The vacuolar proton pyrophosphatase gene (*SbVPPase*) from the *Sorghum bicolor* confers salt tolerance in transgenic Brahmi [*Bacopa monnieri* (L.) Pennell]

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Abstract Plants overcome the effect of Na⁺ toxicity either by excluding Na⁺ at the plasma membrane or by sequestering them into the vacuoles. Influx of Na⁺ ions into the plant vacuoles is usually driven by H⁺ generated by vacuolar-type H⁺-ATPase as well as vacuolar proton pyrophosphatase (VPPase). In the present study, we have developed *Bacopa monnieri* transgenics via *Agrobacterium tumefaciens* containing the recombinant vector pCAMBIA2300-*SbVPPase* gene. Transformants were produced using nodal explants. Transformants were confirmed by PCR and DNA blot analysis. qPCR analysis showed higher transcript levels of *SbVPPase* compared to untransformed control (UC). Higher VPPase activity was recorded in transgenics compared to UC. Under 150 mM salt stress, transgenic shoots showed enhanced Na⁺ accumulation with better biomass production, increased glycine betaine content, and total soluble sugar levels than UC. Transgenic shoots showed 2.9–3.8-folds lower levels of malondialdehyde content indicating lesser membrane damage. Increase in antioxidant enzyme activities (1.4–3.2-folds) was observed in transgenics compared to UC. Transgenics also displayed 7.3–9.0-folds enhanced accumulation of the medicinally important compound bacoside A. Increased biomass production,

accumulation of Na⁺, osmolytes (glycine betaine, sugars etc.), and elevated antioxidant enzyme activities indicate better osmotic adjustment in transgenics by compartmentalization of Na⁺ into the vacuoles under salt stress conditions. Thus, overexpression of *SbVPPase* in *Bacopa* alleviated salt stress by sequestering Na⁺.

Keywords Antioxidant enzymes · *Bacopa monnieri* · Bacoside A · NaCl stress · *SbVPPase* · Transgenic plants

Introduction

Ever increasing soil and water salinity stress results in reduced crop productivity. During NaCl stress, ion imbalance occurs in cytosol with increased Na⁺ accumulation and decreased levels of Ca²⁺, K⁺, and Mg²⁺ (Aleman et al. 2011). Vacuolar H⁺-pyrophosphatase (VPPase), a unique electrogenic proton pump uses pyrophosphate (PPi) as a cheap source of substrate and acidifies vacuoles in plant cells (Maeshima 2000). During salt stress, proton flux across the vacuolar membrane is maintained by VPPase along with vacuolar H⁺ ATPase (VATPase). Besides salt stress, nutrient deficiency also increases both PPi hydrolysis and PPi-dependent proton pump in plants. Under salt stress, increased transcript levels of vacuolar H⁺-PPase was observed in *Suaeda salsa*, *Atriplex halimus*, and barley (Fukuda et al. 2004; Guo et al. 2006; Khedr et al. 2011). Tobacco overexpressing *Thellungiella halophila* VPPase (*TsVP*) displayed salt tolerance along with the accumulation of Na⁺ (Gao et al. 2006). Similarly, transgenic cotton overexpressing *Arabidopsis* vacuolar proton pyrophosphatase *AVP1*, also displayed enhanced tolerance to salt with accumulation of Na⁺ (Pasapula et al. 2011). Overexpression of *AVP1*, *ScVP*, and *KfVP1* genes in

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Arabidopsis thaliana showed salt tolerance due to tonoplast ion transport (Gaxiola et al. 2001; Liu et al. 2011; Yao et al. 2012). Overexpression of *OsVPI* and *OsNHX1* increased tolerance to salt and drought in rice (Liu et al. 2010). Gaxiola et al. (2012) discussed at length about the use of transgenic plants harboring vacuolar H^+ -PPase under phosphorous-deficient soils. Overexpression of *SbVPPase* in finger millet displayed enhanced salt stress tolerance and increased activity of antioxidative enzymes (Anjaneyulu et al. 2014).

Bacopa monnieri (L.) Pennell is a popular neurotonic herb, commonly called as Brahmi. It is a small, common, amphibious plant growing in marshy areas throughout the Indian subcontinent and widely distributed in warmer regions of America and Australia. It has been reported to have the memory-enhancing effects due to the presence of tripterpenoid saponins bacoside A and bacoside B which act as cholinergic system (Nathan et al. 2001; Deepak and Amit 2004). With increasing concentration of NaCl, considerable decrease in fresh and dry weights was observed in *Bacopa* (Ahire et al. 2013). So far, meager information is available on genetic transformation for any specific trait in *B. monnieri*.

In the present study, we report the development of salt tolerant transgenic *B. monnieri* by *Agrobacterium*-mediated genetic transformation using *SbVPPase* gene. Molecular characterization, growth responses, biochemical, and physiological analysis confirm that overexpression of *SbVPPase* improves salt tolerance in transgenic *B. monnieri* when compared to untransformed controls (UC).

Materials and methods

Genetic transformation and selection of the transformants

The nodal explants obtained from in vitro cultures were used for the transformation studies (Ahire et al. 2012). *A. tumefaciens* strain LBA4404 harboring binary vector pCAMBIA2300-*SbVPPase* (NCBI accession no. GQ469975) under the control of *CaMV35S* promoter and

NOS PolyA terminator with *npfII* as selection marker was used for transformation (Fig. 1). The *Agrobacterium* strain was grown in 100 ml of yeast extract, mannitol (YEM) medium supplemented with 50 mg/l kanamycin at 28 °C. The overnight grown bacterial culture was pelleted and resuspended in 100 ml MS medium supplemented with 100 mg/l acetosyringone and used for transformation.

The nodal explants were swirled in *Agrobacterium* culture for 10–15 min and transferred onto the co-cultivation medium (MS with 1 mg/l BA + 100 mg/l acetosyringone). After co-cultivation in dark for 2 days, the nodal explants were washed thoroughly with MS basal medium containing 250 mg/l cefotaxime and transferred onto the selection medium (MS with 1 mg/l BA, 100 mg/l kanamycin, 500 mg/l cefotaxime). Explants were subcultured once in every 15-day for 2 months. The putative transgenics were transferred onto rooting medium (MS medium with 0.2 mg/l α -naphthaleneacetic acid (NAA), 100 mg/l kanamycin and 250 mg/l cefotaxime). Independent putative transgenics were transferred to plastic containers containing autoclaved garden soil and sand in equal ratio. After 3 weeks of acclimatization, plantlets were transferred to the earthen pots containing garden soil and maintained in the glasshouse (25 ± 2 °C temperature; $80 \pm 10\%$ humidity; $200 \mu\text{mol m}^{-2} \text{s}^{-1}$ light intensity). All the putative transgenics were watered three times a day with tap water.

Molecular characterization of the transformants

Genomic DNA was isolated from putative transgenics and UC (Doyle and Doyle 1990). Transgene integration was confirmed by PCR using gene specific primers. The PCR reaction was set to 12.5 μl with 0.5 μl DNA, 2.5 μl 5X PCR buffer, 0.75 μl MgCl_2 , 0.25 μl dNTP, 0.25 μl each of forward (5'-ATCCAGAAGGCCATCTCCGAAGGA-3') and reverse (5'-CTTCATGGTCATGGCTGAGAACCA-3') primers, 0.25 μl Taq polymerase and final volume was made with MQ water. Amplification was performed in a Thermal Cycler (Corbett Research, Australia) with the following program (94 °C for 5 min, 94 °C for 30 s, 59 °C for 30 s, 72 °C for 80 s, 72 °C for 8 min) for 35 cycles.

Fig. 1 Recombinant vector map of pCAMBIA2300-*SbVPPase* gene. *NOS PolyA* terminator. *npfII* neomycin phosphotransferase II (marker gene). *CaMV35S* cauliflower mosaic virus 35S (promoter). *SbVPPase*—*Sorghum bicolor* vacuolar proton pyrophosphatase gene

Southern blot was performed by following Sambrook and Russell (2001). The pCambia2300-*VPPase* plasmid served as positive control. Gene integration was confirmed by digesting genomic DNA (20 µg) with *Hind*III and probed with *SbVPPase* sequence.

Relative quantification of gene expression using qRT-PCR

Total RNA was extracted from the UC and transgenic lines using RNAiso Plus (Total RNA extraction reagent, TAKARA Bio Inc.). One µg of RNA was used for cDNA synthesis (MuMLV-RT, Invitrogen, USA) following manufacturer's instructions. Experiments were repeated twice and each time two replicates were taken. Quantitative real-time PCR (qRT-PCR) was performed using cDNA as template. PCR reaction was performed using *SbVPPase* gene specific PCR primers (F: 5'-TGGTTCTCTGCCATGACCAT-3'; R: 5'-AACAAATGAGGGGAGTGAGCA-3') and 18S gene primers (F: 5'-TAAAGCAGACCCGCGAACAT-3'; R: 5'-TCTTCATCGATGCGAGAGCC-3'). q-PCR was performed with ABI 7900 HT fast Real-Time PCR System (Applied Biosystems, CA, USA) using SYBR Green dye. PCR products and results were analyzed by ABI StepOne Software version 2 (Applied Biosystems, CA, USA). The change in expression of the *SbVPPase* target gene was normalized to 18S. The comparative $2^{-\Delta\Delta CT}$ method was used to calculate the relative quantities of each transcript in the samples (Schmittgen and Livak 2008).

Assay of inorganic phosphate

Free Pi liberated from PPi was measured as described by Ames (1966). The free phosphate liberated by the enzyme resulting in a colorimetric product, was measured at 820 nm, proportional to the enzyme activity present. VPPase activity was defined as nmol/mg protein/min.

Evaluation of transgenic shoots under NaCl stress

For evaluation to salt tolerance, transgenics and UC were treated with 150 mM NaCl in 50 ml liquid MS containing 1 mg/l BA in culture glass bottles for 28-days. About 3 shoots (5 nodes each; viz. total 15 nodes/bottle) of transgenics and UC were inoculated per culture and evaluated after 28-days.

Growth analysis

Morphological observations were recorded for number of shoots per culture, shoot length (cm), fresh weight (FW), and dry weight (DW).

Determination of ion content

Na⁺ and K⁺ contents were analyzed in the transgenics along with UC treated with 150 mM NaCl. Dried shoot biomass was ground to powder and 200 mg powder from each treatment was acid digested in 35% (v/v) HNO₃ at 100 °C for 30 min. Na⁺ and K⁺ ions were measured by atomic absorption spectrophotometer (Lokhande et al. 2011).

Determination of lipid peroxidation and osmolyte content

For determination of lipid peroxidation and osmolyte content, freshly harvested shoot biomass was taken. Lipid peroxidation, total soluble sugars (TSS), and glycine betaine content were measured as described by Lokhande et al. (2011). Proline was estimated according to Bates et al. (1973).

Determination of antioxidant enzyme activities

NaCl treated transgenic and UC shoots (500 mg) were homogenized in 5 ml of ice cold 50 mM sodium phosphate buffer (pH 7.0) containing 0.1 mM EDTA and 1% (w/v) polyvinylpyrrolidone using chilled mortar and pestle. Homogenate was centrifuged at 10,000 g for 20 min at 4 °C. The supernatant was used as a crude enzyme(s) for determination of antioxidant enzyme activities. Soluble protein content in the enzyme extract was determined according to Lowry et al. (1951). Superoxide dismutase (SOD) enzyme (EC 1.15.1.1) activity was assayed according to Becana et al. (1986) by inhibition of the photochemical reduction of nitroblue tetrazolium. Catalase (CAT) enzyme (EC 1.11.1.6) activity was measured by following the decomposition of hydrogen peroxide (H₂O₂) as described by Cakmak and Marschner (1992) with minor modifications (Lokhande et al. 2011). Ascorbate peroxidase (APX) enzyme (EC 1.11.1.11) activity was determined according to Nakano and Asada (1981) and as described by Lokhande et al. (2011). Guaiacol peroxidase (GPX) enzyme (EC 1.11.1.7) activity was assayed according to Hemeda and Klein (1990) with minor modifications to the protocol developed by Ghane et al. (2012).

Estimation of bacoside A

The terpenoid saponin bacoside A was extracted from 1 g of powder samples as per Watoo et al. (2007) with some modifications as described by Parale et al. (2010). HPTLC analysis (CAMAG, Switzerland) was performed on 10 × 10 cm aluminum foil plates coated with 200 µm layer of silica gel 60F254 (E. Merck, Germany). The

standard (Bacoside A; Natural Remedies, Bangalore, India) and samples were loaded with Linomat 5 semiautomatic applicator as bands of 8 mm width. The plates were developed in toluene:ethyl acetate:methanol:glacial acetic acid (4:3:3:1 v/v). The plates were then derivatized in 5% methanolic sulphuric acid followed by drying in an oven for 5 min at 100 °C followed by scanning using CAMAG TLC scanner III at 500 nm. Bacoside A content was quantified by comparing the sample peak area with standard peak area.

Data analysis

A completely randomized design (CRD) was used in all the experiments. All the experiments were repeated at least thrice with ten replicates, unless otherwise mentioned. The data were subjected to analysis of variance (ANOVA) followed by Duncan's multiple range tests (DMRT) at $P \leq 5\%$. The graphs were plotted using GraphPad software V6.01 Version (<http://www.graphpad.com/scientific-software/prism>).

Results

Plant transformation and selection of transformants

Nodal explants obtained from in vitro regenerated shoots were used for transformation. Putative transgenics were obtained on the selection medium after co-cultivation in dark for 2-days. Regeneration frequency in the UC (Fig. 2a) was 100%, but the UC did not survive after 3rd cycle on selection medium (Fig. 2b). Out of 500 nodal explants used for transformation, 378 explants survived after three cycles of selection (Fig. 2c, d) with 75.6% transformation efficiency. Putative transgenic shoots obtained were transferred to selection medium for elongation, growth, and maintenance (Fig. 2e). Well-elongated shoots (5 shoots each from randomly selected 2 events) were transferred to rooting medium. Hundred percent rooting of the shoots was observed on rooting medium (Fig. 2f) and well rooted putative transgenics were transferred to plastic containers for hardening (Fig. 2g). After 3 weeks of acclimatization, the plantlets were transferred to the earthen pots and maintained in the glass house (Fig. 2h).

Molecular characterization of transformants

Putative transgenics were confirmed for the presence of transgene by PCR using gene specific primers. Putative transgenics and plasmid DNA showed the presence of 1555 bp amplicon, which was not observed in UC (Fig. 3). Transgene integration was confirmed by DNA blot, which

showed a distinct hybridizable band in transgenics, but absent in UC (Fig. 4).

Transcript profiling of *SbVPPase* in different transgenics

The expression of *SbVPPase* is higher in T₁ (with RQ 23703.3) followed by T₂ (with RQ 2142.7). Transcript levels were normalized relative to 18s rRNA (Table 1).

Estimation of VPPase

Higher VPPase activity has been recorded in transgenic T₁ (0.328 nmol/mg protein/min) when compared with UC (0.138 nmol/mg protein/min).

Evaluation of transgenics under NaCl stress

After 28-days of 150 mM NaCl stress, transgenics were evaluated for growth, ion analysis, biochemical, antioxidant enzyme activities, and bacoside A content.

Effect of NaCl stress on growth

Significant differences were observed between UC and transgenic shoots when exposed to 150 mM NaCl stress. The shoot number per culture, shoot length, and biomass production was reduced in UC, compared to transgenic lines (Table 2). After 28-days of salt stress, all the transgenic lines exhibited better growth in terms of plant height compared to UC. Shoots of all the transgenics were comparably greener and healthier. T₁ plants exhibited 80.14 ± 4.07 shoots per culture with 7.25 ± 0.65 g of biomass. Shoot length, FW of shoots, and DW among the transgenic lines did not show significant differences compared to UC (Table 2).

Ion analysis under salt stress

Under salt stress, all the transgenic lines accumulated 1.7–2.2-folds more Na⁺ than UC (Fig. 5a). T₁ transgenic line accumulated 2.26-folds more Na⁺ than UC. Similarly, under stress, both the transgenic lines accumulated 1.3–1.6-folds of K⁺ in comparison with UC. T₁ transgenic line accumulated 1.63-folds more K⁺ than UC (Fig. 5b). Devoid of stress, no significant difference in accumulation of Na⁺ and K⁺ was noticed between transgenics and UC (Fig. 5).

Effect of NaCl stress on lipid peroxidation

After 28-days of salt stress, UC accumulated about 2.9–3.8-folds more malondialdehyde (MDA) content compared to transgenics. Transgenic line T₁ showed lower

Fig. 2 Different developmental stages of *Agrobacterium*-mediated transformation of *Bacopa monnieri* using nodal explants. **a** Regeneration of shoots without any selection (*bar* 0.5 mm). **b** Selection of transformed and untransformed explants (100 mg/l kanamycin + 500 mg/l cefotaxime) (*bar* 1.0 mm). **c** Shoot regeneration after 1st cycle of selection on MS + 5 μ M BA + 100 mg/l kanamycin + 500 mg/l cefotaxime (*bar* 1.0 mm). **d** Elongation of shoots of putative transformants on MS medium supplemented with 5 μ M BA and

100 mg/l kanamycin + 500 mg/l cefotaxime (*bar* 1.0 cm). **e** Maintenance of shoots of putative transformants on MS liquid + 5 μ M BA + 100 mg/l kanamycin + 500 mg/l cefotaxime (*bar* 1.0 cm). **f** Rooting of putative transgenic shoots on MS + 1 μ M NAA + 100 mg/l kanamycin + 250 mg/l cefotaxime (*bar* 1.0 cm). **g** Three-week-old in vitro raised plantlets (*bar* 1.5 cm). **h** Three-month-old in vitro raised plantlets in earthen pots containing garden soil (*bar* 2.0 cm)

Fig. 3 PCR confirmation of *SbVPPase* transgenics along with UC. M molecular marker of 1000 bp. UC untransformed control. +C pCAMBIA2300-*SbVPPase* plasmid. T₁ and T₂ transgenic lines

MDA content (Fig. 6). These results indicated that the expression of *SbVPPase* effectively reduced lipid peroxidation caused due to salt stress.

Osmolyte accumulation under NaCl stress

Upon stress imposition, accumulation of proline remained low in the transgenic lines as compared to the UC (Fig. 7a).

Fig. 4 Southern blot analysis of *SbVPPase* transgenics along with UC. UC untransformed control. +C pCAMBIA2300-*SbVPPase* plasmid. T₁ and T₂ transgenic lines

On the contrary, glycine betaine content in transgenic lines significantly increased in comparison with the UC (Fig. 7b). Glycine betaine content was about 1.3-times more in transgenics than in UC (Fig. 7B). Similarly, TSS content increased in transgenics and it was 1.5–1.8-times higher in comparison with UC (Fig. 7c). Devoid of stress, no significant differences in the accumulation of osmolytes were observed in transgenics and UC.

Table 1 Quantification of *SbVPPase* and 18S rRNA *Bacopa monnieri* (endogenous control) gene expressions using $\Delta\Delta Ct$ (threshold cycle) method

Sample	Detector	Threshold cycle (Ct)	Average threshold cycle (ΔCt)	$\Delta\Delta Ct$	Relative quantification (RQ)
UC	<i>SbVPPase</i>	Undetermined	NA	NA	NA
T ₁	<i>SbVPPase</i>	25.19	3.96	– 14.532803	23,703.3
T ₂	<i>SbVPPase</i>	29.49	7.43	– 11.065216	2142.7
UC	18S rRNA	21.50	NA	NA	NA
T ₁	18S rRNA	21.22	NA	NA	NA
T ₂	18S rRNA	22.05	NA	NA	NA

Ct represents mean from two experiments each with two replicates

UC, untransformed control; T₁ and T₂, transgenic lines; NA, not applicable

Table 2 Effect of salt stress on growth and biomass production in UC and transgenic plants

	No. of shoots per culture		Shoot length (cm)		Fresh weight (g)		Dry weight (g)	
	0 mM NaCl	150 mM NaCl	0 mM NaCl	150 mM NaCl	0 mM NaCl	150 mM NaCl	0 mM NaCl	150 mM NaCl
UC	42.71 ± 2.45 ^a	18.57 ± 1.49 ^c	3.57 ± 0.42 ^a	2.58 ± 0.21 ^b	4.89 ± 0.55 ^a	2.83 ± 0.13 ^c	0.36 ± 0.03 ^a	0.25 ± 0.02 ^c
T ₁	43.43 ± 2.93 ^a	80.14 ± 4.07 ^a	3.79 ± 0.30 ^a	6.89 ± 0.41 ^a	5.09 ± 0.44 ^a	7.25 ± 0.65 ^a	0.40 ± 0.02 ^a	0.55 ± 0.04 ^a
T ₂	43.14 ± 2.96 ^a	69.86 ± 6.24 ^b	3.69 ± 0.34 ^a	6.54 ± 0.55 ^a	4.91 ± 0.38 ^a	6.64 ± 0.69 ^b	0.39 ± 0.03 ^a	0.46 ± 0.02 ^b

Data presented in the table are mean ± SE scored from 7 replicates, 28-days after inoculation. Mean followed by different letters within columns are significantly different at $P \leq 0.05$ by Duncan's multiple range test (DMRT)

UC, untransformed control; T₁ and T₂, transgenic lines

Fig. 5 Ion analysis in shoots of *Bacopa monnieri*. **a** Na⁺ content. **b** K⁺ content. The bars with different letters within treatments (0 mM NaCl and 150 mM NaCl) are significantly different according to

Duncan's Multiple Range Test (DMRT). UC untransformed control. T₁ and T₂ transgenic lines. DW dry weight

Effect of NaCl stress on antioxidant enzyme activities

Under salt stress, UC showed lower SOD activity than the transgenic lines. Its activity was enhanced by 1.4–1.5-folds in the shoots of transgenics (Fig. 8a). CAT activity was upregulated by 2.4-folds in transgenics (Fig. 8b). Transgenic lines exhibited 3–3.2-folds higher APX activity than the UC (Fig. 8c). Similarly, GPX activity increased by 1.7–1.8-folds in transgenics (Fig. 8d). However, no

significant differences were detected in transgenics and UC devoid of any stress.

Accumulation of bacoside A, a secondary plant product of medicinal importance under salt stress

Accumulation of medicinally important metabolite bacoside A was 7.3–9-folds higher in transgenic shoots when compared to UC (Fig. 9).

Fig. 6 Effect of salt stress on the accumulation of MDA in shoots of *Bacopa monnieri*. The bars with different letters within treatments (0 mM NaCl and 150 mM NaCl) are significantly different according to Duncan's Multiple Range Test (DMRT). UC untransformed control. T₁ and T₂ transgenic lines. FW fresh weight. MDA Malondialdehyde

Discussion

In the present study, *Bacopa* transgenics were developed using pCambia 2300-*SbVPPase* (Fig. 1). Our transformation results (Fig. 2) corroborate with earlier findings observed by Nisha et al. (2003). Transgene integration

(Figs. 3, 4) into the host plant appears to mitigate the salt stress by sequestering the Na⁺ into the vacuoles. Enhanced transcript levels were observed in transgenics when compared to UC (Table 1). Accordingly, transgenic line showed higher VPPase activity than UC similar to the earlier findings carried out by Darley et al. (1995). Plants when subjected to salinity stress, showed reduced biomass along with the reduced photosynthetic activity. *Bacopa* transgenics exhibited increase in FW, DW, shoot number, shoot length and better biomass than the UC under 150 mM NaCl stress (Table 2). Similar to *SbVPPase*, *AVP1* overexpression in *Arabidopsis* plays an important role in the root development with increase in size and number of rosette leaves (Li et al. 2005). Likewise, Sulian et al. (2008) reported enhanced growth of cotton by over-expressing *TsVP*. Anjaneyulu et al. (2014) reported leaf expansion, reduction (50–70%) in relative water content (RWC), plant height, finger length and width, and grain weight in UC compared with transgenics overexpressing *SbVPPase*.

Na⁺ accumulation increased in the shoots of transgenic lines over the UC under salt stress (Fig. 5a). Upon exposure to salt stress, K⁺ levels decreased in transgenics and UC when compared with the corresponding untreated plants. But, K⁺ levels increased in transgenics in

Fig. 7 Evaluation of transgenic under salt stress. **a** Proline content. **b** Glycine betaine content. **c** TSS in shoots of *Bacopa monnieri*. The bars with different letters within treatments (0 mM NaCl and 150 mM

NaCl) are significantly different according to Duncan's Multiple Range Test (DMRT). UC untransformed control, T₁ and T₂ transgenic lines. FW fresh weight. TSS total soluble sugars

Fig. 8 Activities of antioxidant enzymes. **a** SOD. **b** CAT. **c** APX. **d** GPX in shoots of *Bacopa monnieri*. The bars with different letters within treatments (0 mM NaCl and 150 mM NaCl) are significantly different according to Duncan's Multiple Range Test (DMRT). UC

untransformed control. T₁ and T₂ transgenic lines. SOD superoxide dismutase. CAT catalase. APX ascorbate peroxidase. GPX glutathione peroxidase

Fig. 9 Effect of 150 mM NaCl stress on the accumulation of bacoside A in transgenics and UC. The bars with different letters within treatments (0 mM NaCl and 150 mM NaCl) are significantly different according to Duncan's Multiple Range Test (DMRT). UC untransformed control. T₁ and T₂ transgenic lines. DW dry weight

comparison with UC in presence of NaCl stress (Fig. 5b). Na⁺ ion compartmentation driven by vacuolar Na⁺/H⁺ antiporter in concert with VPPase prevents Na⁺ toxicity and Na⁺ is also used as osmolyte in the vacuole, which alleviates osmotic stress (Li et al. 2011). Bonales-Alatorre et al. (2013) pointed out that Na⁺ also gets sequestered into the vacuoles by the activity of slow (SV) and fast (FV) tonoplast voltage gated channels. Pasapula et al. (2011) also pointed out that accumulation of Na⁺ in vacuoles instead of cytoplasm prevents the toxic effects of excessive

Na⁺ in cytoplasm. Gaxiola et al. (2001) reported the accumulation of Na⁺ and K⁺ in the leaves of transgenic *A. thaliana* overexpressing V-H⁺-PPase. Thus, *SbVPPase* may help the transgenic plants to accumulate easily available inorganic osmotica like Na⁺ and K⁺ for osmotic adjustment. Keeping this in view, Adem et al. (2015) pointed out that plant breeder should incorporate the trait of Na⁺ and K⁺ ion sequestration into the vacuoles of crop plants for better salt stress tolerance.

MDA content was less in transgenic shoots of *Bacopa* than in UC under salt stress conditions (Fig. 6). These results demonstrate that transgenics were subjected to less damage under salt stress. Similarly, reduced MDA content was observed in finger millet transgenics overexpressing *SbVPPase* (Anjaneyulu et al. 2014). The correlation between higher Na⁺ and less membrane damage as evidenced by MDA levels in transgenic *Bacopa* shoots supports the view that the overexpression of *SbVPPase* can enhance the accumulation of Na⁺ in vacuoles and maintain ion homeostasis. Salt stress results in the accumulation of Na⁺ which in turn increase the reactive oxygen species (ROS) leading to oxidative stress. ROS can alter the cellular metabolism and damage the nucleic acids, proteins, and lipids through oxidative stress (Imlay 2003). Plants overcome the oxidative stress by accumulation of osmolytes and effective antioxidative enzyme activities. Proline content in the transgenic shoots was low compared to UC

under stress (Fig. 7a). But, the content of glycine betaine, an important osmolyte was enhanced alongside total sugars (Fig. 7b, c). Thus, it appears that *Bacopa* may not accumulate proline under salt stress, but accumulates glycine betaine and sugars as osmotic agents. Glycine betaine can help to scavenge the ROS in many plants (Chen and Murata 2011). In addition to Na^+ and K^+ as inorganic osmotica, plants accumulate organic osmotica like glycine betaine and sugars under salt stress conditions. *Bacopa* accumulates glycine betaine as an osmoprotectant. Glycine betaine scavenges ROS and increases antioxidative enzyme activities under stress conditions in plants (Islam et al. 2009; Shams et al. 2016). Transgenic *Bacopa* plants perhaps use glycine betaine as an osmoprotectant to enhance antioxidative enzyme activities. Plants contain various K^+ permeable ROS-activated cation channels as pointed out by many studies (Demidchik et al. 2003; Zepeda-Jazo et al. 2011; Velarde-Buendía et al. 2012). Such channels once activated cause K^+ leakage from the cytosol under stress (Shabala 2017). If such an activation comes from H_2O_2 , plants prevent it by enhancing the levels of antioxidative enzyme activities. But, improper scavenging of H_2O_2 results in the interaction of it with transition metals in cell walls causing the formation of hydroxyl radicals. Hydroxyl radicals in turn activate outward K^+ efflux channels that cannot be scavenged by any enzymatic means. Hence, increased levels of soluble sugars may be necessary to counteract hydroxyl radical formation and retention of K^+ in the cell. Accumulation of higher K^+ in *Bacopa* transgenics could be because of better accumulation of sugars. *Bacopa* transgenics showed increased antioxidative enzyme activities when compared with UC shoots under 150 mM NaCl stress (Fig. 8). SOD results (Fig. 8a) corroborate with the transgenic finger millet overexpressing *SbVPPase* (Anjaneyulu et al. 2014). Similarly, increased CAT activity (Fig. 8b) was found at high NaCl concentration in *B. monnieri* (Ahire et al. 2013). APX plays a crucial role in plant defense mechanism against oxidative stress and enhanced APX activity (Fig. 8c) helps in the reduction of H_2O_2 levels in *Bacopa* transgenics similar to finger millet plants (Anjaneyulu et al. 2014). GPXs are one of the vital enzymes of the antioxidant network and are present in various cellular organelles. In transgenic *Bacopa* increased GPX activity (Fig. 8d) was also reported during salt stress treatment similar to maize and sunflower seedlings (Rios-Gonzales et al. 2002; Ahire et al. 2013). Sequestration of Na^+ ions into vacuoles is vital since these ions interfere with normal physiology and reduce the biochemical activities in the cells (Maathuis 2014). Their sequestration into vacuoles on the other hand can help the plants to reduce Na^+ toxicity and in turn improve the antioxidative enzyme activities.

Salt stress effects the overall growth of plants due to enhanced accumulation of Na^+ ions, but a positive effect of salt stress has been noticed on secondary metabolite accumulation in few medicinal plants (Ahire et al. 2013). Salt stress increases the accumulation of secondary metabolites in the callus cultures of soybean (Radhakrishnan et al. 2012). In the present study enhanced accumulation of bacoside A content (Fig. 9) was noticed under the salt stress which corroborates with the earlier findings of Ahire et al. (2013, 2014).

In conclusion, transgenic *Bacopa* overexpressing *SbVPPase* showed increased biomass, higher VPPase activity, higher Na^+ ion accumulation, osmolyte concentration, enhanced antioxidant enzyme activities and lesser lipid peroxidation. Transgenic *Bacopa* overexpressing *SbVPPase* has been found to overcome salt stress by sequestering Na^+ ions into the vacuoles. At the same time, transgenic *Bacopa* plants are able to accumulate higher content of saponin bacoside A, an important medicinal compound under stress.

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Authors' contribution MLA and TDN designed the experiments, and TDN supervised the work. MLA, SAK, DLP and PSM have conducted the laboratory experiments and analysis of the data. The *Agrobacterium tumefaciens* strain with *vacuolar proton pyrophosphatase* (*SbVPPase*) gene was made available by PBK. PBK helped in writing the manuscript.

Compliance with ethical standards

Conflict of interest The authors declare no conflict of interest.

References

- Adem GD, Bose J, Zhou M, Shabala S (2015) Targeting vacuolar sodium sequestration in plant breeding for salinity tolerance. In: Wani SH, Hossain MA (eds) Managing salt tolerance in plants molecular and genomic perspectives. CRC Press, Boca Raton, pp 35–50
- Ahire ML, Patil PP, Kavi Kishor PB, Nikam TD (2012) Micropropagation and assessment of antibiotic selection in vitro of *Bacopa monnieri* (L.) Pennell. Int J Plant Dev Biol 6:34–39
- Ahire ML, Walunj PR, Kavi Kishor PB, Nikam TD (2013) Effect of sodium chloride induced stress on growth, proline, glycine betaine accumulation, antioxidative defense and bacoside A content in in vitro regenerated shoots of *Bacopa monnieri* (L.) Pennell. Acta Physiol Plant 35:1943–1953. <https://doi.org/10.1007/s11738-013-1233-x>
- Ahire ML, Laxmi S, Walunj PR, Kavi Kishor PB, Nikam TD (2014) Effect of potassium chloride and calcium chloride induced stress on in vitro cultures of *Bacopa monnieri* (L.) Pennell. J Plant Biochem Biotech 23:366–378. <https://doi.org/10.1007/s13562-013-0220-z>

- Aleman F, Nieves Cordones M, Martinez V, Rubio F (2011) Root K⁺ acquisition in plants: the *Arabidopsis thaliana* model. *Plant Cell Physiol* 52:1603–1612. <https://doi.org/10.1093/pcp/pcr096>
- Ames BN (1966) Assay of inorganic phosphate, total phosphate and phosphatases. *Methods Enzymol* 8:115–118. [https://doi.org/10.1016/0076-6879\(66\)08014-5](https://doi.org/10.1016/0076-6879(66)08014-5)
- Anjaneyulu E, Reddy PS, Sunita MSL, Kavi Kishor PB, Meriga B (2014) Salt tolerance and activity of antioxidative enzymes of transgenic finger millet overexpressing a vacuolar H⁺-pyrophosphatase gene (*SbVPPase*) from *Sorghum bicolor*. *J Plant Physiol* 171:789–798. <https://doi.org/10.1016/j.jplph.2014.02.001>
- Bates L, Waldren RP, Teare JD (1973) Rapid determination of free proline for water stress studies. *Plant Soil* 39:205–207. <https://doi.org/10.1007/bf00018060>
- Becana M, Moran JF, Iturbe-Ormaetxe I (1986) Iron-dependent oxygen free radical generation in plants subjected to environmental stress: toxicity and antioxidant protection. *Plant Soil* 201:137–147
- Bonales-Alatorre E, Shabala S, Chen ZH, Pottosin I (2013) Reduced tonoplast FV and SV channels activity is essential for conferring salinity tolerance in a facultative halophyte, *Chenopodium quinoa*. *Plant Physiol*. <https://doi.org/10.1104/pp.113.216572>
- Cakmak I, Marschner H (1992) Magnesium deficiency and high light intensity enhance activities of superoxide dismutase, ascorbate peroxidase, and glutathione reductase in bean leaves. *Plant Physiol* 98:1222–1227. <https://doi.org/10.1104/pp.98.4.1222>
- Chen THH, Murata N (2011) Glycine betaine protects plants against abiotic stress: mechanisms and biotechnological applications. *Plant Cell Environ* 34:1–20. <https://doi.org/10.1111/j.1365-3040.2010.02232.x>
- Darley CP, Davies JM, Sanders D (1995) Chill-induced changes in the activity and abundance of the vacuolar proton-pumping pyrophosphatase from mung bean hypocotyls. *Plant Physiol* 109:659–665. <https://doi.org/10.1104/pp.109.2.659>
- Deepak M, Amit A (2004) The need for establishing identities of bacoside A, and B, the putative major bioactive saponins of Indian medicinal plant *Bacopa monnieri*. *Phytomed* 11:264–268. <https://doi.org/10.1078/0944-7113-00351>
- Demidchik V, Shabala S, Coutts K, Tester M, Davies J (2003) Free oxygen radicals regulate plasma membrane Ca²⁺ and K⁺ - permeable channels in plant root cells. *J Cell Sci* 116:81–88. <https://doi.org/10.1242/jcs.00201>
- Doyle JJ, Doyle JL (1990) Isolation of plant DNA from fresh tissue. *Focus* 12:13–15
- Fukuda A, Chiba K, Maeda M, Nakamura A, Maeshima M, Tanaka Y (2004) Effect of salt and osmotic stresses on the expression of genes for the vacuolar H⁺-pyrophosphatase, H⁺-ATPase subunit A, and Na⁺/H⁺ antiporter from barley. *J Exp Bot* 55:585–594. <https://doi.org/10.1093/jxb/erh070>
- Gao F, Gao Q, Duan XG, Yue GD, Yang AF, Zhang JR (2006) Cloning of an H⁺-PPase gene from *Thellungiella halophila* and its heterologous expression to improve tobacco salt tolerance. *J Exp Bot* 57:3259–3270. <https://doi.org/10.1093/jxb/erl090>
- Gaxiola RA, Li J, Undurraga S, Dang LM, Allen GJ, Alper SL, Fink GR (2001) Drought and salt tolerant plants result from overexpression of the *AVP1* H⁺ pump. *Proc Natl Acad Sci USA* 98:11444–11449. <https://doi.org/10.1073/pnas.191389398>
- Gaxiola RA, Sanchez CA, Paez-Valencia J, Ayre BG, Elser JJ (2012) Genetic manipulation of a “vacuolar” H⁺-PPase: from salt tolerance to yield enhancement under phosphorus-deficient soils. *Plant Physiol* 159:3–11. <https://doi.org/10.1104/pp.112.195701>
- Ghane SG, Lokhande VH, Nikam TD (2012) Differential growth, physiological and biochemical responses of niger (*Guizotia abyssinica* Cass.) cultivars to water deficit (drought) stress. *Acta Physiol Plant* 34:215–225. <https://doi.org/10.1007/s11738-011-0820-y>
- Guo S, Yin H, Zhang X, Zhao F, Li P, Chen S, Zhao Y, Zhang H (2006) Molecular cloning and characterization of a vacuolar H⁺-pyrophosphatase gene, *SsVP*, from the halophyte *Suaeda salsa* and its overexpression increases salt and drought tolerance of *Arabidopsis*. *Plant Mol Biol* 60:41–50. <https://doi.org/10.1007/s11103-005-2417-6>
- Hemeda HM, Klein BP (1990) Effects of naturally occurring antioxidants on peroxidase activity of vegetable extracts. *J Food Sci* 55:184–185. <https://doi.org/10.1111/j.1365-2621.1990.tb06048.x>
- Imlay JA (2003) Pathways of oxidative damage. *Ann Rev Microbiol* 57:395–418. <https://doi.org/10.1146/annurev.micro.57.030502.090938>
- Islam MM, Hoque MA, Okuma E, Murata Y (2009) Exogenous proline and glycinebetaine increase antioxidant enzyme activities and confer tolerance to cadmium stress in cultured tobacco cells. *J Plant Physiol* 166:1587–1597. <https://doi.org/10.1016/j.jplph.2009.04.002>
- Khedr AHA, Serag MS, Nemat-Alla MM, Abo-Elnaga AZ, Nada RM, Quick WP, Abogadallah GM (2011) A *DREB* gene from the xero-halophyte *Atriplex halimus* is induced by osmotic but not ionic stress and shows distinct differences from glycophytic homologues. *Plant Cell Tissue Organ Cult* 106:191–206. <https://doi.org/10.1007/s11240-010-9906-2>
- Li J, Krichevsky A, Vaidya M, Tzfira T, Citovsky V (2005) Uncoupling of the functions of the *Arabidopsis* VIP1 protein in transient and stable plant genetic transformation by *Agrobacterium*. *Proc Natl Acad Sci USA* 102:5733–5738. <https://doi.org/10.1073/pnas.0404118102>
- Li W, Wang D, Jin T, Chang Q, Yin D, Xu S, Liu B, Liu L (2011) The vacuolar Na⁺/H⁺ antiporter gene *SsNHX1* from the halophyte *Salsola soda* confers salt tolerance in transgenic alfalfa (*Medicago sativa* L.). *Plant Mol Biol Rep* 29:278–290. <https://doi.org/10.1007/s11105-010-0224-y>
- Liu SP, Zheng LQ, Xue YH, Zhang Q, Wang L, Shou HX (2010) Overexpression of *OsVP1* and *OsNHX1* increases tolerance to drought and salinity in rice. *J Plant Biol* 53:444–452. <https://doi.org/10.1007/s12374-010-9135-6>
- Liu L, Wang Y, Wang N, Dong YY, Fan XD, Liu XM, Yang J, Li HY (2011) Cloning of a vacuolar H⁺-pyrophosphatase gene from the halophyte *Suaeda corniculata* whose heterologous over expression improves salt, saline-alkali and drought tolerance in *Arabidopsis*. *J Integr Plant Biol* 53:731–742. <https://doi.org/10.1111/j.1744-7909.2011.01066.x>
- Lokhande VH, Nikam TD, Patade VY, Ahire ML, Suprasanna P (2011) Effects of optimal and supra-optimal salinity stress on antioxidative defence, osmolytes and in vitro growth responses in *Sesuvium portulacastrum* L. *Plant Cell Tissue Organ Cult* 104:41–49. <https://doi.org/10.1007/s11240-010-9802-9>
- Lowry OH, Roenbrough NJ, Farr AL, Randal EJ (1951) Protein measurement with the Folin phenol reagent. *J Biol Chem* 193:265–275
- Maathuis FJM (2014) Sodium in plants: perception, signalling, and regulation of sodium fluxes. *J Exp Bot* 65:849–858. <https://doi.org/10.1093/jxb/ert326>
- Maeshima M (2000) Vacuolar H⁺-pyrophosphatase. *Biochim Biophys Acta* 1465:37–51. [https://doi.org/10.1016/S0005-2736\(00\)00130-9](https://doi.org/10.1016/S0005-2736(00)00130-9)
- Nakano Y, Asada K (1981) Hydrogen peroxide is scavenged by ascorbate-specific peroxidase in spinach chloroplasts. *Plant Cell Physiol* 22:867–880. <https://doi.org/10.1093/oxfordjournals.pcp.a076232>
- Nathan PJ, Clarke J, Lloyd J, Hutchinson CW, Downey L, Stough C (2001) The acute effects of an extract of *Bacopa monnieri*

- (Brahmi) on cognitive function in healthy normal subjects. *Hum Psychopharmacol* 16:345–351. <https://doi.org/10.1002/hup.306>
- Nisha KK, Seetha K, Rajmohan K, Purushothama MG (2003) *Agrobacterium tumefaciens*-mediated transformation of Brahmi [*Bacopa monniera* (L.) Wettst.], a popular medicinal herb of India. *Curr Sci* 85:85–89
- Parale A, Barmukh R, Nikam T (2010) Influence of organic supplements on production of shoots and callus biomass and accumulation of bacoside in *Bacopa monniera* (L.) Pennell. *Physiol Mol Biol Plants* 16:167–175. <https://doi.org/10.1007/s12298-010-0018-6>
- Pasapula V, Shen G, Kuppu S, Peez-Valencia J, Mendoza M, Hou P, Chen J, Qiu X, Zhu L, Zhang X, Auld D, Blumwald E, Zhang H, Gaxiola R, Payton P (2011) Expression of an *Arabidopsis* vacuolar H⁺-pyrophosphatase gene (*AVP1*) in cotton improves drought- and salt tolerance and increases fiber yield in the field conditions. *Plant Biotechnol J* 9:88–99. <https://doi.org/10.1111/j.1467-7652.2010.00535.x>
- Radhakrishnan R, Leelapriya T, Kumari KBD (2012) Effects of pulsed magnetic field treatment of soybean seeds on calli growth, cell damage, and biochemical changes under salt stress. *Bioelectromagnetic* 33:670–681. <https://doi.org/10.1002/bem.21735>
- Rios-Gonzales K, Erdei L, Lips SH (2002) The activity of antioxidant enzymes in maize and sunflower seedlings as affected by salinity and different nitrogen sources. *Plant Sci* 162:923–930. [https://doi.org/10.1016/s0168-9452\(02\)00040-7](https://doi.org/10.1016/s0168-9452(02)00040-7)
- Sambrook JF, Russell DW (2001) *Molecular cloning: a laboratory manual*, 3rd edn. Cold Spring Harbor Laboratory Press, Cold Spring Harbor
- Schmittgen TD, Livak KJ (2008) Analyzing real-time PCR data by the comparative C(T) method. *Nat Protoc* 3:1101–1108. <https://doi.org/10.1038/nprot.2008.73>
- Shabala S (2017) Signalling by potassium: another second messenger to add to the list? *J Exp Bot* 68:4003–4007. <https://doi.org/10.1093/jxb/erx238>
- Shams M, Yildirim E, Ekinici M, Kul R (2016) Exogenously applied glycine betaine regulates some chemical characteristics and antioxidative defence systems in lettuce under salt stress. *Hortic Environ Biotechnol* 57:225–231. <https://doi.org/10.1007/s13580-016-0021-0>
- Sulian L, Zhang K, Gao Q, Lian L, Song Y, Zhang J (2008) Overexpression of an *H1-PPase* gene from *Thellungiella halophila* in cotton enhances salt tolerance and improves growth and photosynthetic performance. *Plant Cell Physiol* 49:1150–1164. <https://doi.org/10.1093/pcp/pcn090>
- Velarde-Buendía AM, Shabala S, Cvikrova M, Dobrovinskaya O, Pottosin I (2012) Salt-sensitive and salt-tolerant barley varieties differ in the extent of potentiation of the ROS-induced K⁺ efflux by polyamines. *Plant Physiol Biochem* 61:18–23. <https://doi.org/10.1016/j.plaphy.2012.09.002>
- Watoo P, Waraporn P, Hiroyuki T, Kanchalee J, Sakchai W, Kornkanok I (2007) Comparison of various extraction methods of *Bacopa monniera*. *Naresuan Univ J* 15:29–34
- Yao M, Zeng Y, Liu L, Huang Y, Zhao E, Zhang F (2012) Overexpression of the halophyte *Kalidium foliatum* H⁺-pyrophosphatase gene confers salt and drought tolerance in *Arabidopsis thaliana*. *Mol Biol Rep* 39:7989–7996. <https://doi.org/10.1007/s11033-012-1645-5>
- Zepeda-Jazo I, Velarde-Buendía AM, Enríquez-Figueroa R, Jayakumar B, Shabala S, Muñiz J, Pottosin I (2011) Polyamines interact with hydroxyl radicals in activating Ca²⁺ and K⁺ transport across the root epidermal plasma membranes. *Plant Phys* 157:2167–2180. <https://doi.org/10.1104/pp.111.179671>