

Improved salt-tolerance of transgenic soybean by stable over-expression of AhBADH gene from *Atriplex hortensis*

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Research Article

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Abstract

An effective strategy for increasing the productivity of major crops under salt stress conditions is the development of transgenics that harbor genes responsible for salinity tolerance. Betaine aldehyde dehydrogenase (BADH) is a key enzyme involved in the biosynthesis of the osmoprotectant, glycine betaine (GB), and osmotic balance in plants, and several plants transformed with BADH have shown significant improvements in salt and drought tolerance. However, very few field-tested transgenic cultivars have been reported, as most of the transgenic studies are limited to laboratory or green house experiments. In this study, we demonstrated through field experiments that *BADH* from *Atriplex hortensis* (*AhBADH*) confers salt tolerance when transformed into soybean (*Glycine max* L.). *AhBADH* was successfully introduced into soybean by *Agrobacterium* mediated transformation. A total of 256 transgenic plants were obtained, out of which 47 lines showed significant enhancement of salt tolerance compared to non-transgenic control plants. Molecular analyses of the transgenic line TL7 with the highest salt tolerance exhibited stable inheritance and expression of *AhBADH* in progenies with a single copy insertion. TL7 exhibited stable enhanced salt tolerance and improved agronomic traits when subjected to 300mM NaCl treatment. Currently, the transgenic line TL7 with stable enhanced salt tolerance, which has been cleared for environmental release, is under biosafety assessment. TL7 stably expressing *AhBADH* could then be applied in commercial breeding experiments in order to genetically improve salt tolerance in soybean.

Key Message

The salt-tolerance of transgenic soybean cleared for environmental release was improved by stable over-expression of *AhBADH* gene from *Atriplex hortensis*, which was demonstrated through molecular analysis and field experiments.

Introduction

Soil salinity, which affects ~ 20% of cultivated land globally, is the most important abiotic stress severely affecting plant growth and crop production. Owing to climate change, salt-affected agricultural land is on the rise (Ahuja et al. 2010). Relatively high salt concentrations cause low osmotic potential of soil solutions and water deficit to the plants. Excess sodium results in ion homeostasis alteration that is inimical to plant (Zhu 2003; Munns and Tester 2008). Most plant species, except for certain halophytes, cannot thrive under extreme soil saline conditions, as they have limited resistance to salt stress. Exploiting salinity tolerance traits of species such as halophytes by introducing genes responsible for such traits into crop plants is known to be an effective strategy for limiting crop losses from naturally occurring salinity.

Soybean (*Glycine max*) is an important crop and is a major plant source of vegetable protein and oil for human and livestock. Soil salinity seriously affects soybean productivity, as soybean is sensitive to salt (Munns and Tester 2008). Under salt stress, when Cl^- and Na^+ ions accumulate at high concentrations in

soybean plants toxicity occurs; increasing salinity stress (70 mM NaCl) leads to a 40% reduction in soybean yield, whereas high salinity (300 mM NaCl) can result in plant death (Papiernik et al. 2005; Pathan et al. 2007; Phang et al. 2008). Therefore, the improving salt resistance of soybean has been a major objective of soybean breeding experiments. Genetic manipulation using genes responsible for salt tolerance is another approach of addressing the issue of salt stress in soybeans. However, genetic resource variation of soybean in salt tolerance is limited. Therefore, exploring new genetic resources is important to develop salt-tolerant transgenic soybean cultivars.

Plants have developed various tolerance mechanisms in response to salt stress. Among the various salt tolerance mechanisms, the accumulation of osmotic protectants, such as free proline and glycine betaine, is important for balancing the intracellular osmotic potential of plants (Deinlein et al. 2014; Hanin et al. 2016). The accumulation of glycine betaine (GB), a quaternary ammonium compound, can raise cellular osmotic pressure (McCue and Hanson 1992), protect membrane functions by stabilizing the complex protein structures (Rhodes and Hanson 1993), such as those involved in photosystem II (PSII) (Papageorgiou and Murata 1995; Chen and Murata 2011). Exogenous GB application has shown to increase stress tolerance in plants that were unable to synthesize GB (Yang and Lu 2005, 2006; Park et al. 2006; Chen and Murata 2008). In plants, betaine aldehyde dehydrogenase (BADH) is a key enzyme in a two-step oxidation for GB synthesis (Hanson et al. 1985; McNeil et al. 2000; Sakamoto and Murata 2000; Ashraf and Foolad 2007). Till date, *BADH* has been cloned from some plant species such as *Spinacia oleracea* (Weretilnyk and Hanson 1990; Shirasawa et al. 2006), *Beta vulgaris* (McCue and Hanson 1992; Yamada et al. 2015), *Atriplex sapp* (Joseph et al. 2013), *Ammopiptanthus nanus* (Yu et al. 2014), *Lycium ruthenicum* (Liu et al. 2018), *Suaeda liaotungensis* (Li et al. 2003; Zhang et al. 2008; Wang et al. 2013), *Atriplex triangularis* (He et al. 2004), *Atriplex micrantha* (Zhu et al. 2007), *Atriplex hortensis* (Xiao et al. 1995), *Atriplex canescens* (Qin et al. 2017), *Kalidium foliatum* (Zeng et al. 2007), *Arabidopsis* (Missihoun et al. 2011), *Ipomoea batatas* (Chen et al. 2014), mangrove (Hibino et al. 2001), *Zoysia tenuifolia* (Oishi and Ebina 2005), *Sorghum bicolor* (Wood et al. 1996), *Triticum aestivum* (Sun et al. 2019), *Hordeum vulgare* (Ishitani et al. 1995; Zhao et al. 2008; Fujiwara et al. 2008, El-Atroush et al. 2020), and *Oryza sativa* (Tang et al. 2014; Lapuz et al. 2019, Min et al. 2021). Overexpression of *BADH* has shown to significantly increase salt tolerance in transgenic *Arabidopsis* (Yu et al. 2017; Sun et al. 2019), tomato (Jia et al. 2002; Zhou et al. 2007; Goel et al. 2011; Wang et al. 2013; Zhang et al. 2019), potato (Zhang et al. 2011; Ali et al. 2020), tobacco (Nuccio et al. 1998; Holmström et al. 2000; Li et al. 2003, Yang et al. 2008; Zhou et al. 2008; Hasthanasombut et al. 2010), carrot (Kumar et al. 2004), ryegrass (Takahashi et al. 2010), wheat (Liang et al. 2009; He et al. 2010; Li et al. 2019), rice (Guo et al. 1997; Kishitani et al. 2000; Hasthanasombut et al. 2011), maize (Di et al. 2015), alfalfa (Liu et al. 2011; Yan et al. 2012), oilseed rape (Prasad et al. 2000; Chakraborty and Sairam 2017), trifoliate orange (Fu et al. 2011), chicory (Li et al. 2016) and walnuts (Bolagh et al. 2021). The transgenic lines maintained lower Na^+/K^+ ratios, which is characteristic of an osmolyte in maintaining ion homeostasis of leaf cells. The *in vivo* overaccumulation of GB could enhance or stabilize the activity of antioxidant enzymes, which can scavenge reactive oxygen species and mitigate oxidative damage of cell membranes (Liang et al. 2009). In previous studies, transgenic plants with *BADH* showed significant improvements in salt tolerance, but

most of them were limited to laboratory or green house experiments. To date, very few field-tested transgenic cultivars have been reported.

In this study, *AhBADH* was successfully introduced into soybean by *Agrobacterium* mediated transformation, and transgenic plants were developed. We reported a field-tested transgenic line of soybean with enhanced salt tolerance indicating stable expression of *AhBADH*. The stable integration, inheritance, and expression of the exogenous *AhBADH* in T4, T5 and T6 plants of transgenic line TL7 were characterized by molecular analyses, including PCR, Southern blot, RT-PCR, qPCR, western blotting, and ELISA. To evaluate the effect of *AhBADH* overexpression on the salt tolerance of the transgenic line, several salt-related physiological indicators were examined, including GB content and BADH enzyme activity; malondialdehyde (MDA) and proline content; superoxide dismutase (SOD), peroxidase (POD), and catalase (CAT) enzyme activities; Na⁺ and K⁺ contents; and total chlorophyll content and photosynthetic rate (Pn). Under salt stress conditions, the salt tolerance indices (STI) for the progenies of the transgenic line were tested in the laboratory, greenhouse, and field, and the agronomic traits of the transgenic line were evaluated. The transgenic line TL7 with stable enhanced salt tolerance, which has completed environmental release, is currently under biosafety assessment, and would be further subjected to pre-production field trials in Northeast China. The transgenic line TL7 stably expressing *AhBADH* could be applied with great application potential for use in soybean commercial breeding for salt-tolerance improvement by genetic engineering.

Materials And Methods

Agrobacterium mediated transformation of soybean

The cotyledonary nodes of the soybean cultivar Williams 82 were used as explants for the genetic transformation to generate transgenic soybean plants. *Agrobacterium tumefaciens* strain EHA105 harboring the binary vector pCAMBIA3300-35S-*AhBADH* was used for explant infection. The binary vector carried *AhBADH* (GenBank: X69770.1) from *Atriplex hortensis* and a selectable marker gene *BAR* under the control of the cauliflower mosaic virus (CaMV) 35S promoter (Fig. 1). *Agrobacterium*-mediated soybean transformation and recovery of transgenic plants were carried out following the method as previously described (Paz et al. 2006, Yang et al. 2017).

Molecular Identification Of Transgenic Plants

Integration of *AhBADH* gene in the genome of transgenic plants was analyzed by PCR and Southern blot combined with primary screening using LibertyLink® strips (EnviroLogix Inc., USA) and glufosinate selection (1,500 mg/L). For PCR analysis, total genomic DNA was isolated from fresh leaves of transgenic and non-transformed control plants using the Plant Genomic DNA Kit (Tiangen Biotech, Beijing, China) according to the manufacturer's protocol. *E. coli* plasmid DNA was isolated using the Qiagen Plasmid Mini Kit, following the manufacturer's instructions. PCR was performed to amplify a 937 bp fragment using the specific primers BF: 5'-GGCATCTGTGACTTGTCTAGAATTCGG-3' and BR: 5'-

TCAAGGAGACTTGTACCATCCCCATG-3'. For Southern blot analysis, total genomic DNA was extracted from fresh leaves of transgenic and non-transformed plants using a modified CTAB (cetyltrimethylammonium bromide) method, as reported previously (Telzur et al. 1999). The isolated genomic DNA (30µg) was completely digested with *HindIII* restriction enzyme, subjected to electrophoresis on 0.8% (w/v) agarose gel, and subsequently transferred to a positively charged nylon membrane (Amersham, Buckinghamshire, UK) by capillary transfer following the manufacturer's instructions. The expression vector and non-transformed control plants were used as the positive and negative controls, respectively. A DIG-labelled probe (the PCR product with 937 bp amplified from the plasmid as above) was produced by PCR labelling. Subsequent hybridization steps were carried out using the DIG High Prime DNA Labelling and Detection Starter Kit II (Roche, Germany). Hybridization was carried out at 42 °C for 16 h and staining was performed at room temperature using NBT/BCIP as the substrate (following the instructions of the DIG High Prime DNA Labelling and Detection Starter Kit I, NBT/BCIP chromogenic method).

Expression analysis of *AhBADH* gene in transgenic plants

Expression of *AhBADH* in transgenic plants was detected by RT-PCR, qRT-PCR, western blotting, and ELISA. For RT-PCR analysis, total RNA was isolated from leaves of the transgenic and non-transformed plants following the protocol of the Plant RNA Extract Kit (Invitrogen) and then treated with amplification grade DNase I (Invitrogen) to remove all DNA. RNA was reverse-transcribed to cDNA using a TransScript one-step gDNA Removal and cDNA Synthesis SuperMix Kit (TransGen, Beijing, China). The first-strand cDNA was then diluted two-fold to amplify a specific *AhBADH* fragment following the protocol mentioned above for genomic DNA amplification using the same primer pair. *GmACTIN* (GenBank: BW652479) was used as an internal control amplified 405 bp fragment with primers AF: 5'-TTGACTGAGCGTGGTTATTCC-3' and AR: 5'-GATCTTCATGCTGCTGGGTG-3'.

For qRT-PCR analysis, total RNA was extracted from the leaves and roots of transgenic and non-transformed plants using the EasyPure Plant RNA Kit (TransGen Biotech, China) and then treated with RNase-free DNase. First-strand cDNA was synthesized using the ThermoScript RT-PCR system (Invitrogen, USA), according to the manufacturer's instructions. qRT-PCR was performed on an ABI PRISM 7500 Fast Real-Time PCR System (Applied Biosystems). The specific primers used in qPCR for target *AhBADH* gene amplification were as follows: BQF: 5'-CACTCTGGTCTCATCGTGCTAAAT-3', BQR: 5'-CAGGGTGACTGGAGCCTTTTG-3'. Specific primers for the internal reference gene *GmActin* (GenBank: BW652479) were as follows: AQF, 5'-ATCTTGACTGAGCGTGGTTATTCC-3'; AQR, 5'-GCTGGTCCTGGCTGTCTCC-3'. Transcription levels were calculated using the relative quantification ($2^{-\Delta C_t}$) method, and the data were compared with those of the internal control.

For western blot analysis, total proteins were extracted from young leaves of transgenic plants and non-transformed control plants with extraction buffer (100 mM NaCl, 10 mM EDTA, 200 mM Tris-HCl, 0.05% Tween-20, 0.1% SDS, 14 mM β-mercaptoethanol, 400 mM sucrose, and 2 mM phenylmethane sulfonyl fluoride), and quantified by the Bradford method (Bradford 1976). Protein samples were separated using

12% sodium dodecyl sulfate–polyacrylamide gel electrophoresis and transferred onto a PVDF membrane (Amersham™ Hybond™, GE Healthcare, USA). AhBADH protein was detected using a rabbit polyclonal antibody (1:5000 dilution) raised against AhBADH protein (GenScript Co., Ltd. Nanjing, China), and goat anti-rabbit IgG (Abcam, UK) conjugated to horseradish peroxidase (HRP) as the secondary antibody (1:10000 v/v). Protein bands were visualized using the Biodlight Western Chemiluminescent HRP substrate (Bioworld Technology, Inc. USA).

AhBADH protein levels in transgenic and non-transformed control plants were measured using an enzyme-linked immunosorbent assay (ELISA) kit (Meibiao Biotechnology Co., Ltd., Jiangsu, China). Total soluble protein was extracted from the leaves (0.1g) and roots (0.1g) of transgenic and non-transformed plants using a Plant Protein Extraction Kit (CW-biotech, Beijing, China) according to the manufacturer's instructions. The protein assay was performed in a 96-well microtitration plate according to the manufacturer's instructions. Optical density (OD) at 450 nm was measured using a microplate reader (BioTek Elx800). The concentration of BADH in the samples was determined by comparing the optical density (OD) of the samples to the standard curve.

Salt Tolerance Assays Of Transgenic Plants

The salt tolerance of the transgenic plants was assayed at the germination stage in the culture room and seedling stage in the greenhouse and in the field. For the germination stage assay, sterilized seeds of the transgenic and non-transformed plants were germinated in a Petri plate (20 seeds/plate, 9 cm diameter) with filter paper saturated with 300 mM NaCl, which was optimized concentration, for 7 days in a culture room (24°C, darkness). Subsequently, the number of germinated seeds was recorded. Mean values were obtained from three replicates. Germination percentage was calculated as follows:

$$\text{Germination percentage} = \frac{\text{Number of seeds germinated}}{\text{Total number of seeds sown}} \times 100$$

Salt tolerance of the transgenic plants was evaluated at the seedling stage in the greenhouse. Seeds of transgenic and non-transformed plants were germinated separately in a rectangular plastic box (49 · 40 · 20 cm) filled with peat soil and grown under controlled environmental conditions (photoperiod 16/8 h light/dark, temperature 24/20°C day/night, relative humidity 65–75%). The well-grown seedlings were irrigated with 300 mM NaCl solution and water (control) at the V3 stage (three leaves). After salt treatment for 7 d, the dry weight was measured to estimate the salt tolerance index (STI) of the transgenic and non-transformed control plants. Three biological replicates were used in this experiment. Salt tolerance index (STI) was calculated as follows:

$$\text{STI} = \frac{\text{Total dry weight of salt stressed plants}}{\text{Total dry weight of control plants}} \times 100$$

To evaluate the salt tolerance of transgenic plants in the field, a trial was conducted at a field experimental station in the Jilin Province, China. The experiment was arranged in a completely

randomized block design (CRD) with three replicates. Each plot comprised four rows of transgenic and non-transformed plants. At the V3 stage, seedlings of transgenic and non-transformed control plants were saturated with 300 mM NaCl solution and water (control). After salt treatment for 28 d, the dry weight of each sample was measured to estimate the STI of the transgenic and non-transformed control plants. Three biological replicates were used in this experiment. STI was estimated as described above.

Badh Activity Assays In Transgenic Plants

Crude BADH was extracted from the leaves of transgenic and non-transformed plants using a Plant Protein Extraction Kit (ComWin Biotech, Beijing, China) according to the manufacturer's instructions. BADH activity was assayed using an enzyme-linked immunosorbent assay (ELISA) kit (Meibiao Biotechnology Co., Ltd., Jiangsu, China) according to the manufacturer's instructions. The optical density (OD) at 450 nm was measured using a microplate reader (BioTek Elx800), and the activity of BADH in the samples was determined by comparing the OD of the samples to that of the standard curve.

Determination Of Glycine Betaine Content In Transgenic Plants

Sample solutions of glycine betaine (GB) for transgenic and non-transformed plants treated with 300 mM NaCl for seven days were prepared as described by Zhang et al. (2019). Fresh leaf samples (0.5 g) were ground and extracted in 5 ml methanol extraction solution (methanol: chloroform: water = 12:5:1, v/v/v) at 60°C for 30 min. After centrifugation at 1000 rpm for 10 min, the aqueous phase was fractioned using an anionic resin (Dowex-1-OH⁻ and Dowex-50-H⁺ columns; Anland Bio-science, Shanghai, China). The GB fraction was eluted with 4 ml 6M NH₄OH, dried using a rotary evaporator, and dissolved in 2 ml distilled water. GB content was analyzed by HPLC (Agilent 1260 Infinity system with a VWD monitor, Agilent Technology, Waldbronn, Germany) equipped with an SB-C8 column. Commercial betaine (> 98%; Biochemical, Shanghai, China) was used as the reference standard.

Measurements Of Malondialdehyde And Proline In Transgenic Plants

For measurement of malondialdehyde and proline contents in transgenic plants, fresh leaves (0.1 g) both for MDA and PRO from seedlings of transgenic and non-transformed control plants salt-treated for 7 days were homogenized in liquid nitrogen using a mortar and pestle. The MDA and PRO extractions and measurements were carried out using the MDA and PRO Measurement Kit (Jiancheng Bio-Engineering Institute, Nanjing, China) following the manufacturer's instructions. Measurements were performed using a 96-well microtiter plate. The optical densities (OD) at 530 and 520 nm for MDA and PRO, respectively, were measured using a microplate reader (BioTek Elx800). MDA and PRO contents were determined based on standard solutions and calculated using the following formulas:

$$\text{MDA}(\text{nmol/g} \cdot \text{FW}) = \frac{\text{OD}_{\text{ofsample}} - \text{OD}_{\text{ofblankcontrol}}}{\text{OD}_{\text{ofstandard}} - \text{OD}_{\text{ofblankcontrol}}} \frac{10\text{nmol/ml}}{0.1\text{g} \cdot \text{FW/ml}}$$

$$\text{PRO}(\mu\text{g/g} \cdot \text{FW}) = \frac{\text{ODofsample} - \text{ODofblankcontrol}}{\text{ODofstandard} - \text{ODofblankcontrol}} \frac{5\mu\text{g/ml}}{0.1\text{g} \cdot \text{FW/ml}}$$

The samples were measured using three biological replicates.

Sod, Pod, And Cat Activity Assays

For superoxide dismutase (SOD), peroxidase (POD), and catalase (CAT) activity assays, fresh leaves (0.1 g) of transgenic and non-transformed plants salt-treated for 7 days were ground in liquid nitrogen using a mortar and pestle. Crude enzymes were extracted and measured according to the manufacturer's instructions for SOD, POD, and CAT measurement kits (Comin Biotechnology, Suzhou, China). Total SOD, POD, and CAT activities were measured based on the changes in absorbance at 450, 470, and 240 nm, respectively, using a UV/visible spectrophotometer (Hitachi U5100, Japan). Each sample was analyzed using three biological replicates.

Determination Of Na And K Contents In Transgenic Plants

Seeds of transgenic and non-transformed plants were grown and subjected to salt treatment, as described above. After treatment with 300 mM NaCl for seven days, the seedlings of transgenic and non-transformed plants were harvested and rinsed with deionized water. The roots, stems, and leaves were oven-dried overnight at 70°C. Dried and ground plant sample weighing 0.5 g was dissolved in 5 ml HNO₃ (65%) and diluted to 1 ml using a microwave-assisted nitric acid digestion procedure (Kubrakova et al. 1998). The digest was diluted to a final volume of 25 mL with deionized water. Na⁺ and K⁺ contents were measured at 589 nm and 766.5 nm respectively, using an Agilent 240FS-AA atomic absorption spectrophotometer (Agilent Technologies, USA). Triplicate measurements were performed for each treatment group.

Total Chlorophyll Contents Determination In Transgenic Plants

Fresh leaves (0.1 g) from seedlings of transgenic and non-transformed control plants salt-treated for 7 days were homogenized in liquid nitrogen using a mortar and pestle. Chlorophyll extraction and content determination were performed using a chlorophyll measurement kit (Jiancheng Bio-Engineering Institute, Nanjing, China), according to the manufacturer's instructions. Total chlorophyll content was measured using a UV/visible spectrophotometer (Hitachi U5100, Japan) at 645 and 663 nm, and calculated using the following equation:

$$\text{Totalchlorophyll}(\text{mg/g} \cdot \text{FW}) = \frac{(20.21A_{645} + 8.02A_{663})V(\text{ml})}{0.1\text{g} \cdot \text{FW}1000}$$

where V is the volume (mL) of extraction. Each sample was analyzed using three biological replicates.

Photosynthesis Measurement Of Transgenic Plants

The photosynthetic rate (P_n) of transgenic and non-transformed control plants was measured using an LCI portable photosynthetic system (ADC BioScientific, UK), which controls the photosynthetic photon flux density (PPFD) at $1200 \mu\text{mol m}^{-2} \text{s}^{-1}$, temperature at 28°C , and CO_2 concentration at $400 \mu\text{mol mol}^{-1}$ in the leaf chamber. Light was provided by red and blue LED sources. P_n was measured at 9:30–11:00. Triplicate measurements were performed for each treatment and the results were tabulated using mean values.

Agronomic Performance Of The Transgenic Plants In Field

To evaluate the effect of *AhBADH* on soybean productivity, the main agronomic traits of the transgenic and non-transformed control plants under salt-tolerant conditions (seedlings of transgenic and non-transformed control plants were saturated with 300 mM NaCl solution at the V3 stage) in the field were investigated. Plant height, grain number per plant, and grain yield per plant were measured and recorded.

Statistical analysis

A completely randomized design (CRD) were used in the experiments. Each treatment contained three replicates. Data analysis with the paired samples was carried out by t-test to evaluate the statistically significant difference between each transgenic and non-transformed control plant under NaCl treatment, and with more than two treatment levels by ANOVA to evaluate the statistical significance of differences between different generations. Multi-range comparisons were performed using Fisher's least significant difference (LSD) test. Significant levels were defined as a, b between transgenic and non-transformed plants when $P < 0.01$, and as A, B, and C between T4, T5, and T6 generations when $P < 0.05$.

Results And Analysis

Generation and identification of transgenic soybean

To generate *AhBADH*-overexpressing transgenic soybean, *AhBADH* was cloned into the construct pCAMBIA-3300, and the binary vector pCAMBIA3300-35S-*AhBADH* (Fig. 1) was transformed into the soybean cultivar Williams 82 by *Agrobacterium* mediated transformation. A total of 256 independent transgenic lines (Fig. 2A) were produced and detected by PCR (937 bp fragment) and Southern blot analysis combined with phosphinothricin (PPT) selection and the LibertyLink® strips test (Fig. 2B, C, D, E, F). All transgenic plants grew and produced flowers, with no visible differences from that of the non-transformed control plants. Southern blot analysis demonstrated integration of the foreign gene into the soybean genome with 1–4 copies of T-DNA insertion. For the subsequent generations, PCR detection, PPT selection, and the LibertyLink® strips test combined with salt-tolerance screening were applied to produce homozygous transgenic lines. A total of 47 T3 homozygous transgenic lines with salt tolerance

were obtained by NaCl treatment during the germination and seedling stages. The transgenic line 7 (TL7) with a single copy of the T-DNA insertion (Fig. 2E) and salt tolerance to the highest NaCl concentration were the selection criteria used for further stability analysis.

Stable inheritance of AhBADH in transgenic soybean

The progenies of transgenic line TL7 with a single copy of the T-DNA insertion and the highest salt tolerance were selected for stability analysis of *AhBADH* integration. PCR analysis of the T4, T5, and T6 generation plants showed a 937 bp PCR fragment (Fig. 3). Total genomic DNA from T4, T5, and T6 generation plants was digested with *Hind III* and hybridized with a Dig-labelled probe of *AhBADH*. Southern blot analysis showed ~ 5.1 kb hybridization bands for the genomic DNA of T4, T5, and T6 generation plants (Fig. 4). The results of PCR and Southern blot analysis for T4, T5, and T6 generation plants indicated that the *AhBADH* was successfully integrated into the genome of the transgenic line TL7 and showed stable inheritance in subsequent generations.

Stable overexpression of AhBADH in transgenic soybean

Overexpression of *AhBADH* in T4, T5, and T6 plants of transgenic line TL7 under salt stress was analyzed by RT-PCR, q-PCR, western blot, and ELISA. RT-PCR analysis of T4, T5, and T6 plants of TL7 revealed the expression of *AhBADH*; the expected 937 bp band was visible in the transgenic plants (Fig. 5). q-PCR analysis showed that relative expression levels in the leaves and roots of T4, T5, and T6 plants of TL7 were significantly ($P < 0.01$) higher than those of non-transformed plants. No significant differences ($P < 0.05$) were found between T4, T5, and T6 plants of TL7 (Fig. 6). Western blot analysis detected the presence of AhBADH protein as a 55 KDa band in T4, T5, and T6 plants of TL7 (Fig. 7). ELISA analysis showed that AhBADH protein expression levels in the leaves and roots of T4, T5, and T6 plants of TL7 were significantly ($P < 0.01$) higher than those of non-transformed control plants. No significant differences ($P < 0.05$) were found between T4, T5, and T6 generation plants of TL7 (Fig. 8).

Enhanced Salt Tolerance Of Transgenic Soybean

The salt tolerance of T4, T5, and T6 generation plants of TL7 were tested under 300 mM NaCl stress for 7 days at the germination stage in the laboratory, and were evaluated at the seedling stage in a greenhouse and in the field. Germination percentages (GPs) for T4, T5, and T6 generation plants of TL7 were significantly ($P < 0.01$) higher than those of non-transformed control plants. No significant differences ($P < 0.05$) were found between T4, T5, and T6 generation plants after 300 mM 7 days of NaCl stress at the germination stage (Fig. 9A, Fig. 11A). The STI for T4, T5, and T6 generation plants of TL7 was significantly ($P < 0.01$) higher than those of non-transformed control plants, and no significant differences ($P < 0.05$) were found between T4, T5, and T6 generation plants after 300 mM NaCl stress at the seedling stage for 7 d in the greenhouse (Fig. 9B, Fig. 11B) and for 28 d in the field (Fig. 10, Fig. 11C).

Physiological And Biochemical Parameters Of The Transgenic Soybean Related To Salt Tolerance

Physiological and biochemical parameters, including GB content, BADH enzyme activity, MDA content, PRO content, SOD enzyme activity, POD enzyme activity, CAT enzyme activity, Na⁺ and K⁺ content, total chlorophyll content, and photosynthetic rate (Pn), were measured for transgenic soybean related to salt tolerance.

The GB contents from 2.73 to 2.93 µM/g·fw and BADH enzyme activities from 3.31 to 4.53 U/g·fw for T4, T5 and T6 generation plants of TL7 were significantly ($P < 0.01$) higher than those of non-transformed control plants. No significant differences ($P < 0.05$) were found between T4, T5 and T6 generation plants after 300 mM NaCl treatment for 7 days at the seedling stage (Fig. 12A, B).

MDA contents for T4, T5 and T6 generation plants of TL7, with a range of 75.8–90.0 nmol/g·fw, were significantly ($P < 0.01$) lower than those of non-transformed control plants, which ranged from 142.9–176.8 nmol/g·fw. No significant differences ($P < 0.05$) were found between T4, T5 and T6 generation plants after 300 mM NaCl stress for 7 days at seedling stage (Fig. 13A). Proline contents for T4, T5 and T6 generation plants of TL7, ranged from 53.42–61.91 µg/g·fw, were significantly ($P < 0.01$) higher than those of non-transformed plants, which ranged from 24.18–30.07 µg/g·fw. No significant differences ($P < 0.05$) were found between T4, T5 and T6 generation plants (Fig. 13B).

SOD, POD, and CAT enzyme activities in T4, T5 and T6 generation plants of TL7 were significantly ($P < 0.01$) higher than those of non-transformed control plants. No significant differences ($P < 0.05$) were found between T4, T5, and T6 generation plants after 7 days of 300 mM NaCl stress at the seedling stage (Fig. 14A, B, C).

After salt treatment for 7 days at the seedling stage, K⁺ content in roots, stems, and leaves of T4, T5 and T6 generation plants of TL7 were significantly ($P < 0.01$) higher than those of non-transformed control plants. No significant differences ($P < 0.05$) were found between T4, T5, and T6 transgenic plants (Fig. 15A, B, C). However, Na⁺ content in the roots, stems, and leaves for T4, T5 and T6 generation plants of TL7 was significantly ($P < 0.01$) lower than those of non-transformed control plants, and no significant differences ($P < 0.05$) were found between T4, T5, and T6 transgenic plants (Fig. 16A, B, C). The K⁺/Na⁺ ratios of roots, stems, and leaves for T4, T5 and T6 generation plants of TL7 were significantly ($P < 0.01$) higher than those of non-transformed control plants (Fig. 17A, B, C).

Total chlorophyll content and Pn for T4, T5 and T6 generation plants of TL7 were significantly ($P < 0.01$) higher than those of non-transformed control plants, and no significant differences ($P < 0.05$) were found between T4, T5, and T6 transgenic plants after 7 days of 300 mM NaCl stress at the seedling stage (Fig. 18A, B).

The main agronomic traits, including plant height, grain number per plant, and grain yield per plant for T4, T5, and T6 plants of TL7 were investigated under salt stress conditions in the field. The results showed

that plant height, grain number per plant, and grain yield per plant for T4, T5, and T6 plants of TL7 were significantly ($P < 0.01$) higher than those of non-transformed control plants, and no significant differences ($P < 0.05$) were found between the T4, T5, and T6 generation plants (Fig. 19, Fig. 20A, B, C).

Discussion

Plants develop various tolerance mechanisms to survive under salt stress. GB is an important compatible solute that maintains cellular osmotic balance, prevents water loss, maintains cell turgor pressure, and stabilizes the structure of intracellular macromolecules and biological membranes. GB is a osmotic adjustment substance and can be induced by salt stress (Kern and Dyer 2004; Ahmad 2013; Liu et al. 2018). In higher plants, BADH is a key step enzyme involved in the GB biosynthetic pathway (Hanson et al. 1985; McNeil et al. 2000; Ashraf and Foolad 2007). Previous studies have shown that overexpression of *BADH* can improve salt tolerance in many transgenic plants. In transgenic wheat, the salt tolerance of the transgenic lines (T2 and T3 generations) was significantly improved by overexpression of *BADH* gene under 150–200 mM NaCl stress conditions, and the damaging effects of high salinity were significantly reduced (Liang et al. 2009; He et al. 2010; Li et al. 2019). Transgenic rice with *BADH* gene was able to tolerate 100–150 mM NaCl stress (Guo et al. 1997; Kishitani et al. 2000; Hasthanasombut et al. 2011). The salt tolerance of transgenic maize (T4 generation plants) was significantly improved by overexpression of *BADH* gene from *Atriplex micrantha* under 300 mM NaCl stress conditions (Di et al. 2015). However, no studies have reported improvements in salt tolerance in transgenic plants of soybean overexpressing *BADH*.

In the present study, *AhBADH* was successfully introduced into soybean, where 47 transgenic lines with enhanced salt tolerance were obtained. Transgenic lines with 1–4 copies of the T-DNA insertion were confirmed by PCR and Southern blot analyses, and salt tolerance of the transgenic lines was identified under 300 mM NaCl stress conditions. Molecular analyses for three consecutive generations of transgenic line TL7 showed that *AhBADH* was detected in T4, T5, and T6 plants, *AhBADH* gene transcription, AhBADH protein expression in T4, T5, and T6 plants of TL7 were significantly higher than those in non-transformed plants. No significant differences were found between T4, T5, and T6 transgenic plants. Salt tolerance analyses showed that GP and STI for T4, T5, and T6 plants of TL7 were significantly higher than those of non-transformed plants, and no significant differences were found between T4, T5, and T6 transgenic plants under 300 mM NaCl stress conditions at the germination and seedling stages. The results indicated that *AhBADH* gene was stably integrated into the genome of the transgenic line TL7 which was further inherited and expressed in subsequent generations. The salt tolerance of the transgenic line TL7 overexpressing *AhBADH* was also stably inherited in the progenies.

In plants, GB is known as an osmoprotectant that prevents water loss and stabilizes the structure and function of intracellular macromolecular and biological membranes, and has been shown to improve plant salt tolerance (Ahmad et al. 2013; Li et al. 2014). Previous studies have reported that heterologous expression of *BADH* increases BADH activity and synthesis in transgenic plants; enhanced salt tolerance was remarkably correlated with the amount of accumulated GB (Zhou et al. 2007; Yang et al. 2008; Liang

et al. 2009; Fu et al. 2011; Liu et al. 2011; Fan et al. 2012; Yan et al. 2012; Di et al. 2015; Liu et al. 2018; Sun et al. 2019). In the present study, *AhBADH* activity and GB content in T4, T5, and T6 plants of TL7 expressing *AhBADH* were significantly higher than those of non-transformed control plants, and no significant differences were found between T4, T5, and T6 transgenic plants under salt stress conditions. This result is consistent with that of previous studies; in salinized plants, intracellular betaine content is positively correlated with stress tolerance (Ishitani et al. 1993; Nomura et al. 1995; Xu et al. 2001). In the current study, the GB contents in T4, T5, and T6 plants of TL7 were low (2.73–2.93 $\mu\text{M/gFW}$) when compared with GB accumulation (4–40 $\mu\text{M/gFW}$) in certain species (Rhodes and Hanson 1993); such low GB content was not sufficient for significant improvement of osmotic regulation. However, the transgenic line TL7 still showed a significantly enhanced tolerance to salt stress. The increase in salt tolerance of the transgenic line might be associated with other effects that stabilize macromolecular activity and membrane integrity, in addition to osmoregulation (Sakamoto and Murata 2002). Similar results have been reported in transgenic tobacco (Nuccio et al. 2000; Holmström et al. 2000; Yang et al. 2008), rice (Sakamoto and Murata 1998; Su et al. 2006), Arabidopsis (Hayashi et al. 1997; Sakamoto et al. 2000), tomato (Zhou et al. 2007), and alfalfa (Liu et al. 2011).

Salt stress can cause oxidative damage to the cell membranes. MDA is a by-product of the membrane damage process, and the MDA content reflects membrane permeability and membrane lipid peroxidation. Under stress, MDA content is a key indicator of the degree of cellular membrane damage in plants (Tian et al., 2011; Wang et al. 2011; Demirkol 2020). In the case of overexpression of *BADH* in tomato (Zhou et al. 2007), alfalfa (Liu et al. 2011, Yan et al. 2012), rice (Shao et al. 2008), maize (Di et al. 2015), chicory (Zhang et al. 2015; Li et al. 2016), and Arabidopsis (Yu et al. 2017), cell damage and MDA content were lower than those in non-transgenic plants under salt stress, indicating that cell membrane homeostasis was improved in the transgenic plants. In the present study, MDA content in T4, T5, and T6 plants of TL7 overexpressing *AhBADH* was significantly lower than that of non-transformed control plants under 300 mM NaCl stress for 7 days at the seedling stage. This result is in agreement with that of previous studies showing that overexpression of the *BADH* gene prevents damage to the cell membrane by salt stress-induced lipid peroxidation.

Under salt stress conditions, plants often accumulate free PRO, soluble sugars, and soluble proteins in response to high salinity to maintain cell turgor (Nanjo et al. 1999). PRO is a typical compatible osmolyte that can protect subcellular structures and macromolecules in plant cells under osmotic stress (Ashraf and Foolad 2007; Szabados and Savoure 2010). Usually, free proline accumulates in the cytosol of plant cells in response to salt stress (Binzel et al. 1987; Ketchum et al. 1991; Demirkol 2020). Under salt stress conditions, overexpression of *BADH* gene in transgenic plants results in enhanced proline content and salt tolerance, suggesting that GB protects some key enzymes that catalyze the synthesis of free PRO (Shao et al. 2008; Liang 2009; Yan et al. 2012; Zhang et al. 2015; Li et al. 2016; Yu et al. 2017). In our study, PRO content in T4, T5, and T6 plants of TL7 overexpressing *AhBADH* was significantly higher than that in non-transformed plants, and no significant differences were found between T4, T5, and T6 transgenic plants. Our results are consistent with those of previous reports.

Under normal conditions, the generation and scavenging of ROS in plant cells are well regulated in a dynamic balance. However, during salt stress, this balance can be disturbed, leading to the enhanced production of ROS in plant cells (Mittler, 2002). ROS are cytotoxic and can cause oxidative damage to cell membrane (McKersie and Leshem, 1994). To reduce ROS damage to cellular components, plants have developed multiple detoxification mechanisms including synthesis of antioxidant and ROS-scavenging enzymes, such as SOD, POD and CAT (Mittova et al. 2004; Sekmen et al. 2007). In our study, the antioxidase activities of SOD, POD, and CAT in T4, T5, and T6 plants of TL7 overexpressing *AhBADH* gene were significantly higher than those of non-transformed control plants under 300 mM NaCl stress. The results indicate that *AhBADH* catalyzes the oxidation of betaine aldehyde into glycine betaine, as well as influences the expression of *SOD*, *POD* and *CAT* genes; salt tolerance of the transgenic soybean was further enhanced via enhanced antioxidase activities. Similar results were observed in studies on the transformation of *BADH* in *Arabidopsis* (Yang et al. 2015; Yu et al. 2017), alfalfa (Liu et al. 2011), wheat (Liang et al. 2009), and rice (Shao et al. 2008).

Under salt stress conditions, high concentrations of NaCl in the soil cause water deficit in plants and disrupt the balance of K^+ , thereby altering the K^+/Na^+ ratios. In plant cells, excessive Na^+ can inhibit K^+ uptake, resulting in a decrease of the K^+/Na^+ ratio in cytosol. Alteration of the ion ratio leads to the inactivation of important cytosolic enzymes for physiological processes (Zhu 2003; Tuteja 2007). Plant salt tolerance is a complex quantitative trait (Flowers 2004), and a high cytosolic K^+/Na^+ ratio is essential for normal cellular function in plants (Maathuis and Amtmann 1999; Tester and Davenport 2003; Zheng et al. 2008). Different antiporter genes controlling the uptake of toxic Na^+ have been engineered and salt tolerance of transgenic plants has been enhanced (Gaxiola et al. 2001; Zhang and Blumwald 2001; Gao et al. 2003; Xue et al. 2004; Wu et al. 2005; Lu et al. 2005; Zhao et al. 2006; Verma et al. 2007; An et al. 2008; Baisakh et al. 2012). Under salt stress conditions, transgenic plants overexpressing *BADH* in tomato (Zhou et al. 2007), wheat (Liang et al. 2009; Li et al. 2019), trifoliate orange (Fu et al. 2011), and chicory (Zhang et al. 2015; Li et al. 2016) contained higher levels of K^+ , higher K^+/Na^+ ratios, and lower levels of Na^+ . The enhanced salt tolerance of transgenic plants was partly correlated with the stimulation of K^+ , as GB inhibited Na^+ accumulation, maintained normal membrane permeability and integrity. However, the exact mechanism by which GB adjusts K^+ uptake remains unclear. In our study, under 300 mM NaCl stress, K^+ content in non-transformed control plants decreased and Na^+ content increased, whereas K^+ and Na^+ contents in the transgenic line TL7 (T4, T5, and T6 generations) were not remarkably compromised (Figs. 15 and 16). Transgenic line TL7 maintained a significantly higher K^+ content and K^+/Na^+ ratios in the roots, stems, and leaves of three consecutive generations (T4, T5, and T6) than those in non-transformed control plants (Figs. 15 and 17), and maintained a significantly lower Na^+ content in the roots, stems, and leaves of three consecutive generations (T4, T5, and T6) than those in non-transformed plants under 300 mM NaCl stress for 7 days (Fig. 16). Our results are consistent with those of previous studies showing that higher levels of K^+ and higher K^+/Na^+ ratios can be ascribed to the accumulation of GB in transgenic soybean plants with enhanced salt tolerance.

Chlorophyll content and photosynthetic rate are key physiological indicators for the tolerance of plants to abiotic stress (Gitelson et al. 2003; Fu et al. 2011; Wei et al. 2022). Salt stress leads to reduced chlorophyll content in leaves, which reduces the photosynthetic capacity of plants (Kura-Hotta et al. 1987; Sultana et al. 1999; Netondo et al. 2004; Demirkol 2020). In *BADH* gene-expressing transgenic rice (Hasthanasombut et al. 2011), tomato (Zhou et al. 2007; Zhang et al. 2019), tobacco (Yang et al. 2008), ryegrass (Takahashi et al. 2010), trifoliate orange (Fu et al. 2011), and chicory (Li et al. 2016), the chlorophyll content was significantly higher than that in non-transgenic plants under salt stress, which may partly be ascribed to the protection of the photosynthetic system by GB accumulation. However, the exact protective mechanism of GB of the photosynthetic system remains unknown. In our study, the chlorophyll content and photosynthetic rates for three consecutive generations (T4, T5, and T6) of the transgenic line TL7 overexpressing *AhBADH* were significantly higher than those of non-transformed control plants under 300 mM NaCl stress (Fig. 18A, B). Our results are in agreement with those of earlier studies showing that the photosynthetic system is protected from salt damage by overexpression of the *BADH* gene in transgenic plants.

Under salt stress conditions, photosynthesis in plants is easily inhibited, thus influencing plant growth and productivity (Yang et al. 2008). Accumulation of GB in transgenic plants by overexpressing *BADH* has been proven effective in enhancing salt tolerance and therefore improving the agronomic traits of some crop species, such as tomato (Zhou et al. 2007; Wang et al. 2013; Zhang et al. 2019), sweet potatoes (Fan et al. 2012), potatoes (Zhang et al. 2011; Ali et al. 2020), tobacco (Hasthanasombut et al. 2010), wheat (He et al. 2010), and maize (Di et al. 2015). In the present study, the main agronomic traits of the transgenic plants were evaluated. Under 300 mM NaCl stress, the plant height, grain number per plant, and grain yield per plant of the transgenic line TL7 for three consecutive generations (T4, T5, and T6) were stably enhanced in comparison to non-transformed control plants (Fig. 19, Fig. 20A, B, C). These results are consistent with those of the previous findings (Zhou et al. 2007; He et al. 2010; Zhang et al. 2011; Fan et al. 2012; Di et al. 2015; Zhang et al. 2019).

In conclusion, *AhBADH* was successfully introduced into soybean by *Agrobacterium* mediated transformation. Forty-seven transgenic lines with enhanced salt tolerance were obtained. Molecular analyses of the transgenic line TL7 showed that the *AhBADH* gene was stably inherited and expressed in subsequent generations. Under salt stress, the transgenic line TL7 accumulated GB in its progenies by stably overexpressing *AhBADH*, and demonstrated enhanced salt tolerance and improved agronomic traits under field conditions. The transgenic line TL7 with stable enhanced salt tolerance trait has been assessed for biosafety and environmental release, and can be applied in salt tolerance breeding of soybean through genetic engineering. These results indicate that the *AhBADH* can be widely used to improve the salt tolerance of various crops through genetic engineering. To our knowledge, this is the first report of field-tested transgenic soybean with stable enhanced salt tolerance and improved agronomic traits by overexpression of *AhBADH* under salt stress conditions.

Declarations

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Author Contributions

Rui Ma and Xiangdong Yang designed the experiments. Rui Ma and Xiangdong Yang drafted the manuscript and conducted field trial. Zhijing Yu, Lu Niu, Qinan Cai, Jia Wei and Lixia Shang conducted the experiments, field trial, and data analysis.

Conflict of interest The authors claim no conflict of interest in the publication of this paper.

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Figures

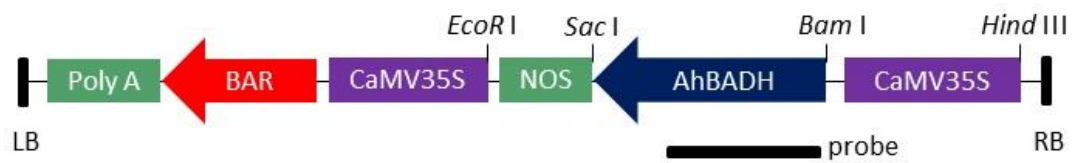


Fig. 1

Figure 1

T-DNA structure of the plasmid pCambia3300-35S-*AhBADH* for transformation in soybean.

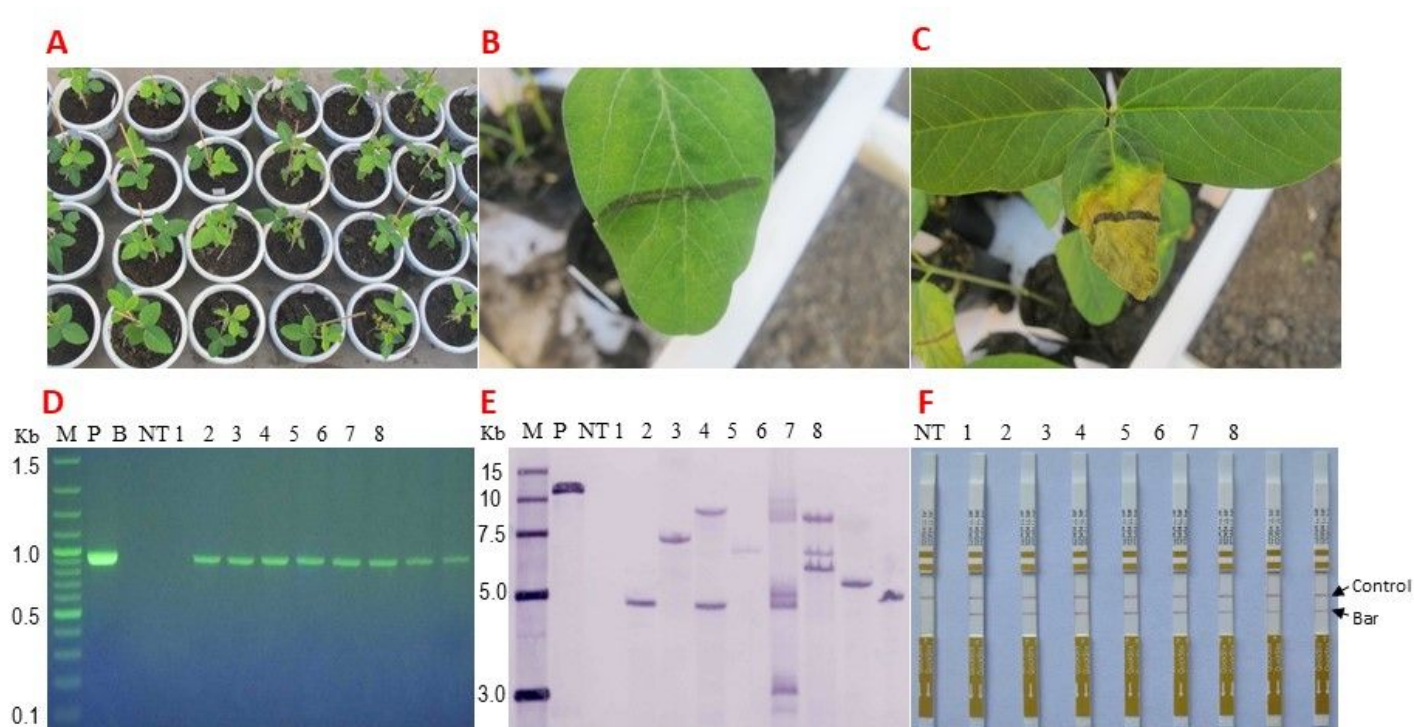


Fig. 2

Figure 2

Transgenic plants of soybean determined by PCR and Southern blot analysis combining with PPT selection and Bar quick stick detection. **A** T0 transgenic plants, **B** T1 transgenic plant detected with PPT, **C** non-transformed plant detected with PPT, **D** PCR analysis showing the expected 937bp band in the positive control and transgenic plants (M DNA molecular weight marker, P positive control of plasmid pCAMBIA3300-*AhBADH*, B negative water control, NT non-transformed control plant, 1-8 transgenic lines), **E** Southern blot analysis showing the hybridized bands in the positive control and transgenic plants, plant genomic DNA and plasmid were digested with *Hind*III and hybridized with digoxigen (DIG) labelled *Ahbadh* probe (M DNA molecular weight marker, P positive control of plasmid pCAMBIA3300-*AhBADH*, NT non-transformed control plant, 1-8 transgenic lines), and **F** Bar protein detection by ENVIROLOGIX quick stick kit for LibertyLink (bar), NT non-transformed control plant, 1-8 transgenic lines showing Bar, transgenic line 7 (TL7).

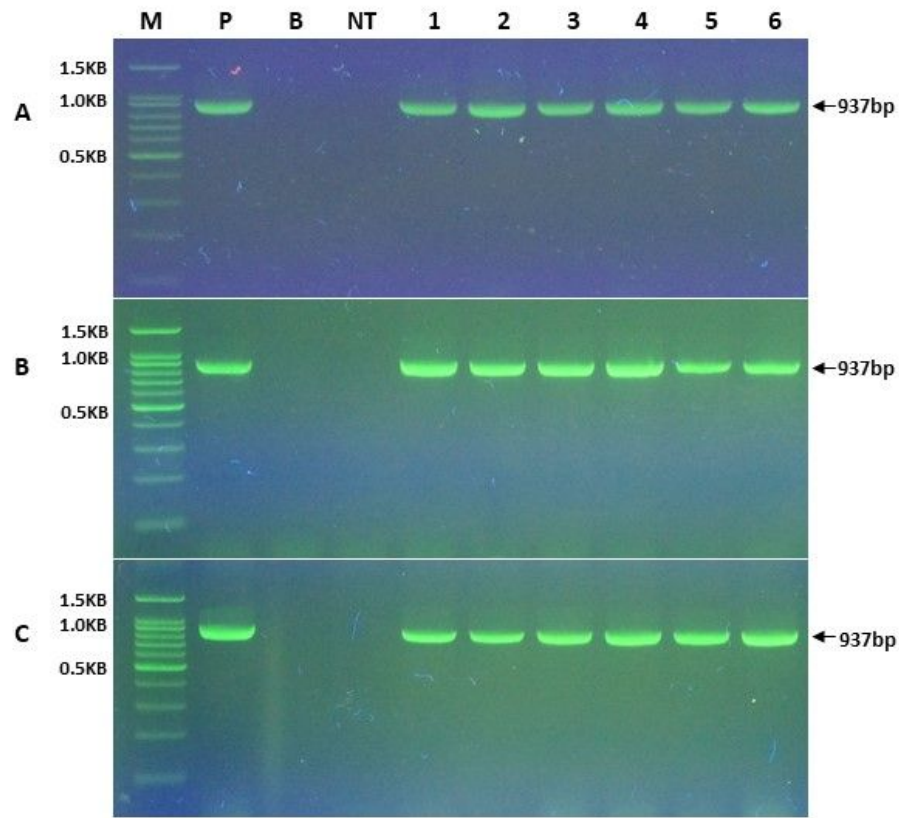


Fig. 3

Figure 3

PCR analysis of *AhBADH* gene in different generations of transgenic line TL7 showing the expected 937bp band. A, B, and C are T4, T5, T6 generation plants of transgenic line 7 (TL7) respectively, M DNA molecular weight marker, P positive control of plasmid pCAMBIA3300-*AhBADH*, B negative water control, NT non-transformed control plant, 1–6 independent plants of TL7.

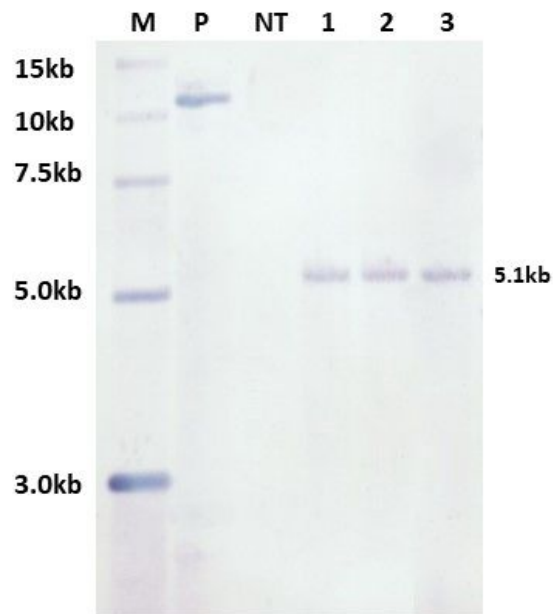


Fig. 4

Figure 4

Southern blot analysis of *AhBADH* gene in different generations of transgenic line TL7 showing the hybridized bands in the positive control and transgenic plants. Plant genomic DNA and plasmid were digested with Hind III and hybridized with digoxigen (DIG) labelled *Ahbadh* probe (M DNA molecular weight marker, P positive control of plasmid pCAMBIA3300-*AhBADH*, NT non-transformed control plant, 1, 2, and 3 are T4, T5, and T6 plants of TL7 respectively).

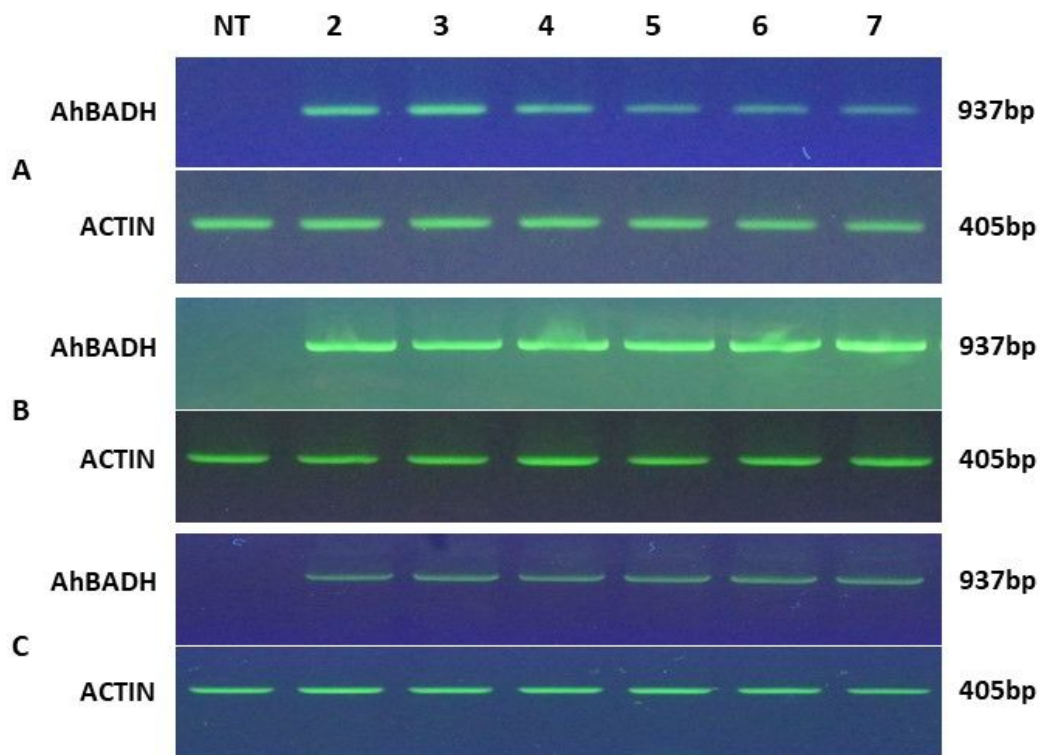


Fig. 5

Figure 5

Expression analysis of *AhBADH* gene in different generations of transgenic line TL7 under salt stress condition (300 mM NaCl treatment for 7 days) at seedling stage by RT-PCR. A, B, and C are T4, T5, and T6 plants of TL7 respectively. The expected 937 bp band of *AhBADH* gene is visible in transgenic plants. The expected 405bp band of reference gene ACTIN of soybean is visible in both non-transgenic control plant and transgenic plants, NT non-transformed control plant, 2–7 independent plants of TL7.

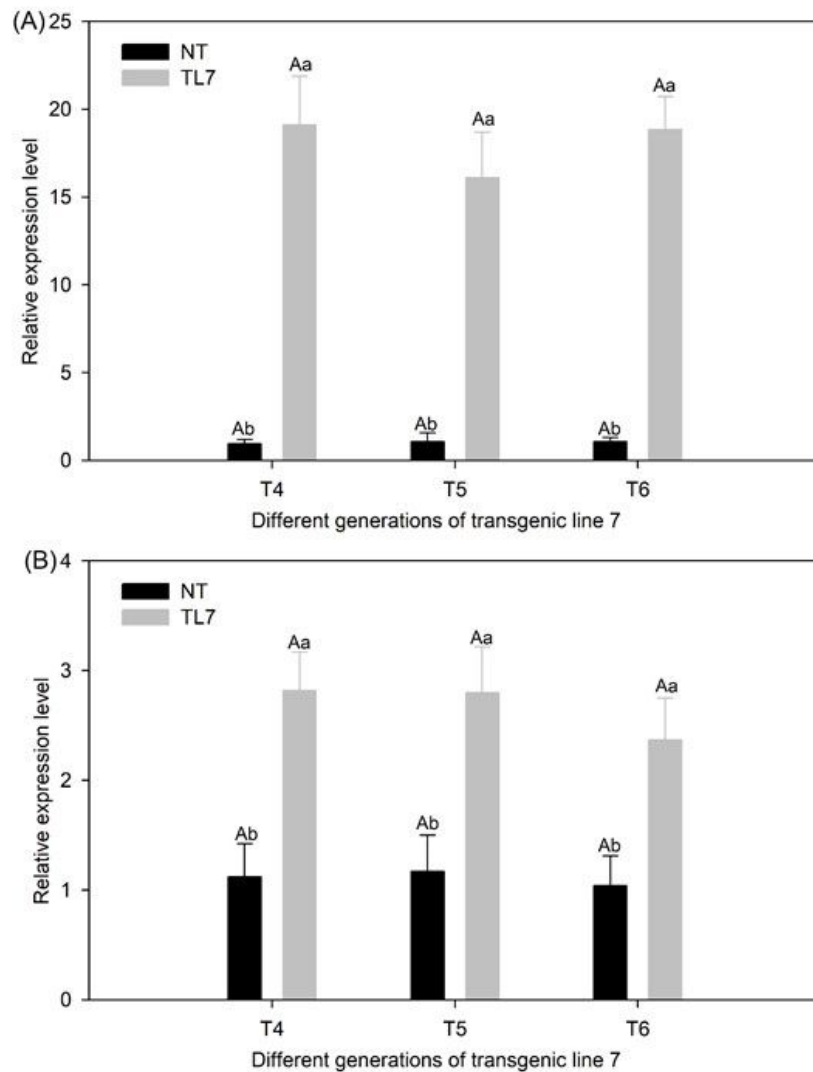


Fig. 6

Figure 6

Expression analysis of *AhBADH* gene in different generations of transgenic line TL7 under salt stress condition (300 mM NaCl treatment for 7 days) at seedling stage by qPCR. (A) relative expression level in leaf at V3 stage and (B) relative expression level in root at V3 stage. Values are the mean $\pm$ SD of three biological replicates, NT non-transformed control plant, TL7 transgenic line 7. A, B, C LSD test at significant level $P < 0.05$ between different generations (T4, T5, T6 generations) of TL7; a, b T-test at significant level $P < 0.01$ between TL7 and NT.

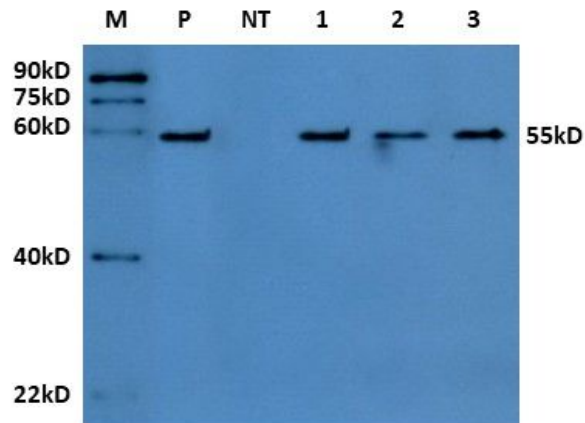


Fig. 7

Figure 7

Expression analysis of AhBADH protein in different generations of transgenic line TL7 under salt stress condition (300 mM NaCl treatment for 7 days) at seedling stage by Western blot. M protein ladder, P recombinant AhBADH protein expressed in *Escherichia coli*, NT non-transformed control plant. 1, 2, 3 are T4, T5, T6 generation plants of TL7 respectively.

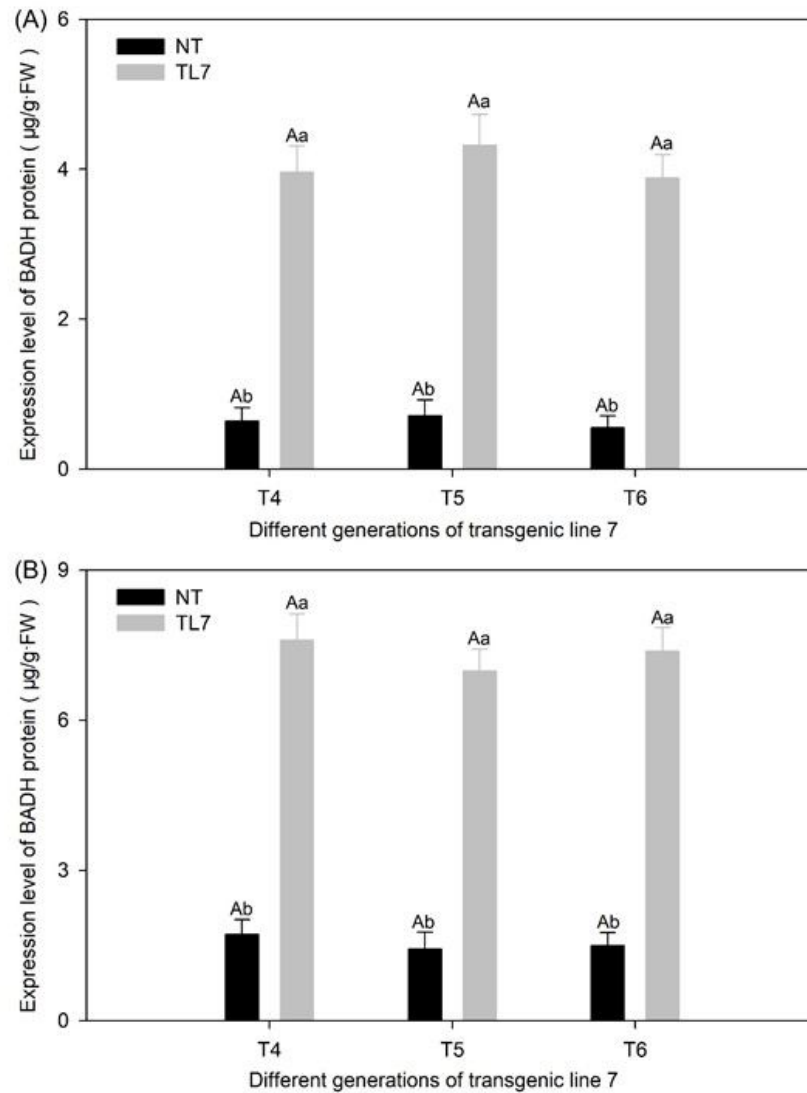


Fig. 8

Figure 8

Expression analysis of BADH protein in different generations of transgenic line TL7 under salt stress condition (300 mM NaCl treatment for 7 days) at seedling stage by ELISA. (A) Expression level in leaf at V3 stage and **(B)** Expression level in root at V3 stage, Values are the mean±SD of three biological replicates, NT non-transformed control plant, TL7 transgenic line 7. A, B, C LSD test at significant level $P < 0.05$ between different generations (T4, T5, T6 generations) of TL7. a, b T-test at significant level $P < 0.01$ between TL7 and NT.

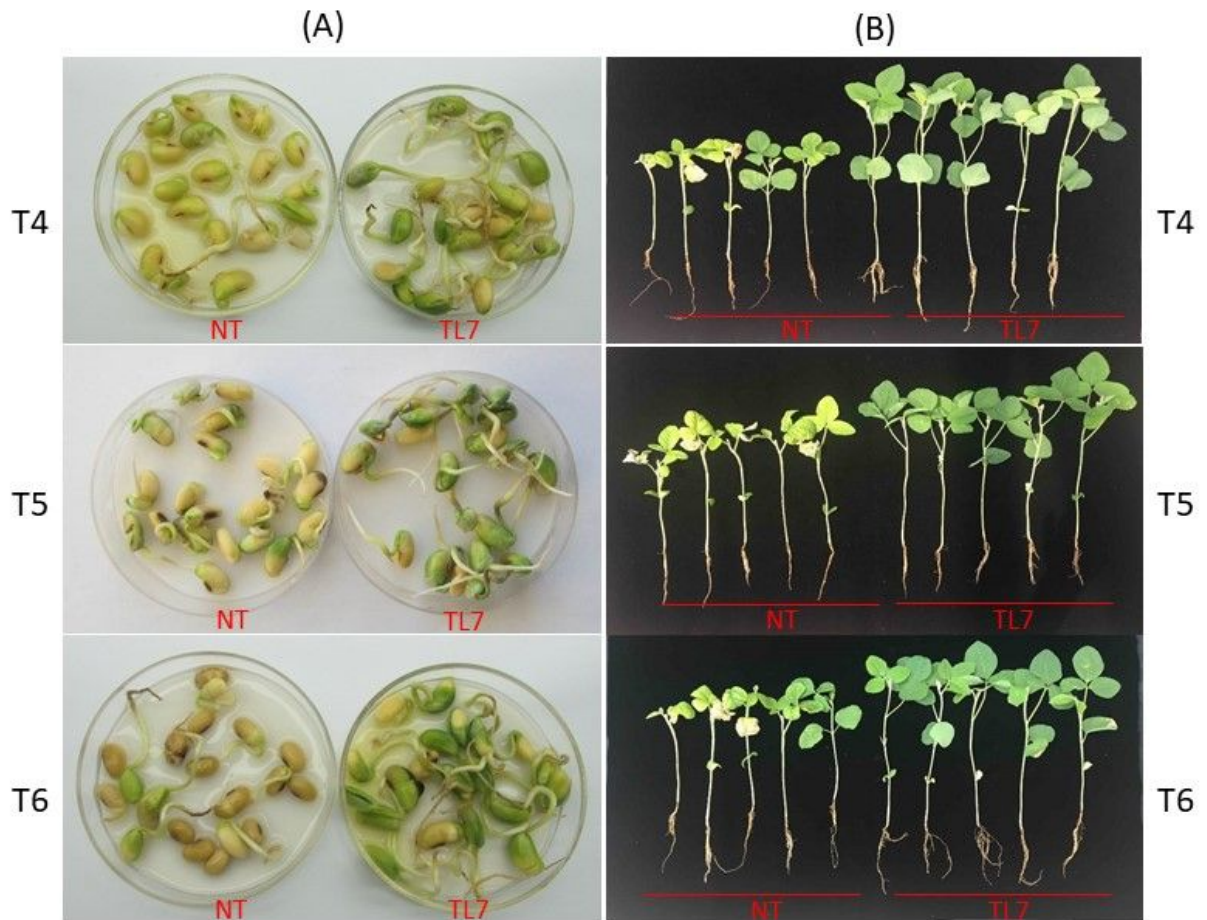


Fig. 9

Figure 9

Salt tolerance of different generations of transgenic line TL7 overexpressing *AhBADH* under salt stress conditions (300 mM NaCl treatment for 7 days). (A) salt tolerance of transgenic line TL7 for different generations (T4, T5, T6 generations) under salt stress condition at germination stage (300 mM NaCl treatment for 7 days) and (B) salt tolerance of transgenic line TL7 for different generations (T4, T5, T6 generations) under salt stress condition at seedling stage (300 mM NaCl treatment for 7 days). NT non-transformed control plant, TL7 transgenic line 7.

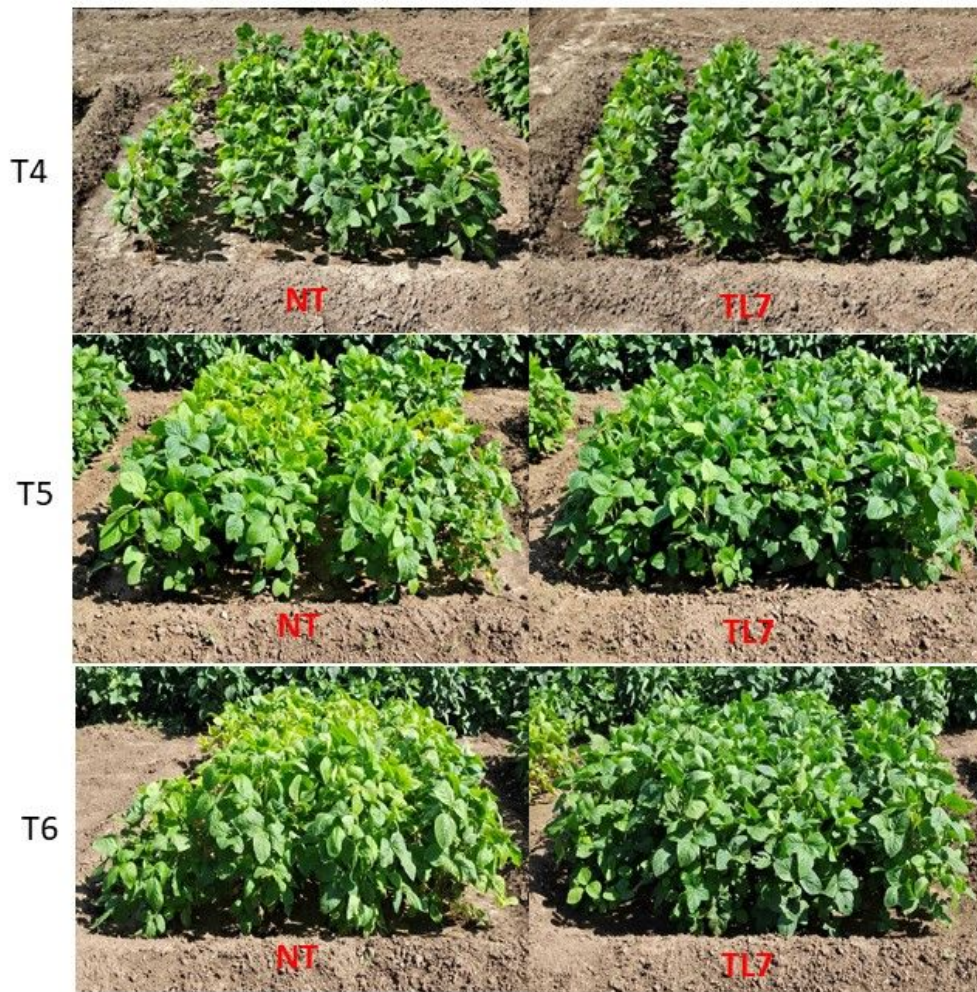


Fig. 10

Figure 10

Growth of T4, T5, and T6 generation plants of TL7 under salt stress condition (300 mM NaCl treatment for 4 weeks) in field trial. NT non-transformed control plant, TL7 transgenic line 7.

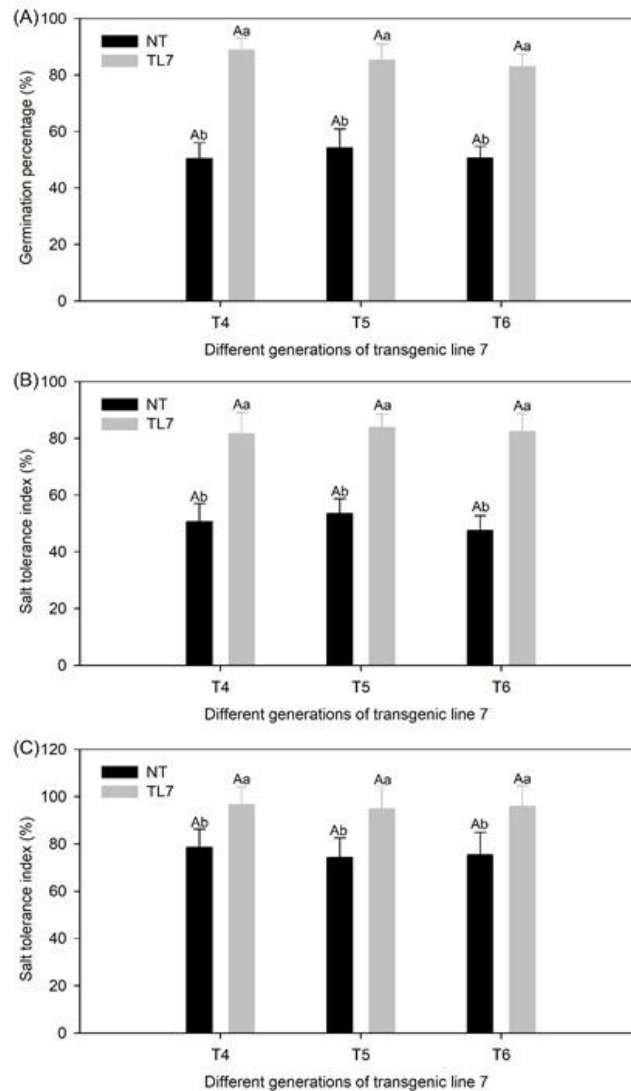


Fig. 11

Figure 11

Salt tolerance of T4, T5, and T6 generation plants of TL7 overexpressing *AhBADH* gene under salt stress condition (300 mM NaCl). (A) germination percentage (GP) of transgenic line TL7 for different generations (T4, T5, T6 generations) under salt stress condition at germination stage (salt treatment for 7 days), (B) Salt tolerance index (STI) of transgenic line TL7 for different generations (T4, T5, T6 generations) under salt stress condition at seedling stage (salt treatment for 7 days), and (C) STI of transgenic line TL7 for different generations (T4, T5, T6 generations) under salt stress condition at seedling stage (salt treatment for 4 weeks). NT non-transformed control plant, TL7 transgenic line 7. A, B, C LSD test at significant level $P < 0.05$ between different generations of TL7. a, b T-test at significant level $P < 0.01$ between TL7 and NT.

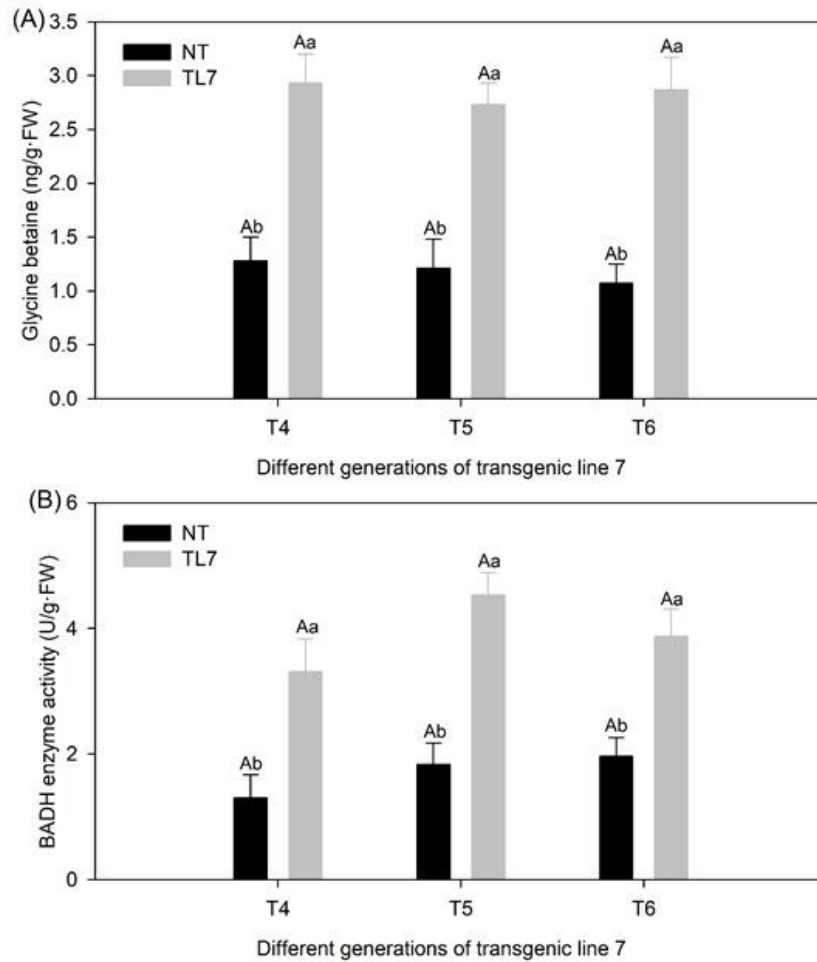


Fig. 12

Figure 12

Glycine betaine content and AhBADH enzyme activity for T4, T5, and T6 generation plants of TL7 under salt stress condition (300 mM NaCl) at seedling stage (salt treatment for 7 days). (A) Glycine betaine content and (B) AhBADH enzyme activity. NT non-transformed control plant, TL7 transgenic line 7. A, B LSD test at significant level $P < 0.05$ between different generations of TL7. a, b T-test at significant level $P < 0.01$ between TL7 and NT.

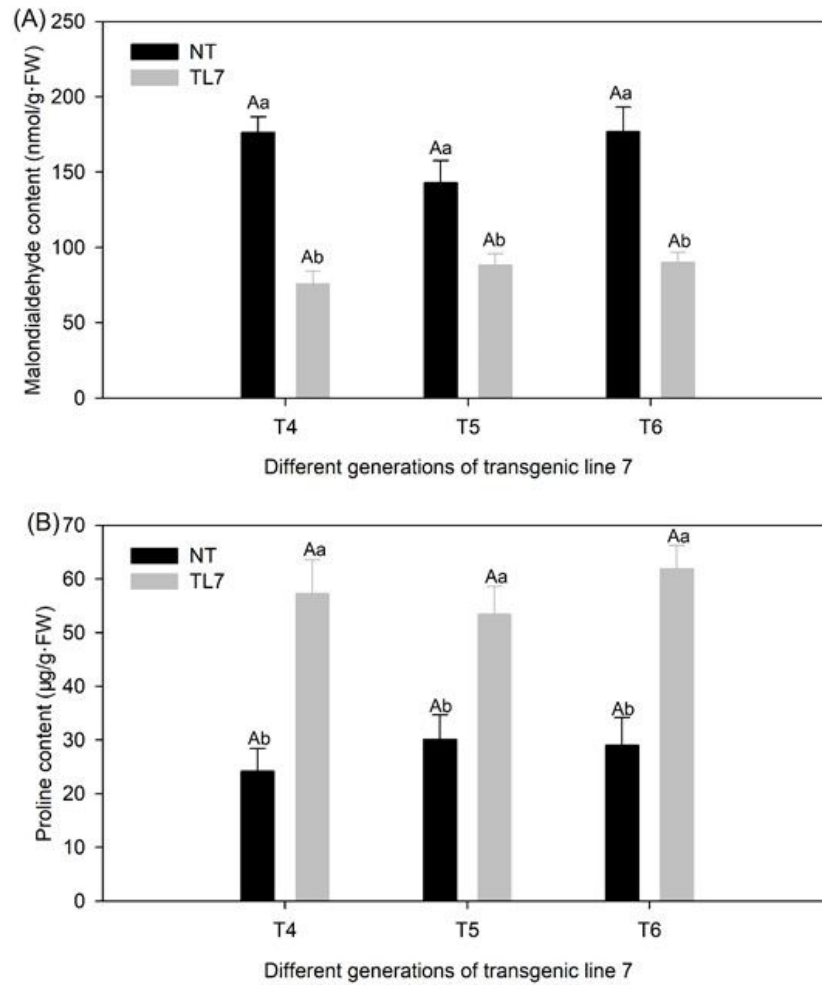


Fig. 13

Figure 13

Malondialdehyde (MDA) and proline (PRO) contents for T4, T5, and T6 generation plants of TL7 under salt stress condition (300 mM NaCl treatment for 7 days) at seedling stage. (A) MDA content and (B) PRO content. NT non-transformed control plant, TL7 transgenic line 7. A, B, C LSD test at significant level $P < 0.05$ between different generations of TL7, a, b T-test at significant level $P < 0.01$ between TL7 and NT.

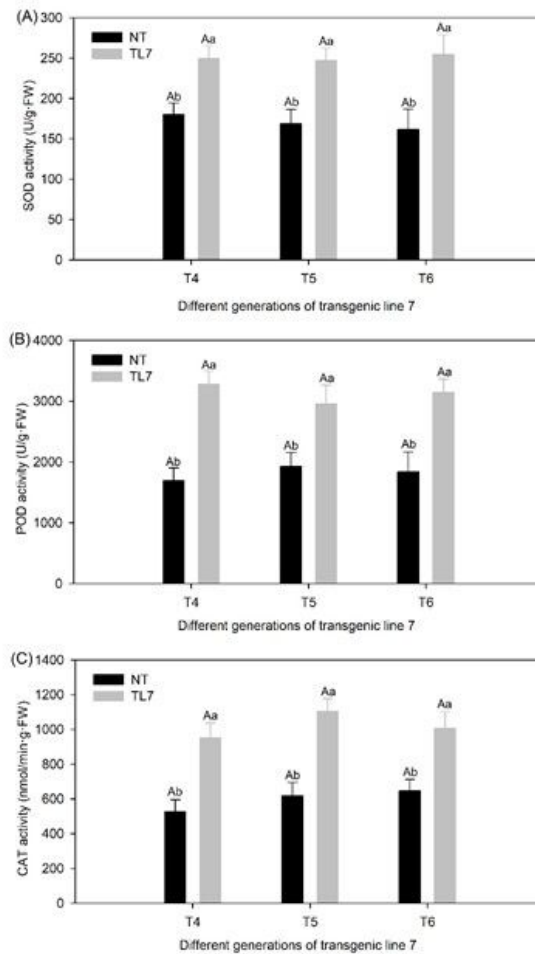


Fig. 14

Figure 14

SOD, POD, and CAT enzyme activities for T4, T5, and T6 generation plants of TL7 under salt stress condition (300 mM NaCl treatment for 7 days) at seedling stage. (A) SOD enzyme activity, (B) POD enzyme activity, and (C) CAT enzyme activity. NT non-transformed control plant, TL7 transgenic line TL7. A LSD test at significant level $P < 0.05$ between different generations of TL7, a, b T-test at significant level $P < 0.01$ between TL7 and NT.

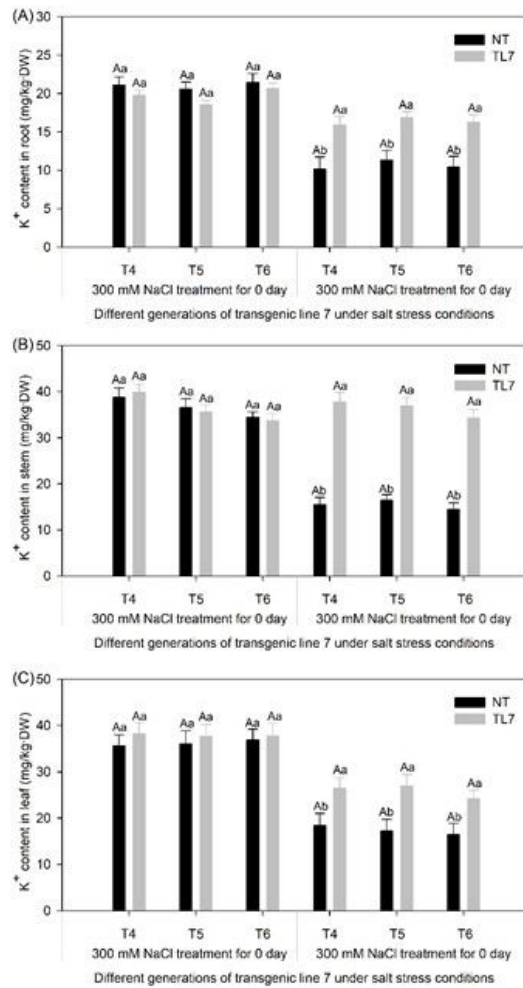


Fig. 15

Figure 15

K⁺ contents of transgenic soybean for T4, T5, and T6 generation plants of TL7 under salt stress conditions (300 mM NaCl for 0 day and 7 days) at seedling stage. (A) K⁺ contents in root, (B) K⁺ contents in stem, and (C) K⁺ contents in leaf. NT non-transformed control plant, TL7 transgenic line 7. Data are means±SD of three independent measurements. A, B, C LSD test at significant level $P < 0.05$ between different generations of TL7 within different salt treatments, a, b T-test at significant level $P < 0.05$ between TL7 and NT of different generations within different salt treatments.

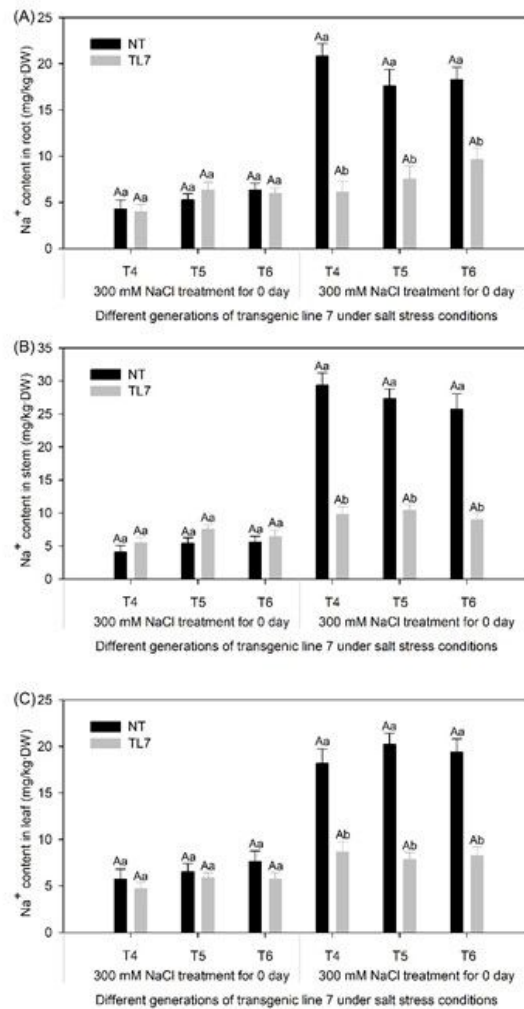


Fig. 16

Figure 16

Na⁺ contents of transgenic soybean for T4, T5, and T6 generation plants of TL7 under salt stress conditions (300 mM NaCl for 0 day and 7 days) at seedling stage. (A) Na⁺ contents in root, (B) Na⁺ contents in stem, and (C) Na⁺ contents in leaf. NT non-transformed control plant, TL7 transgenic line 7. Data are means $\pm$ SD of three independent measurements. A, B, C LSD test at significant level $P < 0.05$ between different generations of TL7 within different salt treatments, a, b T-test at significant level $P < 0.01$ between TL7 and NT for different generations within different salt treatments.

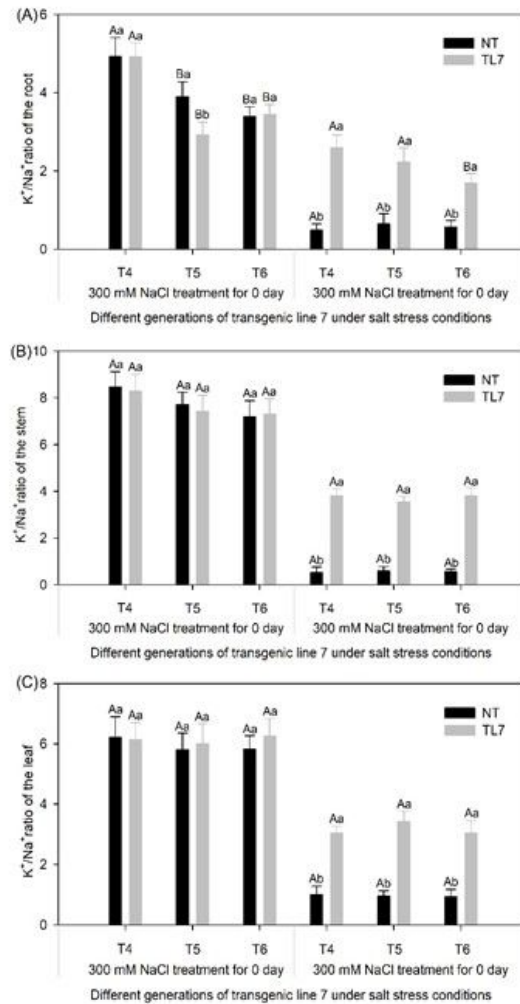


Fig. 17

Figure 17

K^+/Na^+ ratios of transgenic soybean for T4, T5, and T6 generation plants of TL7 under salt stress conditions (300 mM NaCl for 0 day and 7 days) at seedling stage. (A) K^+/Na^+ ratios of the root, (B) K^+/Na^+ ratios of the stem, and (C) K^+/Na^+ ratios of the leaf. NT non-transformed control plant, TL7 transgenic line 7. Data are means $\pm$ SD of three independent measurements. A, B, C LSD test at significant level $P < 0.05$ between different generations of TL7 within different salt treatments, a, b T-test at significant level $P < 0.01$ between TL7 and NT of different generations within different salt treatments.

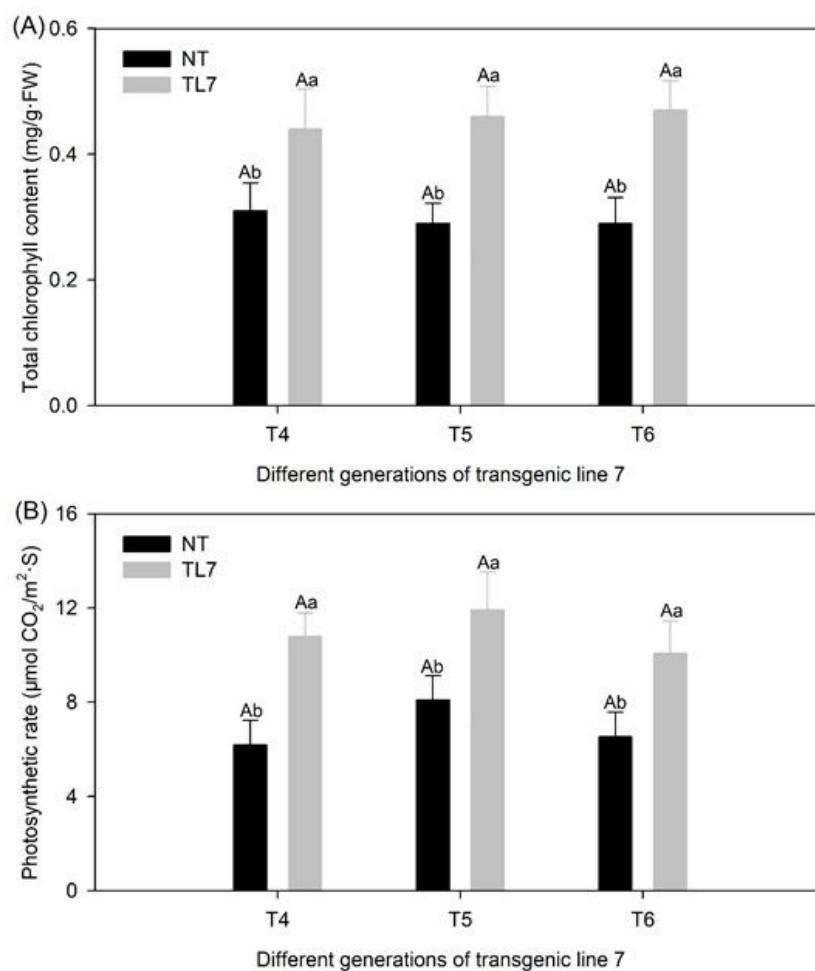


Fig. 18

Figure 18

Total chlorophyll contents and photosynthetic rates (Pn) for T4, T5, and T6 generation plants of TL7 under salt stress condition (300 mM NaCl treatment for 7 days) at seedling stage (salt treatment for 7 days). (A) total chlorophyll contents and (B) photosynthetic rates. NT non-transformed control plant, TL7 transgenic line 7. A, B,C LSD test at significant level $P < 0.05$ between different generations of TL7, a, b T-test at significant level $P < 0.01$ between TL7 and NT.

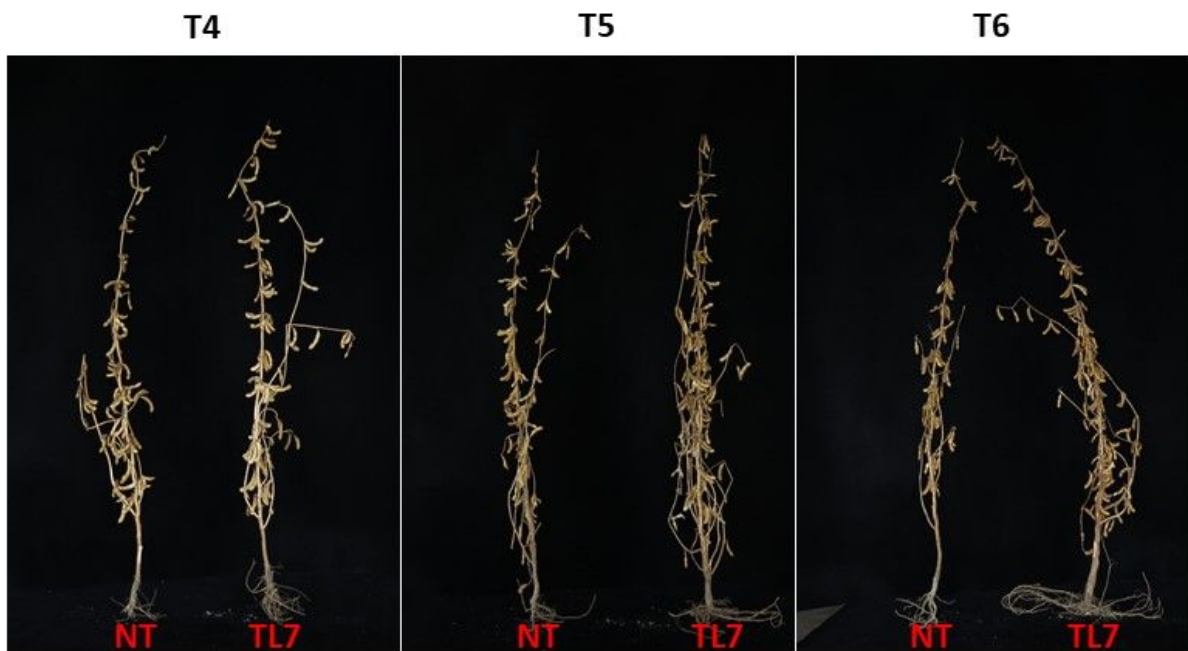


Fig. 19

Figure 19

T4, T5, and T6 generation plants of transgenic line TL7 under salt stress condition (300 mM NaCl treatment) in field trial. NT non-transformed control plant, TL7 transgenic line 7.

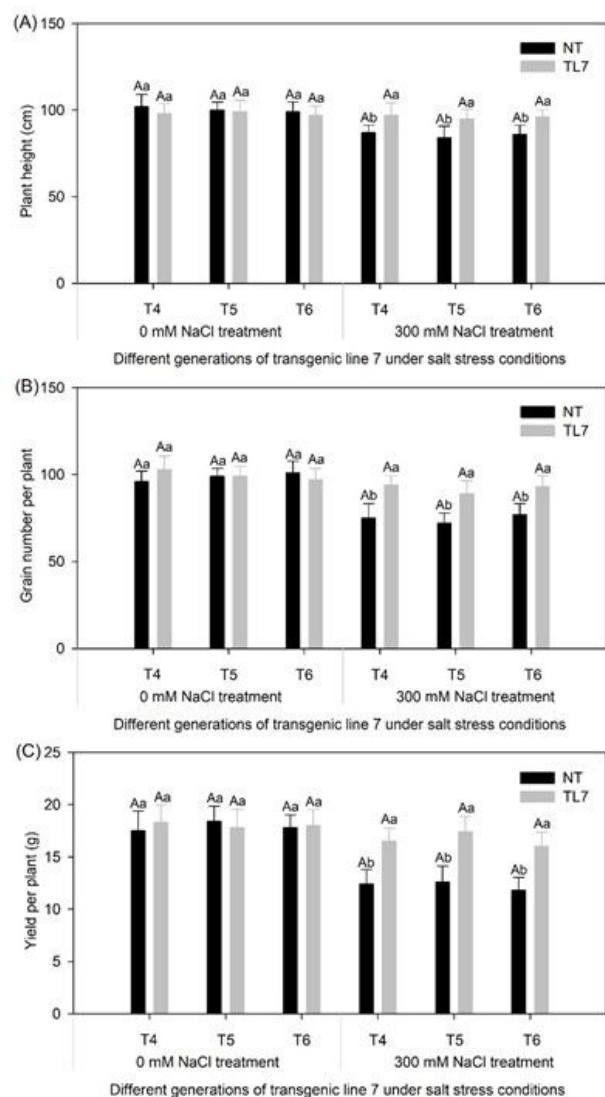


Fig. 20

Figure 20

Agronomic traits for T4, T5, and T6 generation plants of TL7 under salt stress conditions (300 mM NaCl) in field trial. (A) plant height, **(B)** grain number per plant, and **(C)** grain yield per plant. NT non-transformed control plant, TL7 transgenic line 7. Data are means $\pm$ SD of three replicates. A, B LSD test at significant level $P < 0.05$ between different generations of TL7 within different salt treatments, a, b T-test at significant level $P < 0.01$ between TL7 and NT of different generations within salt treatments.