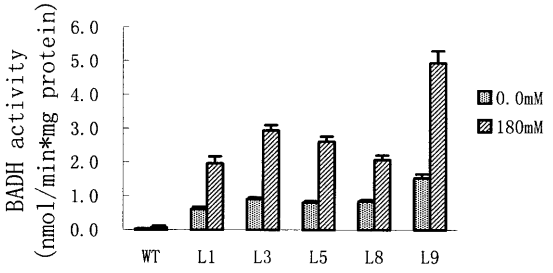


Fig. 4 BADH activity in cv. Bailichun wild-type (*WT*) and transgenic (*L1*, *L3*, *L5*, *L8*, *L9*) tomato lines under standard watering conditions and salt stress. The results are expressed as averages (± standard errors) from three independent experiments

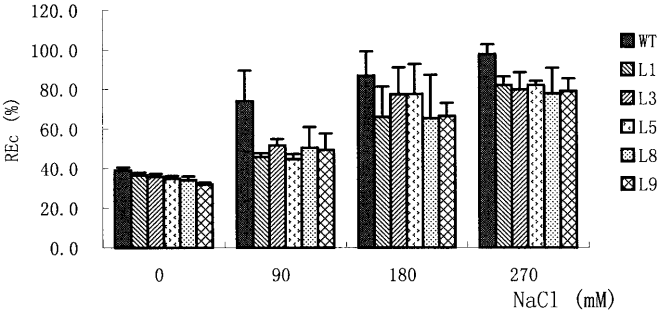


Fig. 5 Effects of NaCl on plasmalemma permeability of cv. Bailichun wild-type (*WT*) and transgenic (*L1*, *L3*, *L5*, *L8*, *L9*) tomato lines. The results are expressed as averages (± standard errors) from three independent experiments

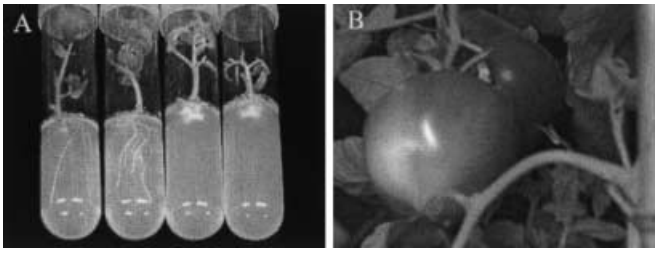


Fig. 6A, B Salt tolerance of transgenic lines. **A** Shoots of transgenic lines (*left two*) rooted normally on RM medium containing 90 m*M* NaCl, while the control (*right two*) could only form a swelling. **B** Bailichun transgenic plants produced fruit under 90 m*M* NaCl stress

(180 m*M*) stress conditions. As illustrated in Fig. 4, under normal conditions the transgenic plants tested exhibited varying levels of BADH activity, and when they were stressed by 180 m*M* NaCl this activity increased approximately 2.4- to 3.2-fold. Similar results were found in tobacco (Holmstrom et al. 1994) and *Synechococcus* (Nomura et al. 1995).

The status of the cell membrane is related to the function of the whole cell and whole plants. Membrane permeability has been thought to be an important index of the physiological function of the cell. Adversities such as drought, salinity, and high and low temperatures initially damage the structure of the cell membrane, thereby affecting its function; this leads to an increase in membrane permeability, which results in leakage of the intracellular contents. By minimizing other factors affecting the growth of plants, it is possible to assess the degree of damage of the membrane by measuring the REc or leakage rate of macromolecules under a certain kind of stress (Leopold and Toenniessen 1984; Guo et al. 1997; Liu et al. 1997). In our investigation, small differences in REc were detected among plants of the wild-type and different transgenic lines when watered with MS nutritional solution without NaCl supplementation (Fig. 5); the REc increased in both wild-type and transgenic plants when NaCl was added to the MS nutritional watering solution. The REc values of all of the transgenic lines under 90 m*M* and 270 m*M* NaCl stress were significantly lower than that of the wild type (Fig. 5). These results suggest that the integration of *BADH* cDNA resulted in glycinebetaine biosynthesis in cv. Bailichun. The resultant glycinebetaine and its protective effects on the photosystem and enzymes could enhance the stability of the proteins and membranes of plant cells in adverse environments (Csonka 1989; Yi et al. 1999).

Figure 6A shows the effects of salt stress on the growth of shoots in MS medium. The wild-type and transgenic shoots were placed on RM medium containing 90 m*M* NaCl. All of the transgenic shoots rooted normally 8–10 days later, while the wild type ceased to grow, turned yellow after about 1 week, and ultimately died. Rooted transgenic plants were transplanted to pots, grew well while watered with a MS nutritional solution supplemented with 90 m*M* NaCl, and produced fruit normally 2 months later (Fig. 6B). Most of the transgenic

plants maintained a normal growth when the concentration of NaCl supplemented to the nutritional solution was increased to 120 m*M* during the vegetative stage.

Most reports available on the metabolic engineering of salt tolerance improvement are focused on tobacco and crops of the grass family, and there are still very few reports on vegetables. Introduction of the glycinebetaine biosynthesis pathway by introducing the *BADH* gene cloned from different plants (Rathinasabapathi et al. 1994; Guo et al. 1997; Liu et al. 1997; Trossat et al. 1997; Li et al. 2000) or the *CodA*, *BetA*, or *B* genes that encode a bifunctional enzyme in bacterium (Lamark et al. 1991; Hayashi et al. 1997; Sakamoto et al. 1998) has increased the salt tolerance of the resultant transgenic plants. One problem with glycinebetaine engineering is that the inadequacy of the endogenous choline supply is usually a limiting factor in the accumulation of glycinebetaine in tobacco, *Arabidopsis thaliana*, and *Brassica napus* (Nuccio et al. 1998; Huang et al. 2000). This calls for an up-regulated de novo synthesis of choline (Nuccio et al. 1998) and metabolic engineering of the cholinebetaine network using a systematic approach (Huang et al. 2000).

Improving salt tolerance of plants by metabolic engineering is still a challenge. To date, commercialized salt-tolerant transgenic vegetable crops with a high genetic stability have not been reported because of gene silencing and transgene loss at a high frequency in progenies of transgenic plants. We are continuing to study the genetic stability of *BADH* transgenic tomato and attempting multi-gene transformation to improve the salt tolerance of tomato.

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