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Overexpression of a nascent polypeptide associated complex gene (*SaβNAC*) of *Spartina alterniflora* improves tolerance to salinity and drought in transgenic *Arabidopsis*

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ABSTRACT

Salinity and drought are the most important environmental constraints limiting crop growth and productivity. Here, we have characterized a gene '*SaβNAC*' encoding the β subunit of nascent polypeptide associated complex from a halophyte *Spartina alterniflora* and investigated its role toward abiotic stress regulation. Expression of *SaβNAC* was differentially regulated by abiotic stresses, including salinity, drought, cold, and ABA in leaves and roots of *S. alterniflora*. Constitutive over-expression of *SaβNAC* in *Arabidopsis* exhibited normal growth under non-stress conditions but enhanced tolerance to salt and drought stresses. Transgenic *SaβNAC Arabidopsis* retained more chlorophyll, proline, and showed improved ionic homeostasis with less damage under stress conditions compared to WT plants. This is a first report to demonstrate the involvement of *βNAC* in imparting abiotic stress tolerance which might be due to protection of the newly synthesized polypeptides involved in various stress tolerance mechanisms from abiotic stress induced damage and inhibition of cell death in plant.

1. Introduction

Abiotic stresses severely affect the growth, development, and metabolism of plants. Plants have developed an extensive framework of regulatory pathways at each level of cellular hierarchy and a network of genes are co-expressed at a particular time of stress to cope with the environmental stresses.

Ribosomes are molecular machines inside the cytosol that synthesize polypeptides under different environmental conditions. Newly synthesized polypeptides on ribosome get attached with nascent polypeptide associated complex (NAC), also known as basal transcription factor 3 (*Btf3*), which plays an important role in protecting newly formed proteins from interaction with inappropriate cytosolic factors [1–3]. The nascent polypeptide associated complex directly interacts with signal recognition particle and reported to be involved in translocation of newly synthesized proteins to the endoplasmic reticulum to prevent the mistargeting of nonsecretory proteins [4,5]. It is a highly conserved heterodimeric complex composed of α and β subunits [3]. Unequal expression of either NAC subunit has been reported to be associated with transcription regulation and cell differentiation, suggests subunit specific functions of α and β subunits [6–8]. α-NAC has transcriptional activating activity and bind to DNA, rRNA, and tRNA, whereas βNAC can bind to RNA polymerase II, although it does not activate transcription [3,9]. In *Saccharomyces cerevisiae*, both α and β subunits of NAC associates with nascent polypeptide directly, however only βNAC is responsible for binding to the ribosome [9]. βNAC of *Arabidopsis* interacts with eIF4E, a major component of the translational initiation complex and might be associated with the regulation of translation initiation [10]. All NAC proteins contain a NAC domain, which is suggested to be responsible for their dimerization [1]. NAC has several important functions in the cellular environment: (a) it promotes interaction of ribosomes with the mitochondrial surface and maintain efficient targeting of mitochondrial proteins such as fumarase and malate dehydrogenase [11], (b) it provides a protective environment for newly synthesized polypeptides destined for endoplasmic reticulum and mitochondria [11–13], and (c) it interacts with unfolded polypeptide chains independent of their amino acid sequence like a typical chaperone and aids in folding of nascent polypeptide chains [14,15]. Inhibitor of cell death-1 gene (*ICD-1*) of *Caenorhabditis elegans* encodes for *βNAC* and its overexpression prevents apoptosis, while RNAi mediated underexpression of *ICD-1* induces inappropriate apoptosis [16]. In *Nicotiana benthamiana*, virus induced silencing of *NbBTF3*, a homolog of *βNAC* caused leaf yellowing, abnormal leaf morphology, and simultaneously reduced the chloroplast sizes and chlorophyll content [17]. Recently, Huh et al. [18] reported involvement of βNAC (*CaBtf3*) isolated from *Capsicum annuum* in regulation of transcription for pathogenesis re-

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lated genes during hypersensitive response to tobacco mosaic virus infection. However, the function of *βNAC* in abiotic stress regulation in plants is not yet studied. In the present work, we characterized the gene "*SaβNAC*" from a halophyte *Spartina alterniflora* and investigated its role toward abiotic stress response. *SaβNAC* was transcriptionally regulated by salt, drought, cold, and ABA. Constitutive overexpression of *SaβNAC* into *Arabidopsis* improved salinity and drought stress tolerance by regulating the ionic homeostasis, osmolyte, and chlorophyll accumulation.

2. Materials and methods

2.1. Plant materials and stress treatment

Three to four-leaf stage uniform, clonally propagated plants of *S. alterniflora* cv. 'Vermilion' grown in sand culture inside a greenhouse with 14 h light and 10 h dark at 26 °C/18 °C day/night temperature, were used for stress experiments [19]. Plants were supplied with Hoagland's nutrient solution [20]. For salinity stress, 5% (w/v) solution of commercial synthetic sea salts (Instant Ocean, Aquarium Systems, OH, USA) dissolved in Hoagland's solution was used. Drought stress was imposed by keeping uprooted plants on Whatman paper under normal growth condition of greenhouse. For cold stress, pots containing plants were kept at 4 °C under dim light. Leaves and roots were collected at 1, 8, and 24 h of stress from at least three representative plants, washed thoroughly with distilled water, wiped with tissue paper, frozen immediately in liquid nitrogen and stored at −80 °C till further use. Unstressed samples at 0 h were harvested as control.

2.2. Bioinformatic sequence analysis

An expressed sequence tag (EST#1588) of *S. alterniflora* obtained from a salt stressed EST library [19], was found to be similar with the NAC β subunit gene hereafter, referred as '*SaβNAC*'. Deduced amino acid sequences corresponding to ORF of *SaβNAC* were used for multiple sequence alignment with orthologs from different organisms using ClustalW program (www2.ebi.ac.uk/clustalw), and phylogenetic analyses were performed in MEGA 4 using the neighbor-Joining method [21]. The bootstrap consensus tree inferred from 1000 replicates was used to represent the evolutionary history of the selected species.

2.3. RNA isolation and cDNA synthesis

Total RNA was isolated using an RNeasy plant midi kit (Qiagen, USA) followed by on-column DNase I digestion to avoid the possible contamination of genomic DNA. Quality of total RNA was checked in a 1.2% formamide-denaturing agarose gel and quantification was done using ND-1000 spectrophotometer (Nanodrop Technologies, USA). First strand cDNA synthesis was carried out using iScript™ cDNA synthesis kit (Bio-Rad, USA).

2.4. Quantitative real time PCR (qRT-PCR)

Quantitative RT-PCR was performed [22] to evaluate the expression levels of *SaβNAC* under different stress treatments in the leaves and roots of *S. alterniflora*. Briefly, each 10 μl of PCR contained 5 μl 2× SYBR Green Mix (Quanta Bioscience, USA), diluted cDNA, and 0.4 μM of gene specific primers, *SaβNAC*RTF and *SaβNAC*RTR (Supplementary Table S1), while tubulin gene of *S. alterniflora* amplified by primers, *SaTUB*RTF and *SaTUB*RTR (Supplementary Table S1), was used as an internal control for normalization of expression in different cDNA samples. Melt curve analysis was performed to check the specificity of amplified product and relative gene expression levels were determined using the $2^{\Delta\Delta C_T}$ method [23]. The C_T (cycle threshold) values for both the target and internal control genes were means of three technical replicates. At least two biological replicates were used for the analysis of gene expression in different samples.

2.5. Generation of transgenic plants

The complete ORF of *SaβNAC* was amplified by PCR using forward primer *SaβNACBglIIF* and reverse primer *SaβNACSpeIR* (Supplementary Table S1) containing the *Bgl*II and *SpeI* restriction sites, respectively, with *Pfu* DNA polymerase (New England Biolab, USA). The obtained PCR product was digested with *Bgl*II and *SpeI* and cloned into pCAMBIA1304 vector (CAMBIA, Australia), to generate the binary vector *35S:SaβNAC*. The identity and orientation of *SaβNAC* in *35S:SaβNAC* was further verified by DNA sequencing. *35S:SaβNAC* construct was introduced into *Agrobacterium* strain LBA4404 by freeze thaw method and transferred into wild type (WT) Columbia ecotype of *Arabidopsis* by floral dip method [24]. Positive transgenic lines were screened on 40 mg/L hygromycin containing MS medium [25] and integration of transgene in the genome of *Arabidopsis* was confirmed by PCR using vector specific forward primer pCAMF, and *SaβNAC* specific reverse primer *SaβNACSpeIR*. Expression of the transgene was confirmed by RT-PCR using cDNA made from total RNA isolated from positive *SaβNAC* transgenic plants. Transgenic *Arabidopsis* lines #1 and #3 of T3 generation were further used for stress related experiments.

2.6. Salinity and drought tolerance assay

Seeds of WT and *SaβNAC Arabidopsis* were sown in *Arabidopsis* potting medium PM-15-13 (Lehle seeds, USA) for stress experiments. Two week old plants were supplied with 150 mM of NaCl for salinity stress, and drought stress was provided by withholding water. Photographs were taken after 15 and 12 days of salinity and drought stress, respectively. Rosette leaves harvested at different time points were used for various physiological and biochemical assays. At least, three independent experiments with three replicates for each WT and *SaβNAC* plants were performed.

2.7. Measurement of electrolyte leakage (EL), total chlorophyll and proline

Two week old WT and transgenic *Arabidopsis*, grown under non-stress and stress (salinity and drought) conditions for next 10 days, were harvested and used for physiological and biochemical measurements. Electrolyte leakage was measured as suggested by Bajji et al. [26]. Briefly, 100 mg leaves were placed in 25 mL distilled water, shaken on a gyratory shaker (200 rpm) at room temperature for 2 h, and the initial conductivity ($C1$) was measured with a conductivity meter (VWR, USA). The samples were then boiled for 10 min to induce maximum leakage and when the sample cooled down at room temperature, the final conductivity ($C2$) was measured. Relative electrical conductivity ($C\%$) was calculated as $(C1/C2) \times 100$. Total chlorophyll from WT and *SaβNAC* lines was estimated following the protocol of Arnon [27]. About 100 mg of fine powder of leaf tissue was homogenized in 1 mL of 80% acetone and kept for 15 min at room temperature in dark. The crude extraction was centrifuged for 20 min at 10,000 rpm (rotation per minute) at room temperature, and the resultant supernatant was used for assessing absorbance at 663 and 645 nm with a spectrophotometer (Shimadzu UV-1600, Japan), and total chlorophyll content was computed in terms of fresh weight (FW). For Proline estimation, protocol of Bates et al. [28] was used. Free proline contents of WT and transgenic plants were measured using fresh leaf tissues of non-stress, salinity and drought stressed plants. Around

100 mg of tissues were used and extracted in 5 mL of 3% sulfosalicylic acid at 95 °C for 15 min. After filtration, 2 mL of supernatant was transferred to a new tube containing 2 mL of acetic acid and 2 mL of acidified ninhydrin reagent. After 30 min of incubation at 95 °C, samples were kept at room temperature for 30 min, and 5 mL of toluene was added to the tube with shaking at 150 rpm to extract red products. The absorbance of the toluene layer was determined at 532 nm using spectrophotometer. Standard curve prepared using different concentrations of proline was used for measuring free proline content in experimental samples from three independent experiments.

2.8. Na^+ and K^+ estimation

Leaf tissues harvested from unstressed, salt-stressed plants (2 week old plants treated with 150 mM NaCl for 10 days) of WT and transgenic *Arabidopsis* plants, oven-dried at 65 °C for 48 h were used for ion estimation. Around 50 mg of oven dried tissues were digested with 0.1% HNO_3 at 100 °C for 45 min. Ions were extracted in distilled H_2O and Na^+ and K^+ were measured using inductively coupled plasma-mass spectrometry (ICP-MS, Perkin-Elmer Plasma 400 emission spectrometer).

3. Results

3.1. Molecular characterization of SaβNAC

The full-length *SaβNAC* cDNA clone was 727 bp in length with 129 bp of 5′ untranslated region, 477 bp of open reading frame (ORF), and 121 bp of the 3′ untranslated region (Fig. 1A). The ORF encoded a polypeptide of 158 amino acid residues with a predicted molecular mass of 17.16 kD and p*I* value of 9.33. The NAC domain was present between 36 and 92 amino acid residues. The deduced amino acid sequence of SaβNAC showed higher identity (89–92%) with βNAC of *Brachypodium distachyon*, *Hordeum vulgare*, *Sorghum bicolor*, and *Oryza sativa*, and lower identity (68–76%) with *Zea mays*, *Medicago truncatula*, *Musa acuminata*, *Glycine max*, *Lotus japonicas*, *Populus trichocarpa*, *Solanum lycopersicum*, *G. max*, and *Arabidopsis thaliana*, while very low identity with *Homo sapiens* (48%) and *S. cerevisiae* (31%) (Fig. 1B). Phylogenetic analysis using MEGA software reflected that SaβNAC grouped with monocots and shared high degree of homology with counterpart proteins from dicots, human and yeast, indicating the evolutionary conserved relationship of βNAC proteins and possibly evolved from a common ancestor (Fig. 1C). Tertiary structure prediction using SWISS MODEL revealed that the SaβNAC sequence forms three alpha-helices and four beta-pleated sheets (Fig. 1D).

3.2. Expression of SaβNAC is regulated by multiple stress conditions

Quantitative RT-PCR was used to analyze the expression patterns of *SaβNAC* in leaves and roots of *S. alterniflora*. It was constitutively expressed in both leaves and roots but differentially expressed under abiotic stresses such as salt, drought, cold, and ABA. In leaves, salinity and drought increased its expression within 1 h of stress, while cold downregulated its expression up to 8 h before reaching to basal level and ABA did not alter the expression. Under salt stress, *SaβNAC* expression was gradually increased in both tissues, but transcript upregulation rate was more in leaves starting from 8 h up to 24 h of stress. In roots, salt stress, and ABA gradually upregulated its expression up to 24 h, while drought stress showed immediate two fold upregulation and maintained its expression level until 24 h of stress. Cold stress slightly upregulated its expression in roots only at 24 h (Fig. 2). Above expression analysis indicated stress regulated organ specific expression of *SaβNAC* in *S. alterniflora*.

3.3. Overexpression of SaβNAC improved salinity tolerance of Arabidopsis

SaβNAC ORF was cloned into binary vector and constitutively expressed under 35S promoter (Fig. 3A) in *Arabidopsis* ecotype Columbia to investigate its role toward salinity tolerance in plant. T3 homozygous *SaβNAC Arabidopsis* lines (T#1 and T#3) with higher expression of *SaβNAC* were analyzed for stress experiments. When plants were irrigated with 150 mM of NaCl, leaves of WT plants started yellowing (Fig. 3B), while *SaβNAC* plants showed less bleaching, i.e., more chlorophyll content than WT plants on 15 days of stress (Fig. 3C). Transgenic *Arabidopsis* plants also retained more proline and showed improved ionic homeostasis under salinity stress (Fig. 3D and E).

3.4. Overexpression of SaβNAC improved drought tolerance by maintaining membrane integrity

Drought tolerance of *SaβNAC Arabidopsis* plants was tested by continuous withholding of water. Leaves of WT plants completely lost turgidity after 12 days of stress compared to transgenic plants (Fig. 4A). *SaβNAC Arabidopsis* leaves showed more chlorophyll and proline accumulation under continuous drought stress (Fig. 4B and C) and maintained its membrane integrity as revealed by less electrolyte leakage percentage in comparison to WT plants (Fig. 4D).

4. Discussion

Nascent polypeptide associated complex (NAC) plays an important role in binding with newly synthesizing polypeptides emerging from the ribosome for nascent peptide targeting, and preventing the peptides from incorrectly binding with other cytosolic factors [2]. Functions of NAC have been studied in control of protein translocation from ribosome to endoplasmic reticulum or into mitochondria. In eukaryotes, NAC is a heterodimer of two different subunits, α-NAC and βNAC encoded by different genes, while archaebacterial genomes have only one gene homologous to α-NAC and forms homodimer [2,29]. NAC mutation in mice, fruit fly, and *C. elegans* induced embryonically lethal phenotypes [16,30,31], which suggested important role of NAC in embryonic development. Similarly, variation in relative concentration of two NAC subunits observed in patients of Alzheimer's disease, Down's syndrome, malignant brain tumors, AIDS, and ulcerative colitis [32–35], indicated the role of NAC in disease manifestations. Although the function of NAC has been studied in animal system, very few reports are available in plants. Silencing of *NbBtf3* in *N. benthamiana* showed abnormal developmental phenotype by repressing the transcript levels of some plastid and mitochondria encoded genes [17], and a recent report on involvement of *CaBtf3* in hypersensitive response such as cell death during tobacco mosaic virus infection in *C. annuum* [18]. In the present study, we studied a *SaβNAC* gene of a halophytic plant, *S. alterniflora* and investigated its possible role in abiotic stress regulation.

SaβNAC is nascent polypeptide associated domain containing protein and its deduced proteins sequence analysis showed high homologies (~90%) with corresponding proteins in monocots except *Z. mays*, relatively less (~70%) homologies with dicots, and around 30–40%, with yeast and human. Furthermore, phylogenetic analysis revealed a clade containing monocots except *Z. mays*, which could be seen with another clade of dicots, whereas yeast and human separated earlier from the monocots and dicots. Thus,

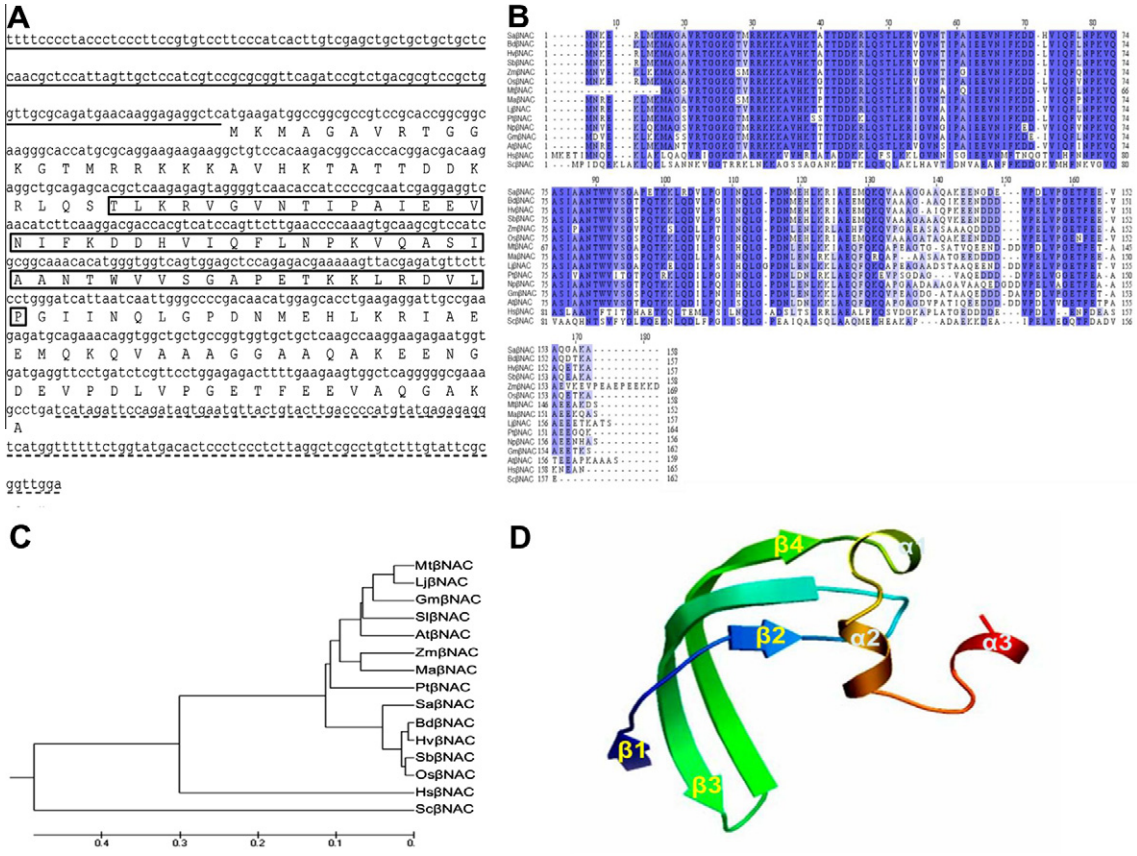

Fig. 1. Sequence analyses, phylogenetic tree, and predicted tertiary structure of SaβNAC protein. (A) Nucleotide sequence and the deduced amino acid sequence of *SaβNAC*. The NAC domain is shown within rectangle. 5′ and 3′ UTR are indicated with solid and dashed underlines, respectively. (B) Multiple sequence alignment of SaβNAC protein with βNAC proteins from different organisms. Accession numbers of sequences are, Sa: *Spartina alterniflora*; Bd: *Brachypodium distachyon* (XP_003562301); Hv: *Hordeum vulgare* (BAJ84846); Sb: *Sorghum bicolor* (XP_002466094); Zm: *Zea mays* (ADD91323); Os: *Oryza sativa* (NP_001051911); Mt: *Medicago truncatula* (XP_003606991); Ma: *Musa acuminata* (AAT67244); Lj: *Lotus japonicus* (CAE45592); Pt: *Populus tricocarpa* (XP_002305248); Sl: *Solanum lycopersicum* (NP_001234229): Gm: *Glycine max* (NP_001237824); At: *Arabidopsis thaliana* (NP_177466); Hs: *Homo sapiens* (NP_001198); Sc: *Saccharomyces cerevisiae* (NP_015288). (C) Phylogenetic tree of SaβNAC. The deduced amino acid sequences were subjected to Bootstrap test of phylogeny by the MEGA 4.0 program, using neighbor-joining method with 1000 replicates. (D) Model of predicted tertiary structure was performed using SWISS-MODEL based on crystallographic data deposited in the Swissprot.

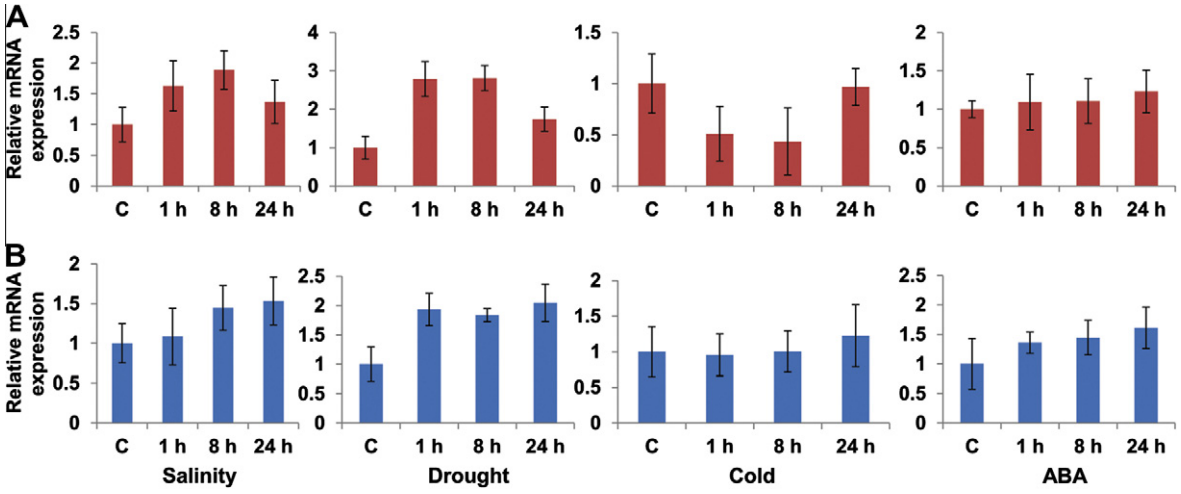

Fig. 2. Expression kinetics of *SaβNAC* in leaves and roots of *S. alterniflora* in response to various stresses. Expression patterns of *SaβNAC* at different time intervals in leaves (A) and roots (B) under salinity stress (5% sea salt), drought (kept on Whatman paper), cold (at 4 °C) and ABA (100 μM). Samples were harvested at indicated time intervals, i.e., 1, 8, and 24 h. C: represents control samples harvested before stress treatments. Tubulin gene of *S. alterniflora* was used as an internal control for normalization of different cDNA samples. Error bars are mean ± standard deviation of three independent reactions. h represents time in hour.

SaβNAC may evolve along with monocots and possibly shares function with other organisms.

Differential regulation of *SaβNAC* by salt, drought, cold, and ABA in leaves and roots of *S. alterniflora* suggested its stress responsive behavior. We also tested its putative function toward abiotic stress tolerance in model plant *Arabidopsis*. Constitutive overexpression of *SaβNAC* in *Arabidopsis* increased chlorophyll and proline content under both salinity and drought stress indicating its possible role

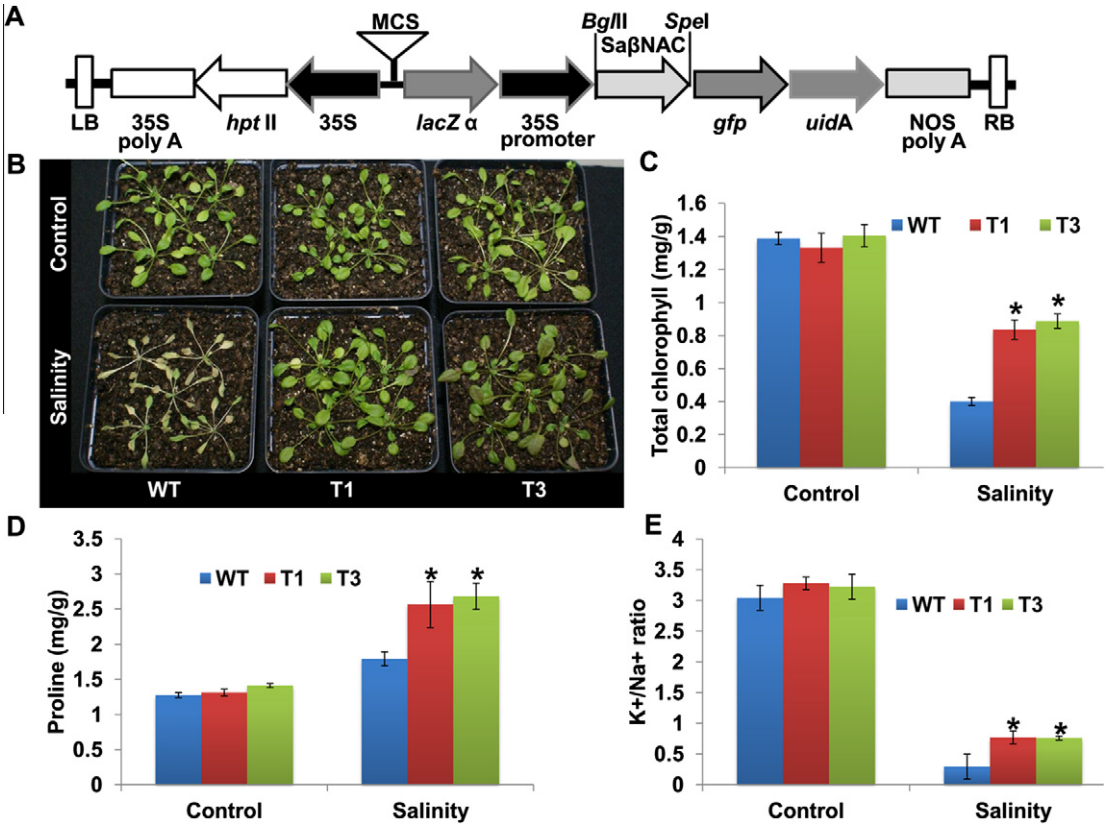

Fig. 3. Salinity stress tolerance assay of *SaβNAC* transgenic *Arabidopsis* plants. (A) map of binary vector used for *Arabidopsis* transformation (B) Two week old WT (Columbia ecotype) and transgenic plants grown under normal conditions were irrigated with 150 mM of NaCl once in a day and photographs were taken after fifteen days of stress. (C) Chlorophyll, (D) proline, and (E) K^+/Na^+ ratio, were estimated in rosette leaves of 2 week old WT and transgenic plants grown under normal conditions after seven days of 150 mM NaCl stress. Comparison was made between WT and individual transgenic lines under control condition or salinity stress by paired t-test. *Indicates significant differences in comparison with the control at $P < 0.05$. T1 and T3 represent two independent SaβNAC transgenic *Arabidopsis* lines.

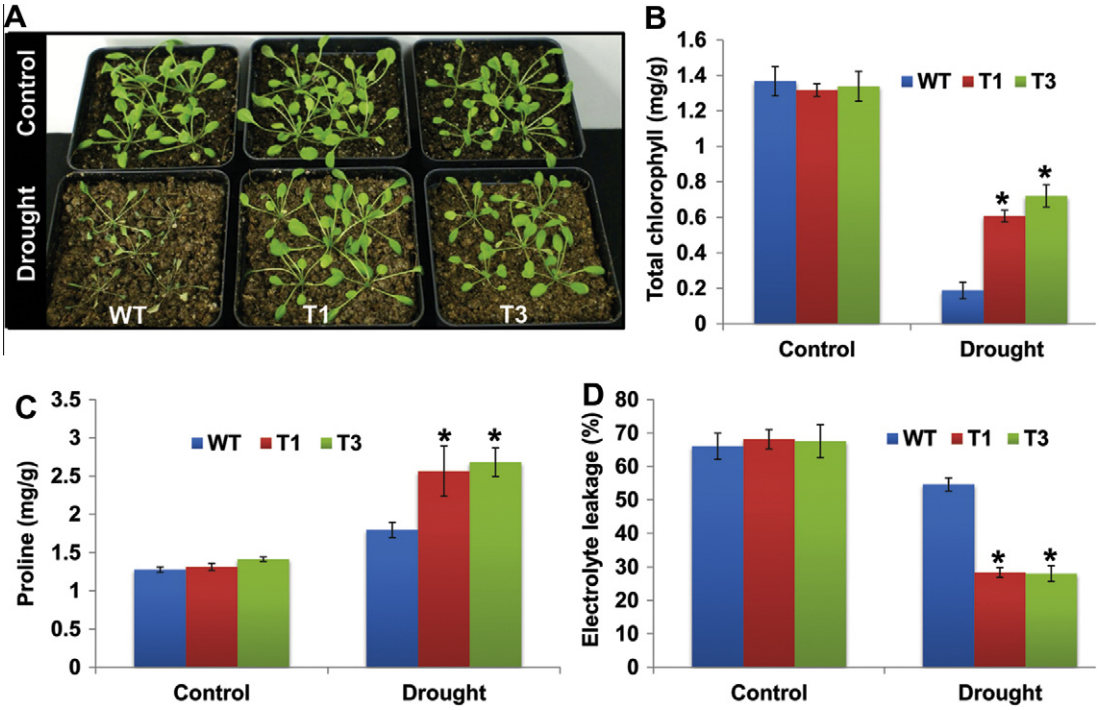

Fig. 4. Drought tolerance assay of *SaβNAC* transgenic *Arabidopsis* plants. (A) Two week old WT (Columbia ecotype) and transgenic plants were withheld for irrigation for 12 days and photograph was taken. (B) Chlorophyll, (C) proline, and (D) electrolyte leakage, were estimated from the rosette leaves of WT and transgenic plants after seven days of continuous drought. Comparison was made between WT and individual transgenic lines under control condition or drought stress by paired *t*-test. *Indicates significant differences in comparison with the control at $P < 0.05$. T1 and T3 represent two independent *SaβNAC* transgenic lines.

in protecting photosynthetic apparatus and production of osmolytes. *SaβNAC* expression in *Arabidopsis* regulated the K^+/Na^+ ratio and leakage of electrolyte under salinity and drought stress respectively. Thus, *SaβNAC* may have a indirect role in maintenance of ionic homeostasis and protection of plasma membrane against damage of stress. Maintenance of K^+/Na^+ homeostasis is an important aspect of salinity tolerance and transgenic plants having higher K^+/Na^+ levels are able to tolerate more salinity stress [36–38]. Proline is an important osmolyte and its increased accumulation under stress is an important mechanism for stress adaptation in plants [39]. Our results suggest that enhanced abiotic stress tolerance might be due to the important role played by *SaβNAC* in protecting the newly synthesized polypeptides involved in various stress tolerance mechanisms from abiotic stress induced damage and inhibiting cell death in plant. This is a first report demonstrating the involvement of *βNAC* in abiotic stress response in plant. Further research is needed to provide insight into the functions of *βNAC* and the associated mechanisms that are responsible for enhancing abiotic stress tolerance in plants.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2012.07.023.

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