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ORIGINAL RESEARCH PAPER

Improved salt tolerance in tobacco plants by co-transformation of a betaine synthesis gene *BADH* and a vacuolar Na^+/H^+ antiporter gene *SeNHX1*

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Abstract Three types of transgenic tobacco plants were acquired by separate transformation or co-transformation of a vacuolar Na^+/H^+ antiporter gene, *SeNHX1*, and a betaine synthesis gene, *BADH*. When exposed to 200 mM NaCl, the dual gene-transformed plants displayed greater accumulation of betaine and Na^+ than their wild-type counterparts. Photosynthetic rate and photosystem II activity in the transgenic plants were less affected by salt stress than wild-type plants. Transgenic plants exhibited a greater increase in osmotic pressure than wild-type plants when exposed to NaCl. More importantly, the dual gene transformed plants accumulated higher biomass than either of the single transgenic plants under salt stress. Taken together, these findings indicate that simultaneous transformation of *BADH* and *SeNHX1* genes into tobacco plants can enable plants to accumulate betaine and Na^+, thus conferring them more tolerance to salinity than either of the single gene transformed plants or wild-type tobacco plants.

Keywords *BADH* · Betaine · Dual gene transformation · Salt tolerance · *SeNHX1* · Transgenics

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Introduction

Accumulation of osmoprotectants and compartmentation of intracellular Na^+ are two important mechanisms underlying plant tolerance to salt stress (Chen and Murata 2002; Zhu 2003). Betaine, which plays vital roles in osmotic adjustment, stabilization of complex proteins and membrane in vivo, and protecting enzyme activities, is one of the most frequently documented osmoprotectants in many organisms (Sakamoto and Murata 2001). Exogenous application of betaine confers plants tolerance to various abiotic stresses and increase yield under salt conditions (Park et al. 2004; Yang and Lu 2005). In this context, Saneoka et al. (1995) found a positive correlation between the level of endogenous betaine and the degree of salt tolerance in maize. However, some plant species, such as tomato, *Arabidopsis* and tobacco, cannot synthesize betaine (Weretilnyk and Hanson 1990; Nuccio et al. 1998). In higher plants, betaine is synthesized in two steps from choline via betaine aldehyde (Nuccio et al. 1998). The two steps are catalyzed by choline monooxygenase (CMO,

E.C. 1.1.1.) and betaine aldehyde dehydrogenase (BADH, E.C.1.2.1.8.), respectively. Plants over-expressing genes responsible for betaine synthesis have enhanced tolerance to a wide range of abiotic stresses including salt, heat, drought, and chilling (Park et al. 2004; Quan et al. 2004; Su et al. 2006).

Sequestering excessive cytoplasmic Na^+ into the vacuole through the vacuolar Na^+/H^+ antiporter to maintain ionic homeostasis is another important strategy to deal with salt stress (Zhu 2003). The vacuolar Na^+/H^+ antiporter plays an important role in controlling plant salt tolerance as evidenced by the enhanced tolerance to salt stress in various plant species overexpressing Na^+/H^+ antiporters (Zhang et al. 2001; Chen et al. 2007). Moreover, the *Arabidopsis* vacuolar Na^+/H^+ antiporter mutant *nhx1* is more sensitive to salt stress than its wild-type counterpart, and the overexpression of *AtNHX1* complements the phenotype (Apse et al. 2003), suggesting that the vacuolar Na^+/H^+ antiporters are of critical importance in regulating ion homeostasis under salt stress.

Previous studies in plant salt tolerance engineering are mostly restricted to single gene transformation. Actually, tolerance of plants to salt stress is often involved in multigenes rather than single gene, and requires an interplay of many genes (Brini et al. 2007). In the present study, we constructed a binary plasmid containing a vacuolar Na^+/H^+ antiporter gene *SeNHX1* and a betaine synthesis gene *BADH*, which were isolated from halophyte *Salicornia europaea* and salt tolerant plant *Atriplex hortensis*, respectively. Then, the two genes were introduced into tobacco plants via separate transformation or co-transformation, and the salt tolerance of the resultant transgenic plants was evaluated.

Materials and methods

Binary plant expression vector construction and regeneration of tobacco plants

Agrobacterium tumefaciens (LBA4404) carrying a binary vector pBinBS was used for transformation. *Xba*I fragments carrying the *SeNHX1* cDNA from pBI121-Se were recovered and inserted into the *Xba*I site of the plasmid pRTL2, with the recombinant plasmid labeled as pRTL2-Se. *Xho*I digestion was used to determine the sense orientation of the *SeNHX1* gene relative to the 35S promoter of cauliflower mosaic virus (CaMV35S) in pRTL2-Se. The *SeNHX1* expression cassette [*CaMV35S::SeNHX1::35S poly(A)*] was released by the *Hin*dIII restriction enzyme and subcloned into the unique *Hin*dIII site of the binary vector pBin438. The T-DNA sequence of pBin438 contains the *BADH* expression cassette (*CaMV35S::BADH::Nos*) and the kanamycin resistance gene. The resultant plasmid containing *SeNHX1* and *BADH* was named as pBinBS (Fig. 1). This construct was testified by DNA sequencing and transferred into *A. tumefaciens* strain LBA4404. Thereafter, it was introduced into tobacco (*Nicotiana tabacum* cv Wisconsin 38) using the *Agrobacterium*-mediated transformation. As control, genes of *BADH* and *SeNHX1* driven by CaMV35S promoter in binary vector pBin438 and pBI121-Se, respectively, were also introduced into tobacco plants by separate transformation. The regenerated T_0 plantlets were grown into a greenhouse. Seeds were harvested, and then grown on MS medium containing 100 mg l^{-1} kanamycin for selection.

Characterization of transgenic plants

The probes used for Northern hybridization were the full cDNA fragments of *SeNHX1* and *BADH* labeled with α-[^{32}P]-dCTP via the PCR method from plasmid pBinBS (Fig. 1). Hybridization was carried out following standard procedures (Sambrook et al. 1989). The specific primer sets for PCR analysis of *SeNHX1* and *BADH* were: 5′-TCTAGATGGAGGGAATTTGGAGGAGC-3′/5′-GGATCCTGCCTAACTGCCTCGGATT-3′ (for *SeNHX1*); 5′-AGAATGGCGTTCCCAATTCCTGCTC-3′/5′-TTCAAGGAGACTTGTACCATCCCCA-3′ (for *BADH*).

The extraction and purification of betaine were carried out as described by Hitz and Hanson (1980). HPLC was used to determine betaine concentration as described by Chen et al. (2000).

Salt tolerance analysis

The positive transgenic plants were grown on medium containing kanamycin. When the plants

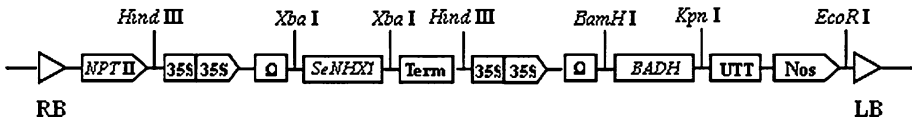

Fig. 1 Schematic representation of the T-DNA region of the binary vector pBinBS harboring genes of *BADH* and *SeNHX1*. RB, right border; LB, left border; *NPT II*, neomycin phosphotransferase; 35S, cauliflower mosaic virus 35S promoter; Ω, TMV translation enhancer; UTT, termination sequence of transcription; Nos, nopaline synthase terminator; Term, 35S poly (A) signal

grew to a height of about 6 cm, they were transplanted to plastic pots containing a mixture of vermiculite, turf and humus (1:1:1; by vol.) in a greenhouse, acclimated for 4 weeks and irrigated with half-Hoagland solution. Thirty days after the transplant, the plants were watered with half-Hoagland solution containing 200 mM NaCl. The plants were irrigated at 5 day intervals up to 4 weeks. The half-Hoagland solution without NaCl was used as control.

At the end of the salt treatment, the top fully expanded leaves were used for measurement of photosynthetic rate, photosystem II (PSII) activity, Na^+ contents, and cell sap osmolality. The net photosynthesis rate was determined with an LI6400 portable apparatus (LI-COR, USA). The PSII activity was measured using a pulse modulation fluorometer (PAM-2000, Walz, Germany), and expressed as Fv/Fm to assess damage to the photosynthetic apparatus in leaves. After measuring the cell sap osmolality using a vapor pressure osmometer (model 5520, Wescor Inc., Logan, Utah, USA), the plants were harvested, dried, and then weighted. Na^+ contents were determined according to Volkov and Amtmann (2006) using a flame photometer (Corning Ltd, Essex, England). The greenhouse average photosynthetically active radiation was above 1000 μmol m^{-2} s^{-1} and temperature was 30 ± 2°C day/28 ± 1°C night. Humidity ranged from 40 to 85%. Six replicates were performed for each treatment.

Results

Transformation of *BADH* and *SeNHX1* genes into tobacco plants

Transgenic tobacco plants overexpressing both *BADH* and *SeNHX1* were generated by infecting leaf discs with engineered *A. tumefaciens* that harbors a binary vector pBinBS (Fig. 1). As control, single genes of *BADH* and *SeNHX1* were also introduced into tobacco plants by separate transformation. DNA blotting showed that genes of *BADH* and/or *SeNHX1* had been integrated into tobacco genome (see Supplementary Figure). As shown in Fig. 2a, the two

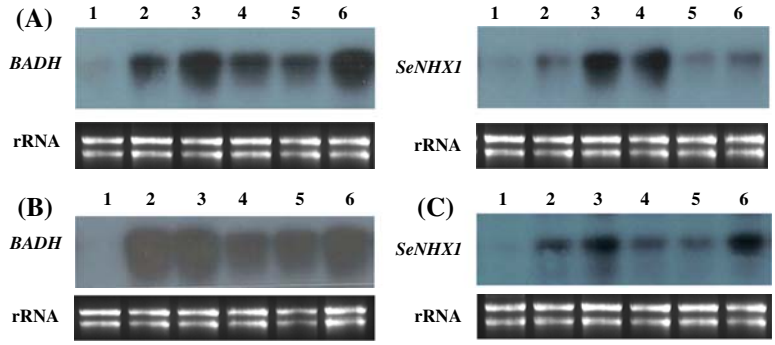

Fig. 2 RNA blotting of *BADH* and *SeNHX1* in tobacco plants. Total RNA was hybridized with α-[32P]-labeled probe for *BADH* or *SeNHX1*. (**a**) Tobacco plants simultaneously transformed with *BADH* and *SeNHX*1. Left, RNA blotting of *BADH*; Right, RNA blotting of *SeNHX1*. Lane 1, wild-type plants; Lanes 2–6, five independent transgenic lines of BS1, BS5, BS15, BS21 and BS23, respectively. (**b**) Tobacco plants transformed with *BADH*. Lane 1, wild-type plants; Lanes 2–6, five independent transgenic lines of B1, B8, B15, B19 and B23, respectively. (**c**) Tobacco plants transformed with *SeNHX1*. Lane 1, wild-type plants; Lanes 2–6 five independent transgenic lines of S1, S8, S11, S15 and S21, respectively

transgenes were simultaneously expressed in each transgenic tobacco plant. Meanwhile, plants stably overexpressing *BADH* or *SeNHX1* were also obtained (Fig. 2b, Fig. 2c). In the subsequent salt tolerance assay, two transgenic lines were used for each transgenic type (lines of S1 and S15 transformed with *SeNHX1*; lines of B8 and B23 transformed with *BADH*; lines of BS5 and BS15 co-transformed with *BADH* and *SeNHX1*).

Transgenic plants exhibit greater amounts of betaine and/or Na^+ under salt stress

To test whether the transformed *BADH* gene is functional, we examined the effect of salt stress on betaine contents in the transgenics. Betaine contents in wild-type and transgenic lines were low and comparable under control conditions (Fig. 3a). However, when these plants were exposed to 200 mM NaCl, the plants overexpressing *BADH* displayed a 6- to 30-fold increase in betaine contents with line B8 being the highest (7.2 μmol/g). By contrast, wild-type plants and plants overexpressing *SeNHX1* alone showed little change in betaine contents upon exposure to the same salt stress (Fig. 3a). These results indicate that the transformed *BADH* gene is functional and underpins the observed betaine accumulation under salt stress.

The function of *SeNHX1* gene is to facilitate Na^+ accumulation into the vacuoles thus minimizing toxic effect of cytosolic Na^+ on plant cells. To test whether the transgenic plants differ from their wild-type plants in terms of Na^+ accumulation under salt stress, Na^+ contents in leaves of the transgenic and wild-type plants were measured. As shown in Figure 3b, there were no significant differences in Na^+ contents between the transgenic lines and the wild-type plants grown in the absence of NaCl. An increase in Na^+ contents in both the transgenic and wild-type plants was observed when exposed to 200 mM NaCl (Fig. 3B). When the plants were exposed to NaCl, the accumulation of Na^+ in plants overexpressing *SeNHX1* was significantly greater than that of the wild-type plants or plants overexpressing *BADH* alone (Fig. 3B). For instance, exposure to NaCl led to increases in Na^+ contents in wild-type leaves by 3.5 fold, whereas the Na^+ contents in leaves of the dual gene transformed lines of BS5 and BS15 increased by 5.7- and 6.7-fold when treated by the same concentration of NaCl, respectively.

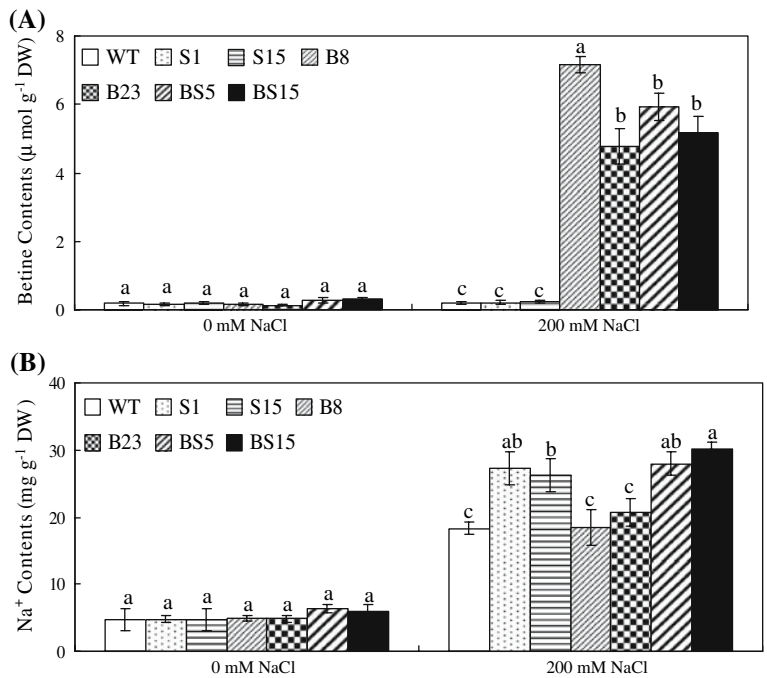

Fig. 3 Effects of NaCl stress on betaine and Na^+ contents in leaves of wild-type and the transgenic tobacco plants. Tobacco plants were treated with 200 mM NaCl for 4 weeks, and betaine (**a**) and Na^+ (**b**) contents were determined. WT, wild-type plants; S1 and S15 plants transformed with *SeNHX1*; B8 and B23 plants transformed with *BADH*; BS5 and BS15 plants simultaneously transformed with *SeNHX1*and *BADH*. Values having the same letter in each NaCl level are not significantly different at $P \leq 0.05$, by LSD test. Values shown are means ± SE of three replicates

Transgenic plants have greater osmotic pressure

The greater accumulation of Na^+ and betaine in the dual gene-transformed plants than the wild-type plants would contribute to maintenance of osmotic balance, thus facilitating water flow into roots under salt stress. As shown in Table 1, no significant difference in osmotic pressure of wild-type and transgenic plants was observed under control conditions. When challenged by NaCl, the osmotic pressure of the transgenic plants was increased by approx. 2-fold with dual gene transformed plants being the highest (Table 1), while the osmotic pressure of the wild-type plants was increased by only 56% under the same salt treatment. These results suggest that the enhanced accumulation of Na^+ and betaine in the dual gene transformed plants under salt stress may contribute to lowering the osmotic pressure of plants, thus maintaining turgor pressure for plant growth under salt stress.

Transgenic plants display greater photosynthetic rate and PSII activity

The decrease in productivity in many plants subjected to excessive salinity is often associated with a diminished photosynthetic capacity and irreversible damage to the photosynthetic apparatus. As shown in Fig. 4a, under normal conditions, both the wild-type and transgenic plants exhibited comparable photosynthetic rate. When the plants were treated with 200 mM NaCl, photosynthetic rate in the wild-type plants was reduced by approx. 46% (Fig. 4a). By contrast, the same treatment decreased the photosynthetic rate in the dual gene transformed lines of BS5 and BS15 by 6% and 19%, respectively. Plants transformed with *BADH* or *SeNHX1* alone had a moderate reduction in photosynthetic rate. Like the photosynthetic rate, PSII activity reflected by Fv/Fm was comparable between the transgenic and wild-type plants grown in the absence of NaCl (Fig. 4b). Although values of Fv/Fm for both the transgenic and wild-type plants were reduced when suffered from salt stress, reduction in Fv/Fm of wild-type plants was greater than those of transgenic plants (Fig. 4b). This indicates that the transgenic plants maintain a greater PSII activity than the wild-type plants under salt stress.

Table 1 Effect of NaCl stress on leaf cell sap osmolality of wild-type and the transgenic tobacco plants. Tobacco plants were treated with 200 mM NaCl for 4 weeks, and the leaf cell sap osmolality was determined

Lines	Cell sap osmolality (MPa)	
	0 mM NaCl	200 mM NaCl
WT	1.19 ± 0.36a	1.86 ± 0.01c
S1	1.16 ± 0.03a	2.42 ± 0.19b
S15	1.12 ± 0.02a	2.33 ± 0.18b
B8	1.14 ± 0.02a	2.1 ± 0.25bc
B23	1.19 ± 0.06a	2.13 ± 0.27bc
BS5	1.2 ± 0.26a	3.35 ± 0.13a
BS15	1.21 ± 0.08a	3.29 ± 0.28a

WT, wild-type plants; S1 and S15, plants transformed with *SeNHX1*; B8 and B23 plants transformed with *BADH*; BS5 and BS23 plants simultaneously transformed with *SeNHX1* and *BADH*

Values at the same NaCl level having the same letter are not significantly different at $P \leq 0.05$, by LSD test. Values shown are means ± SE ($n = 4$)

Transgenic plants exhibit different sensitivities to salt stress

To test whether transformation of *BADH* and *SeNHX1* genes has impact on plant growth, biomass of the transgenics and wild-type was determined under both control and stress conditions. 200 mM NaCl stress decreased the whole-plant dry weight in both the transgenics and the wild-type (Table 2), however, the reduction was more remarkable in the wild-type than in the transgenics. Dual gene transformed lines of BS5 and BS15 exhibited the highest dry weight, followed by *SeNHX1* transformed lines and *BADH* transformed lines. The results demonstrate that dual gene transformed plants displayed the highest salt tolerance, and genes of *SeNHX1* and *BADH* could act additively in the transgenic plants.

Discussion

Plant tolerance to salt stress is a complex trait that is modulated by multiple genes and requires the coordinated action of many genes. Although transformation of multiple genes into a plant can act

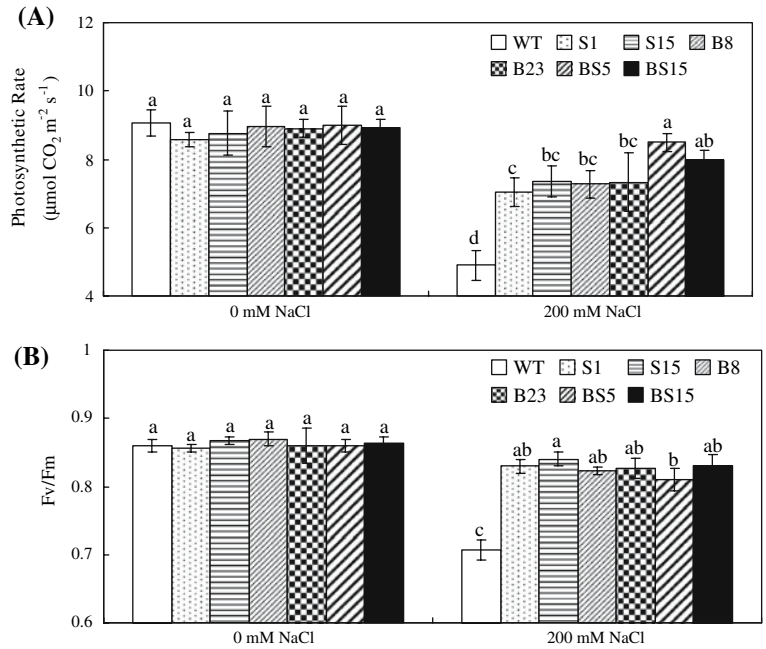

Fig. 4 Effects of NaCl stress on photosynthetic rate and PSII activity of wild-type and the transgenic tobacco plants. Tobacco plants were treated with 200 mM NaCl for 4 weeks, and photosynthetic rate and PSII activity were determined. (**a**) Photosynthetic rate. (**b**) PSII activity. WT, wild-type plants; S1 and S15 plants transformed with *SeNHX1*; B8 and B23 plants transformed with *BADH*; BS5 and BS15 plants simultaneously transformed with *SeNHX1*and *BADH*. Values having the same letter in each NaCl level are not significantly different at $P \leq 0.05$, by LSD test. Values shown are means ± SE of three replicates

Table 2 Effect of NaCl stress on biomass of wild-type and the transgenic tobacco plants. Tobacco plants were treated with 200 mM NaCl for 4 weeks, and the dry weight (DW) was determined

Lines	Biomass (g/plant DW)	
	0 mM NaCl	200 mM NaCl
WT	15 ± 0.7a	9.2 ± 1d
S1	14.8 ± 1.1a	12.6 ± 0.96abc
S15	14.9 ± 1.3a	12.4 ± 1.5bc
B8	15.2 ± 1.1a	11.6 ± 0.91c
B23	14.5 ± 1.1a	11.9 ± 0.74bc
BS5	15.2 ± 0.9a	13.9 ± 1.6a
BS15	14.8 ± 1.1a	13.1 ± 0.84ab

WT, wild-type plants; S1 and S15 plants transformed with *SeNHX1*; B8 and B23 plants transformed with *BADH*; BS5 and BS23 plants simultaneously transformed with *SeNHX1* and *BADH*

Values at the same NaCl level having the same letter are not significantly different at $P \leq 0.05$, by LSD test. Values shown are means ± SE ($n = 6$)

additively or synergistically to enhance tolerance to salt stress (Bohnert et al. 1996), there has been few report on transformation of multigenes into an individual higher plant and evaluation of their physiological performance under salt stress. In the present study, we successfully co-introduced two genes (*BADH* and *SeNHX1*) that underlie different mechanisms of salt tolerance into tobacco plants for the first time: The two transgenes functioned additively in the transgenics. The transgenic plants exhibit enhanced accumulation of betaine and/or Na^+ under salt stress, suggesting that the two genes were functionally expressed. The greater accumulation of betaine and Na^+ due to the expression of *BADH* and *SeNHX1* genes is likely to underpin the improved tolerance of the transgenic plants to salt stress.

Plants have developed elaborate mechanisms to protect them from damage caused by salt stress. These mechanisms include accumulation of low molecular weight compatible solutes and Na^+ sequestration (Volkov and Amtmann 2006; Wang et al. 2006). Sequestration of excessive cytoplasmic

Na^+ in the vacuole to maintain turgor is a typical feature of halophytes (Flowers et al. 1977). In this study, the transgenic plants overexpressing *SeNHX1* displayed characteristics of halophytes as indicated by greater accumulation of Na^+ in leaves (Fig. 3a). The vacuolar compartmentation of Na^+, together with the enhanced accumulation of betaine (Fig. 3b), would enable the transgenic plants to combat excessive cytosolic Na^+ and maintain a physiological osmotic pressure for growth under salt stress. Furthermore, our findings that *BADH* and *SeNHX1* co-transformed plants had higher osmotic pressure than either of the single gene transformed plants under salt stress (Table 1), suggests that the two transgenes act additively in tobacco plants. And the dual gene transformed plants can regulate osmotic pressure more effectively. The greater osmotic pressure in the transgenics would allow plants to maintain a favorable water status under salt stress, thus contributing to the enhanced tolerance to salt stress.

Our data also revealed that treatment with 200 mM NaCl led to a greater inhibitory effect on photosynthetic rate in wild-type than in transgenic plants (Fig. 4a). This effect is likely to be explained, at least partially, by the relative higher PSII activity in the transgenic than in wild-type plants (Fig. 4b). Betaine protects the photosynthetic machinery in betaine-accumulating transgenic plants (Holmström et al. 2000). The protective role of betaine may lie in strong stabilizing effects of betaine on the structure and function of oxygen-evolving PSII complex (Ohnishi and Murata 2006). Betaine also can counteract the inhibitory effects of salt stress on the turnover of the D1 protein, a constituent of the PSII reaction center, during photoinhibition in *Synechococcus* (Ohnishi and Murata 2006).

In conclusion, we succeed in transforming tobacco plants with *BADH* and *SeNHX1*genes. The two genes were functional expressed in the transgenics as evidenced by greater accumulation of betaine and/or Na^+ under salt stress. Under salinity, the dual gene transformed plants showed higher salt tolerance than those transformed with *BADH* or *SeNHX1* alone, and accumulated the highest biomass. The binary vector we constructed is likely to be applicable to other important crops for breeding salt tolerance crops.

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