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ORIGINAL ARTICLE

Expression of Indica rice *OsBADH1* gene under salinity stress in transgenic tobacco

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Abstract Glycine betaine has been reported as an osmoprotectant compound conferring tolerance to salinity and osmotic stresses in plants. We previously found that the expression of betaine aldehyde dehydrogenase 1 gene (*OsBADH1*), encoding a key enzyme for glycine betaine biosynthesis pathway, showed close correlation with salt tolerance of rice. In this study, the expression of the *OsBADH1* gene in transgenic tobacco was investigated in response to salt stress using a transgenic approach. Transgenic tobacco plants expressing the *OsBADH1* gene were generated under the control of a promoter from the maize ubiquitin gene. Three homozygous lines of T_2 progenies with single transgene insert were chosen for gene expression analysis. RT-PCR and western blot analysis results indicated that the *OsBADH1* gene was effectively expressed in transgenic tobacco leading to the accumulation of glycine betaine. Transgenic lines demonstrated normal seed germination and morphology, and normal growth rates of seedlings under salt stress conditions. These results suggest that the *OsBADH1* gene could be an excellent candidate for producing plants with osmotic stress tolerance.

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Introduction

Abiotic stresses are major factors that reduce crop productivity and quality, and cause food shortages in many areas of the world. Salinity is one of the abiotic stress problems which reduce arable land. Nowadays, salinity areas of the world are expanding due to unreasonable irrigation, environmental pollution and use of chemical fertilizers. Although plants have evolved a number of protective mechanisms to overcome such abiotic stresses (Tester and Davenport 2003; Bartels and Sunkar 2005), many plant species are still susceptible to such environmental hazards. Therefore, development of suitable salt-tolerant plants is one of the alternative ways to increase agricultural production in salinity areas (Dudal and Purnell 1986).

The active accumulation of solutes compatible with cellular metabolism is thought to play a central role in osmotic protection (Yancey et al. 1982), and the accumulation of compatible solutes is stimulated under salinity and water stresses (Hasegawa et al. 2000). Such solutes, when accumulated in the cytoplasm, can lower the osmotic potential for cells without interfering with the metabolic processes or protein structuring and functioning and, consequently, maintain the water content of the cells under stress conditions. The exogenous application of various compatible solutes reduces the salt-induced K^+ efflux which is the main effect of salinity stress (Cuin and Shabala 2007). These small molecules include a quaternary

ammonium compound, glycine betaine, which is reported as one of the most effective osmoprotectant compounds (Ashraf and Foolad 2007). Glycine betaine is widely distributed in bacteria, algae, higher plants (e.g., sugar beet and cotton) and animals, and is frequently detected in those plant species that are exposed to drought and salinity stresses (Rhodes and Hanson 1993; Reda et al. 2004). Glycine betaine is known to protect protein structure and enzyme activities and also stabilize membranes during osmotic and ionic stresses (Rhodes and Hanson 1993). Moreover, it functions to stabilize both PSII complex and Rubisco during photosynthesis under stress conditions (Holmström et al. 2000). In higher plants, glycine betaine is synthesized via two-step oxidation of choline by ferredoxin-dependent choline monooxygenase (CMO) and betaine aldehyde dehydrogenase (BADH) (Scott et al. 2000). The expression level of the *BADH* gene which encodes for the BADH enzyme was found to increase when plants were subjected to salt and water stresses (Weretilnyk and Hanson 1990). In addition, genetic engineering of the *BADH* gene conferring accumulation of glycine betaine in plants could enhance tolerance to osmotic stresses (Guo et al. 2000; Holmström et al. 2000).

Although rice was considered as a non-accumulator of glycine betaine in the past (Rathinasabapathi et al. 1993), the presence of *CMO* and *BADH* genes in the rice genome was subsequently proved (International Rice Genome Sequencing Project 2005). This finding signifies that rice should have the capacity of synthesizing glycine betaine. The recent rice genome sequence revealed that rice contains *CMO* on chromosome 6 and two *BADH* homologs, *OsBADH1* on chromosome 4 and *OsBADH2* on chromosome 8, respectively. OsBADH2 shares 75% homology with OsBADH1 at the amino acid level and 92% similarity at the catalytic domain. Both OsBADH1 and OsBADH2 contain SKL signal peptide at the C terminus, which targets the peroxisome (Chen et al. 2008; Fitzgerald et al. 2008). Studies on the transcription of *OsBADH1* and *OsBADH2* in response to salt and drought stresses revealed that the constitutive expression of *OsBADH2* was demonstrated under normal and stress conditions, but that *OsBADH1* expression level was up-regulated by salt and drought stresses (Niu et al. 2007; Fitzgerald et al. 2008). Furthermore, the positive correlation between BADH enzyme activity and glycine betaine content with the salt tolerant ability of rice was demonstrated (Hasthanasombut et al., unpublished). Consequently, it is now possible to use *OsBADH1* gene as a new source of genetic material to engineer various species of plants for resistance to osmotic stresses. The objective of this study was to investigate whether over-expression of the *OsBADH1* gene cloned from the Indica rice cultivar could generate osmotic stress-resistant phenotypes in transgenic tobacco.

Materials and methods

Vector construction

A 1.5-kb open reading frame fragment of *OsBADH1* gene from *Oryza sativa* L. ssp. *indica* 'Homjan,' which is a native Thai rice cultivar, was cloned previously (Hasthanasombut et al., unpublished). *OsBADH1* gene (Genbank accession no. DQ234301) showed 99% homology to *OsBADH1* gene in ssp. *japonica*. *Agrobacterium* binary vector was constructed using easy cloning binary vector system (EBis) (Nakamura et al., unpublished). *OsBADH1* fragment was isolated from *Xba*I and *Sal*I site of plasmid pET29/*OsBADH1*. The *gus* gene in the plasmid pS221S, which was derived from pBI221 (Clontech, NJ, USA), was replaced by this fragment at the corresponding site. Due to the highly effective nature of the ubiquitin promoter over the 35S promoter for monocot plants (McElroy and Brettell 1994), maize ubiquitin promoter was sub-cloned from the plasmid pAHC17 (Christensen et al. 1992) to the plasmid pS221S containing the *OsBADH1* gene. Then, pS221S/Ubi::*OsBADH1* plasmid was cut and ligated into *Sse*8387I site of binary vector, pEKH2, which contains kanamycin and hygromycin resistance genes. Finally, the plasmid pEKH2 containing the chimeric Ubi::*OsBADH1* gene under the control of the maize ubiquitin promoter was transformed into *Agrobacterium tumefaciens* strain EHA101 by the triparental mating method.

Plant material and transformation

Nicotiana tabacum 'Petit Havana SR1' was co-cultivated with *A. tumefaciens* containing the Ubi::*OsBADH1* gene using the leaf disc method (Horsch et al. 1985). The infected leaf discs were cultured and selected on MS medium containing α-naphthaleneacetic acid (0.1 mg l^{-1}), 6-benzylaminopurine (1.0 mg l^{-1}), hygromycin (20 mg l^{-1}), and meropenem (25 mg l^{-1}). Transformants with single transgene insertion were selected using Southern blotting analysis, and transferred to a growth chamber for seed production. Homozygous seeds at T_2 generation were used for further analyses.

PCR and Southern blot analysis

Total DNA was isolated according to CTAB method (Doyel and Doyle 1987). The 610 kb of *hptII* gene in putative T0 transformants was amplified using the following primer pairs: forward 5′-ACAGCGTCTCCGACCTGATGCA-3′ and reverse 5′-AGTCAATGACCGCTGTTATGCG-3′. The amplification reaction was carried out with initial denaturation at 94°C for 3 min, followed by 35 cycles of denaturation at 94°C for 1 min, annealing at 59°C for 1 min, extension at

72°C for 2 min, and final extension for 5 min at 72°C. Then, the amplified fragment was detected by staining with ethidium bromide after electrophoresis on 1.2% agarose gel.

In order to confirm the stable integration of transgene in hygromycin resistant plants, genomic DNA of T_1 transgenic plants and control plant, which were extracted as described above, were digested with *Hin*dIII enzyme. Digested DNA was separated by electrophoresis on 0.8% agarose gel. After transfer to nylon membrane and hybridizing with Digoxigenin-labeled *hptII* probe, the insertion copy number of the transgene was observed on X-ray film.

Segregation analysis

T_1 seeds were germinated on MS medium containing hygromycin (20 mg l^{-1}). The number of hygromycin resistance seedlings was counted. Segregation patterns were analyzed with the chi-square test (χ^2) against the expected Mendelian ratio of 3:1 for single locus insertion.

Gene expression analysis by RT-PCR

Total RNA was extracted from leaves of 2% (342 mM) NaCl-treated T_2 plants using the hot phenol buffer method (Sacco et al. 1988). Then, RNA was treated with DNase to get rid of DNA contamination. Superscript III RNase H^- RT reverse transcription system (Invitrogen, Auckland, New Zealand) was used to synthesize first stranded cDNA of the *OsBADH* gene. PCR was carried out using gene specific primers for the *OsBADH1* gene (forward: 5′-GC CATCTGAGCTTGCTTCCCTGAC-3′ and reverse: 5′-GT GGCACCTTCACATCTTGCTG TT-3′) (Fig. 1) and KOD FX DNA Polymerase enzyme (TOYOBO, Japan). PCR was performed using the conditions, 94°C for 2 min, 98°C for 10 s, 55°C for 30 s, 68°C for 1 min, and 68°C for 7 min with 35 cycles of amplification.

Measurement of OsBADH1 protein by western blot analysis

The expression of integrated *OsBADH1* gene at the translation level was evaluated using western blot analysis. Salinity stress treatments as described previously were applied to 4-week-old control and transgenic tobacco plants before analysis. Leaf tissue was ground in liquid nitrogen and homogenized with extraction buffer [62.5 mM Tris–HCl, pH 6.8, 2% (v/v) SDS (sodium dodecyl sulphate), 10% (v/v) glycerol and 0.2% β-mercaptoethanol]. The homogenized samples were boiled for 3 min followed by incubation on ice for 2 min. Then, the samples were centrifuged (20,000*g*) for 10 min at 4°C. Fifty micrograms of total proteins from each samples were separated on 12.5% SDS–PAGE and electroblotted onto a polyvinylidene difluoride (PVDF) membrane (Amersham BioScience, USA). Detection of the OsBADH1 protein was performed using polyclonal antisera (primary antibody, 1:1,250 v/v) raised in rabbit against two synthetic peptides (LETFDSGKPL and AVISNDLERC) of the OsBADH1 protein and goat-anti-rabbit IgG (Amersham BioScience) conjugated to horseradish peroxidase (HRP) as secondary antibody (1:40,000 v/v).

Evaluation of glycine betaine production

The seedlings of control and transgenic plants after growth for 4 weeks were subjected to salt treatment (342 mM NaCl) for 3 days. Glycine betaine was isolated from 2 g of fresh leaf samples as described previously (Cha-um et al. 2007a). The content was determined using high-performance liquid chromatography (HPLC; JASCO 2080 series, Japan). The column is a stainless steel (250 mm long, 4.6 mm wide) packed with Partilsil 10 SCX. The mobile phase was nanopure-water containing 5% (v/v) methanol buffered with 50 mM KH_2PO_4 at pH 4.6 with a flow rate at 1.0 ml min^{-1}. A UV detector was used and wavelength was set at 195 nm. Betaine hydrochloride (Merck, Darmstadt, Germany) was used as a standard, and glycine betaine content was calculated using a standard curve equation. This compound was mixed with the leaf-extract sample as an internal standard.

Salinity stress treatment

To evaluate transgenic plants for resistance to salinity stress, stress (NaCl) was applied at two different growing

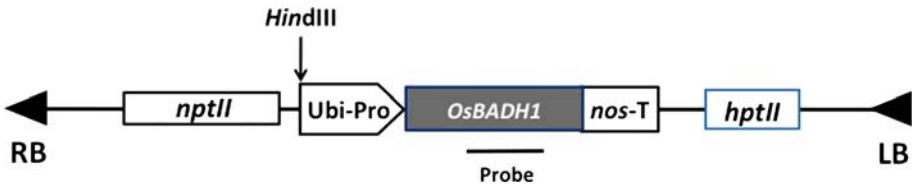

Fig. 1 Schematic diagram of T-DNA region of the transforming vector Ubi::*OsBADH1*. The *arrow* and the *solid bar* indicate the *Hin*dIII site and the region corresponding to the amplification size of RT-PCR, respectively. *nptII* Neomycin phosphotransferase II gene, *Ubi-Pro* maize ubiquitin promoter, *OsBADH1* rice betaine aldehyde dehydrogenase 1 gene, *hptII* hygromycin phosphotransferase II gene, *RB* right border, *LB* left border

stages; germination and seedling stage. Germination rate of transgenic seeds was compared with the control line after application of 100 mM NaCl. In order to investigate the effect of salinity stress at seedling stage, 2 week-old transgenic and control seedlings were transferred to plug tray, kept in the growth chamber, and applied with water containing 0, 171, and 342 mM NaCl every 2 days. Growth rates of control and transgenic seedlings were investigated by measuring the fresh weight and dry weight at 0, 1, 2 and 4 weeks after initiating the NaCl treatments.

Results

Transformation of tobacco plants with *OsBADH1* gene

Transgenic tobacco over-expressing *OsBADH1* gene under the control of the maize ubiquitin promoter was generated by infecting leaf discs with *A. tumefaciens* harboring the binary vector Ubi::OsBADH1/pEKH2 (Fig. 1). Twenty-five independent transformants were selected on 20 mg l^{-1} hygromycin-containing medium. The integration of transgene was confirmed by the PCR technique. A positive band of hygromycin gene at 610 bp was detected in all transgenic lines (Fig. 2a). The stable integration of the transgene was investigated at the T_1 generation using Southern blot analysis and 1–3 insertion sites were found (Fig. 2b). The integration of transgene was further confirmed by segregation analysis with respect to Mendelian's law in the T_1 generation. Three lines having single locus insertion showed 3:1 ratio of hygromycin-resistant phenotypes whereas one line with 2 loci insertions showed a 15:1 ratio in the T_1 generation (Table 1). In order to produce homozygous plants for further analyses, single locus insertion lines were chosen and advanced to T_2 progenies.

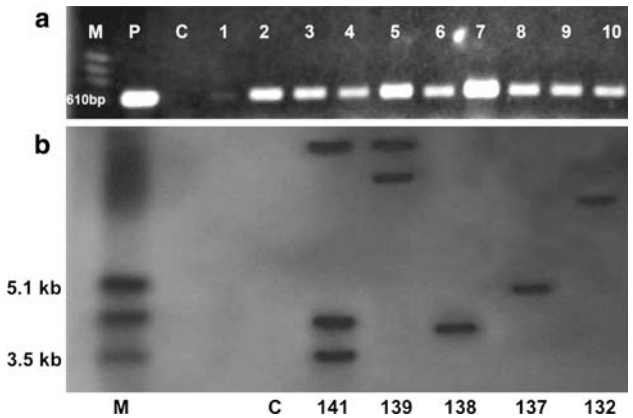

Fig. 2 Molecular analysis of *OsBADH1* transgenic plants. **a** PCR amplification of the 610 bp of *hptII* gene. *Lane M* molecular weight marker, *lane P* plasmid DNA, *lane C* DNA from untransformed plant, *lane 1–10* DNA from transformed plants. **b** Confirmation of stable integration of transgene into the tobacco genome by Southern blot analysis in T_1 plants. Genomic DNA was digested with *Hin*dIII, and hybridized with *hptII* probe. *Lane M* DNA molecular weight marker, *Lane C* genomic DNA from untransformed plant, *Lanes 141*, *139*, *138*, *137,* and *132* genomic DNA from transformed plants, numbers 141, 139, 138, 137, and 132, respectively

Expression of *OsBADH1* under salt stress

Expression of *OsBADH1* gene in transgenic tobacco was investigated using RT-PCR analysis. The result revealed expression of *OsBADH1* gene in all three lines of transgenic T_2 plants whereas the expression was not detected in the control line. The expression was moderate in transgenic line numbers 132 and 137, while transgenic line number 138 showed extremely high expression level (Fig. 3).

Analysis of the accumulation of OsBADH1 protein in T_2 transgenic plants

A single OsBADH1 polypeptide band cross-reacting with anti-OsBADH1 antiserum was detected in each protein sample from all lines of transgenic T_2 plants but not from the control line. The size of the polypeptide band was 54 kDa (Fig. 4), which is close to our predicted molecular mass (54.5 kDa) of the *OsBADH1* gene product.

Glycine betaine production in transgenic plants

Level of glycine betaine in control and transgenic plant was analyzed via HPLC analysis. The glycine betaine content in transgenic leaves was 0.14, 0.20 and 0.34 μmol g^{-1} fresh weight in transgenic lines 132, 137 and 138, respectively. The increase in glycine betaine accumulation was observed in transgenic lines 132 and 138 after NaCl stress application, while the production of this compound was stable in transgenic line 137. In contrast, no glycine betaine was detected in either NaCl-treated or non-treated control plants (Table 2).

Germination of *OsBADH1* transgenic tobacco seeds under salt stress

To investigate the response of transgenic tobacco over-expressing *OsBADH1* gene to salt stress, transgenic seeds of T_2 progenies were germinated on a filter paper containing 100 mM NaCl. Germination rate of the control line under the NaCl stress condition decreased to less than half when compared with normal conditions (without NaCl). On the other hand, germination rate of transgenic plants (line 132 and 138) was not affected by NaCl stress, although transgenic line 137 showed a slight reduction in germination rate (Table 3). Moreover, transgenic lines exhibited normal morphological phenotypes under salt

Table 1 Segregation analysis of T_1 plants derived from the *OsBADH1* T_0 transgenic lines

T_1 line	No. of T_1 plants tested	Hygromycin-resistant plants	Hygromycin-sensitive plants	Ratio	X^2 value	*P* range
141	102	98	4	15:1	0.94	0.3–0.5
132	98	80	18	3:1	2.29	0.05–0.1
137	108	86	22	3:1	1.23	0.3–0.5
138	104	75	29	3:1	0.46	0.3–0.5

Scoring for the number of transgenic plants was done on the basis of germination on 20 mg l^{-1} hygromycin-containing medium

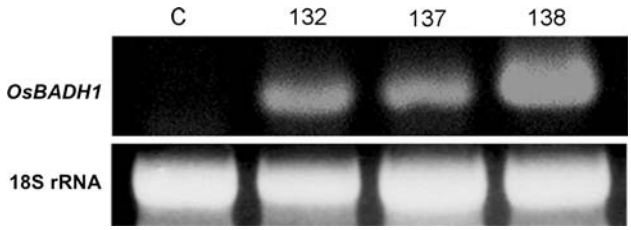

Fig. 3 Detection of the transcription for *OsBADH1* transgene in T_2 transgenic plants by RT-PCR. Non-transformed plant (*lane C*) gave no product of RT-PCR, whereas all transgenic lines (*lane 132*, *137* and *138*) gave positive signals of RT-PCR. The *lower panels* in all cases show ethidium bromide-stained 18S rRNA for equivalent loading and RNA quality

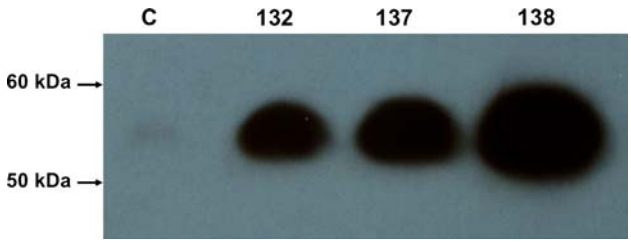

Fig. 4 Immunoblot analysis of OsBADH1 protein in NaCl-treated T_2 transgenic tobacco. A band of 54 kDa of polypeptide was detected in transgenic lines (*lane 132*, *137* and *138*) but not in the control line (*lane C*)

stress conditions when compared with the control line under normal condition. Transgenic seedlings were bigger and greener than the control seedlings under the salt stress conditions (Fig. 5).

Salt tolerance of *OsBADH1* transgenic tobacco seedlings

Effect of NaCl stress on growth at seedling stage was also investigated by using three transgenic lines containing single transgene insert. Growth parameters such as fresh and dry weights were observed after application of 0, 171 and 342 mM NaCl to control and transgenic seedlings. From the results, there were no significant differences in fresh weight between control and transgenic lines under normal conditions (0 mM NaCl) (Fig. 6a). When 171 or 342 mM NaCl was applied, fresh weight of the control line decreased sharply in the first week and then slightly increased thereafter. On the other hand, fresh weight of transgenic lines gradually increased in the first and second weeks, and then dramatically increased in the fourth week, especially lines 137 and 138 (Fig. 6b, c). In the same way, dry matter of control line reduced to 1/3 after treatment with 171 mM NaCl for 4 weeks compared with the no

Table 2 Glycine betaine (*GB*) levels in leaves of transgenic plants

Plant	NaCl treatment[a]	GB in leaves[b] (μmol g^{-1} FW[c])
Control	−	N.D.[d]
	+	N.D.
Transgenic line 132	−	0.14 ± 0.01
	+	0.22 ± 0.02
Transgenic line 137	−	0.20 ± 0.01
	+	0.20 ± 0.03
Transgenic line 138	−	0.34 ± 0.02
	+	0.56 ± 0.01

[a] 342 mM for 3 days

[b] Mean and SE of three replicates

[c] Fresh weight

[d] Not detected

Table 3 Germination percentage of control and transgenic plants under salt stress

Plant	NaCl concentration (mM)	Germination[a]
Control	0	84.7 ± 1.5 b
	100	33.4 ± 1.8 c
Transgenic line 132	0	93.3 ± 0.9 a
	100	93.3 ± 0.7 a
Transgenic line 137	0	93.7 ± 1.2 a
	100	91.0 ± 0.6 a
Transgenic line 138	0	92.7 ± 0.9 a
	100	95.1 ± 1.8 a

[a] Germination percentage ± standard error (SE). The experiment was repeated 3 times, each with 50 seeds. Means with the same letter in a given vertical array are not significantly different ($P > 0.05$) according to Duncan's multiple range test

NaCl treatment, whereas dry matter of transgenic line 137 and 138 were not much affected by salt stress application (Fig. 7a–c).

Discussion

Salt stress acts as osmotic and oxidative stresses, which affect plant growth by interfering with photosynthetic and transpiration rates (Sharma et al. 2005). Genetic manipulation is one of the challenging strategies to improve stress tolerance in plants, and *BADH* genes isolated from spinach and sugar beet were used to confer the enhanced salt tolerance (Trossat et al. 1997; Liang et al. 1997; Rathinasabapathi et al. 1994). Although the *BADH* gene has also been isolated from *Atriplex hortensis*, *Suaeda liaotungensis* and *Sorghum bicolor* and used for the improvement of salt tolerance of plants (Jia et al. 2002; Li et al. 2003; Moghaieb et al. 2000), the variation of genetic resources for salinity-tolerant phenotypes is still limited. Therefore, exploring new genetic resources would be important for expanding the knowledge of genetically engineered salinity-tolerant plants.

In rice, the functions of OsBADH1 and OsBADH2 enzymes have been extensively studied (Bradbury et al. 2008; Fitzgerald et al. 2008; Niu et al. 2008). Bradbury et al. (2008) reported that the OsBADH1 and OsBADH2 enzymes are substrate-specific; for example, OsBADH2 have higher specificity to betaine aldehyde, which is the intermediate substrate for the glycine betaine biosynthesis pathway, than OsBADH1 enzyme. Although OsBADH2 has higher affinity to betaine aldehyde than OsBADH1, OsBADH1 has been reported to exhibit significantly increased transcriptional level when exposed to salt and drought stresses (Niu et al. 2007; Ishitani et al. 1994; Liang et al. 1997), whereas no consistent relationship between *OsBADH2* transcription level and salt treatment was observed (Fitzgerald et al. 2008). This evidence suggests that only the *OsBADH1* gene is probably involved in protecting plants against these stresses. Therefore, in this study, we chose a traditional Indica rice cultivar 'Homjan' for the isolation of the *OsBADH1* gene to generate transgenic tobacco plants because of its strong drought and salt tolerance ability proven previously (Cha-um et al. 2007b). In the regenerants obtained after infection with *A. tumefaciens* harboring the *OsBADH1* gene, the integration of the chimeric *OsBADH1* transgene was confirmed by PCR analysis, suggesting that the T-DNA of the binary plasmid vector was successfully integrated into the genome of

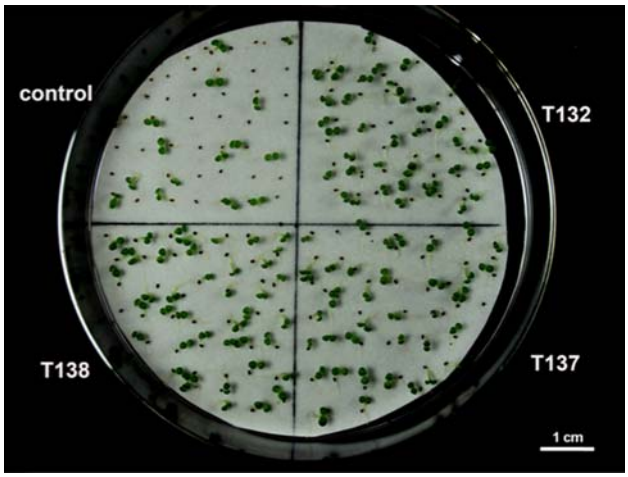

Fig. 5 Germination of transgenic tobacco seeds compared with control seeds on a filter paper wetted with 100 mM NaCl solution. Seeds were germinated at 25°C under photoperiod with 16 h light and 8 h dark cycles. Photograph was taken 2 weeks after sowing. The control showed scanty germination compared to the transgenic lines

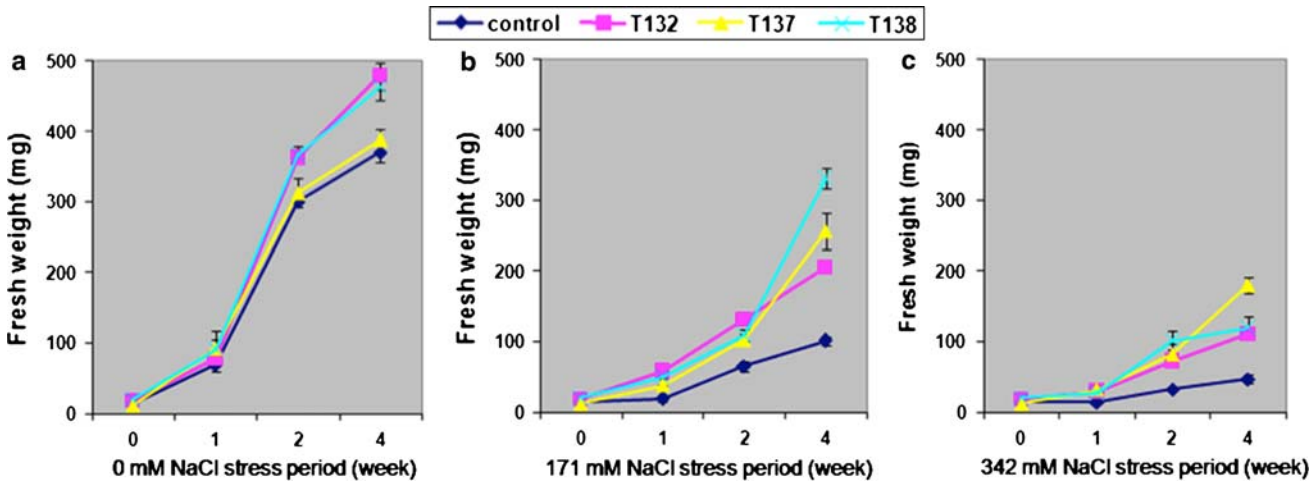

Fig. 6 Fresh weight of control and transgenic T_2 tobacco seedlings grown under 0 (**a**), 171 (**b**), and 342 (**c**) mM NaCl stress conditions. Values are the mean ± SE (*vertical bar*) of the three replications

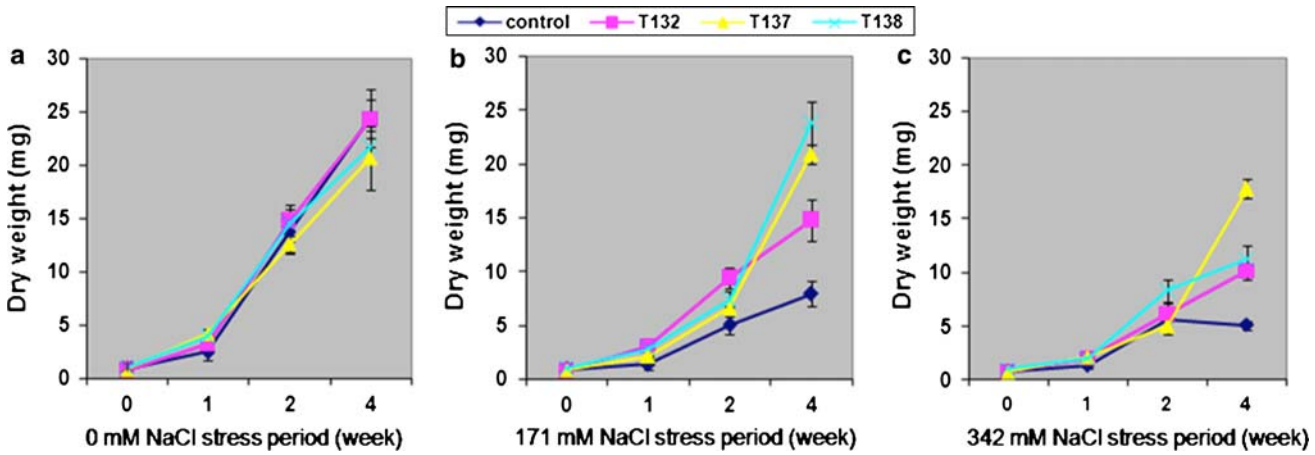

Fig. 7 Dry weight of control and transgenic tobacco T_2 seedlings grown under 0 (**a**), 171 (**b**), and 342 (**c**) mM NaCl stress. Values are the mean ± SE (*vertical bar*) of the three replications

transgenic tobacco plants. The unique banding pattern observed in Southern blot analysis indicates that the transgenic clones have originated from independent transformation events with 1–3 transgene integration sites. Moreover, segregation analysis of hygromycin-resistant lines 132, 137 and 138 showed a 3:1 ratio (Table 1), which indicates that the transgene is inherited in a simple Mendelian manner.

The result of RT-PCR denoted the correct and stable expression of chimeric *OsBADH1* in transgenic tobacco plants without silencing phenomena and growth retardation. These results proved that monocot-derived ubiquitin promoter can control transgene expression effectively in dicot plants. A similar conclusion was reached by Khan et al. (2001). Moreover, a western blotting result showed the positive band of OsBADH1 protein at the predicted molecular mass, thus confirming the stable expression of the *OsBADH1* gene in transgenic lines. Additionally, transgenic line 138 exhibited the highest expression level of *OsBADH1*, which correlated with the amount of OsBADH1 protein and the level of glycine betaine production (Figs. 3 and 4, and Table 2).

Recently, an unusual post-transcription of *CMO* and *BADH* genes according to deletion and truncation at the 5′ exonic region was reported in rice (Luo et al. 2007; Niu et al. 2007). Therefore, generation of extremely few mRNAs result in the difficulty of producing normal CMO and BADH proteins, leading to loss of functional enzyme to drive the pathway of glycine betaine biosynthesis in rice. Interestingly, the incorrect transcriptional process was not found in dicotyledonous species such as spinach. In the present study, glycine betaine compound was produced in transgenic *OsBADH1* tobacco, implying the occurrence of the correct transcription of the *OsBADH1* gene, leading to the production of active BADH enzyme for the conversion of betaine aldehyde to glycine betaine. This suggests that the phenomenon of incorrect splicing which was previously found in rice (Niu et al. 2007) did not occur in transgenic tobacco plants.

Bradbury et al. (2008) reported that the OsBADH1 enzyme has much lower specificity to the substrate, betaine aldehyde, than the OsBADH2 enzyme. In this study, the level of glycine betaine production in transgenic tobacco over-expressing *OsBADH1* was higher than the control plant which did not produce glycine betaine under normal and stress conditions. From the result, glycine betaine was produced at a lower level (0.1–0.5 μmol g^{-1} FW) when compared with a previous report (Yang et al. 2008) which showed over-expression of spinach *BADH* gene in tobacco at 0.44–4.92 μmol g^{-1} FW in response to salt stress. Since the transgenic *OsBADH1* tobacco still showed significantly enhanced tolerance to salinity stress, the effective conversion of betaine aldehyde to glycine betaine was achieved by the OsBADH1 enzyme, which is more stable with a longer half-life than the OsBADH2 enzyme (Bradbury et al. 2008). Surprisingly, the elevation of glycine betaine production response to salt stress was not marked in all transgenic lines, especially in line 137 which showed stable production of glycine betaine in both normal and salinity stress conditions. This may be because of the use of a constitutive ubiquitin promoter. In the past, genetic modification of the glycine betaine pathway in tobacco plants was done by over-expressing various genes such as *E. coli betA* (Lilius et al. 1996), spinach *CMO* (Nuccio et al. 1998) and spinach *BADH* (Yang et al. 2008). Transgenic tobacco over-expressed spinach *BADH* showed the highest production of glycine betaine (0.44–4.92 μmol g^{-1} FW), whereas *betA* and *CMO* transgenic plants produced less than 66 nmol g^{-1} FW and 0.02–0.05 μmol g^{-1} FW, respectively. These results indicate that BADH is the most

effective enzyme for glycine betaine synthesis. Our study proved that rice *BADH* gene had more efficiency to drive the glycine betaine biosynthesis pathway than the *betA* and *CMO* genes but less efficiency than spinach *BADH* gene.

To test the salt tolerance of *OsBADH1* transgenic plants, control and transgenic plants were subjected to salt stress at different concentrations of NaCl. The ability of tobacco plants to survive in the presence of NaCl was enhanced with the introduction of the *OsBADH1* gene from Indica rice. From a previous report, germination and growth of tobacco seedlings were inhibited when 50 mM NaCl was applied (Niknam et al. 2004). In our study, germination of transgenic *OsBADH1* tobacco seeds was not affected by 100 mM NaCl stress, whereas germination of the control seeds decreased to less than half under the same conditions. This indicates that the expression of *OsBADH1* in transgenic tobacco could overcome the injuries caused by salt stress, and promote seed germination under salt stress conditions (Table 3).

Since osmotic stress causes immediate reduction in cell expansion of roots and young leaves (Vijayan 2009), fresh and dry weights of transgenic plants under salt stress conditions were used as indicators to demonstrate salt tolerance of transgenic plants. From the result, the decrease in whole plant dry weight and fresh weight by application of NaCl in transgenic lines was less than that of the wild-type line, suggesting that transgenic lines were more tolerant to salt stress than the control line. Furthermore, transgenic lines exhibited normal morphology, which indicates that the *OsBADH1* gene does not have a negative effect on the physical appearance of transgenic lines. Moreover, wilting and necrotic symptoms under salt stress conditions were not observed, whereas the control line showed yellowish leaves (data not shown).

In conclusion, this is the first report highlighting that transgenic tobacco plants expressing the stress-responsive *OsBADH1* gene from *Oryza sativa* ssp. *indica* showed favorable tolerance to salinity stress. The results presented in this study could be valuable information for genetic modification of other plants especially crop plant species that are recalcitrant to genetic engineering. However, further studies are necessary to demonstrate the expression and biological function of this gene in crop plants.

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