Plant Mol Biol (2008) 66:73–86
DOI 10.1007/s11103-007-9253-9

Genetic engineering of the biosynthesis of glycinebetaine leads to increased tolerance of photosynthesis to salt stress in transgenic tobacco plants

Xinghong Yang · Zheng Liang · Xiaogang Wen · Congming Lu

Received: 8 September 2007 / Accepted: 23 October 2007 / Published online: 2 November 2007

Abstract Genetically engineered tobacco (*Nicotiana tabacum* L.) with the ability to synthesis glycinebetaine (GB) in chloroplasts was established by introducing the BADH gene for betaine aldehyde dehydrogenase from spinach (*Spinacia oleracea* L.). The genetic engineering resulted in enhanced tolerance of growth of young seedlings to salt stress. This increased tolerance was not due to improved water status, since there were no significant differences in accumulation of sodium and chloride, leaf water potential, and relative water content between wild type and transgenic plants under salt stress. Salt stress resulted in a decrease in CO_2 assimilation and such a decrease was much greater in wild type plants than in transgenic plants. Though salt stress showed no damage to PSII, there were a decrease in the maximal PSII electron transport rate in vivo and an increase in non-photochemical quenching (NPQ) and these changes were greater in wild type plants than in transgenic plants. In addition, salt stress inhibited the activities of ribulose 1,5-bisphosphate carboxylase/oxygenase, chloroplastic fructose-1,6-bisphosphatase, fructose-1,6-bisphosphate aldolase, and phosphoribulokinase and such a decrease was also greater in wild type plants than in transgenic plants, suggesting that GB protects these enzymes against salt stress. However, there were no significant changes in the activities of phosphoglycerate kinase, triose phosphate isomerase, ribulose-5-phosphate isomerase, transketolase, and sedoheptulose-1,7-bisphosphatase in both wild type and transgenic plants. The results in this study suggest that enhanced tolerance of CO_2 assimilation to salt stress may be one of physiological bases for increased tolerance of growth of transgenic plants to salt stress.

Keywords Glycinebetaine · Photosynthesis · Salt stress · Transformed tobacco

X. Yang · Z. Liang · X. Wen · C. Lu (✉)
Photosynthesis Research Center, Key Laboratory of Photosynthesis and Environmental Molecular Physiology, Institute of Botany, Chinese Academy of Sciences, Beijing 100093, China
e-mail: lucm@ibcas.ac.cn

X. Yang
College of Life Science, Key Laboratory of Crop Biology of Shandong Province, Shandong Agricultural University, Taian 211018, China

C. Lu
National Plant Gene Center, Beijing 100093, China

Introduction

It is estimated that worldwide about one third of the irrigated arable land is already salt affected and that salinization of land is still expanding (Szabolcs 1989; Tanji 1990). Salt stress is one of the major environmental factors that restrict plant growth and the worldwide productivity (Epstein et al. 1980; Boyer 1982). The decline in growth observed in many plants subjected to excessive salinity is often associated with a decrease in their photosynthetic capacity (Long and Baker 1986). The decrease in photosynthesis in salt-stressed plants can be associated with the partial stomatal closure and/or the non-stomatal limitation which is involved in the dark enzymatic processes of CO_2 assimilation, e.g. the decrease in Rubisco activity and content (Downton et al. 1985; Brugnoli and Björkman 1992). The decrease in photosynthesis in salt-stressed plants may be also associated with the inhibition of PSII activity (Mishra et al. 1991; Belkhodja et al. 1994; Everard et al. 1994) though some studies have indicated that PSII is

high resistant to salinity stress (Morales et al. 1992; Abadía et al. 1999; Lu and Zhang, 1998; Lu et al. 2002, 2003a, b).

Although salt stress significantly affects growth of plants, plants have evolved a variety of mechanisms for adapting to salt stress. One of the mechanisms is the accumulation of glycinebetaine (GB), a so-called "compatible solute," in response to salt stress (Rhodes and Hanson 1993). GB is also accumulated under other environmental stress, such as drought and cold stress (Rhodes and Hanson 1993). Numerous studies have suggested that GB plays an important role in plants under various types of environmental stresses (see review e.g. Sakamoto and Murata 2002). GB increases not only salt tolerance (Hayashi et al. 1997; Holmström et al. 2000) but also cold tolerance (Hayashi et al. 1997; Alia et al. 1998a; Park et al. 2004), and freezing tolerance (Sakamoto et al. 2000). In addition, GB may relieve high temperature stress of plants (Alia et al. 1998b; Yang et al. 2005).

The mechanisms of GB in protecting plants against salt stress have been investigated by many studies. GB may protect plants by acting as an osmolyte maintaining the water balance between the plant cell and the environment during salt stress (Saneoka et al. 1995). GB may also protect PSII activity by stabilizing the PSII complexes at high salt concentrations (Papageorgiou and Murata 1995). In addition, it has been shown that transformed rice and tobacco with accumulation of GB enhances tolerance of PSII against photoinhibition (Hayashi et al. 1997; Sakamoto et al. 1998; Holmström et al. 2000). It has also been reported that the accumulation of GB in a cyanobacterium *Synechococcus* by genetic engineering of *bet* genes protects ribulose-1,5-bisphosphate carboxylase/oxygenase from inactivation under salt stress (Nomura et al. 1998).

Several studies have shown that the accumulation of GB in *Arabidopsis* and rice in the chloroplast by transferring *codA* gene responsible for GB synthesis improves their growth under salt stress condition (Hayashi et al. 1997; Sakamoto et al. 1998). Recently, it has been demonstrated that several GB-producing transgenic rice lines obtained by transferring choline oxidase gene show greater salt tolerance than wild type plants (Su et al. 2006). However, it remains unknown what is the physiological basis of GB in vivo for this increased tolerance of growth to salt stress. Since photosynthesis is sensitive to salt stress (Long and Baker 1986), we propose a hypothesis that the physiological basis for enhanced tolerance of growth to salt stress induced by accumulation of GB in vivo may be associated with increased tolerance of photosynthesis to salt stress. To our knowledge, no report is available whether the accumulation of GB in vivo can enhance photosynthesis against salt stress.

We have established the system for the biosynthesis of GB in vivo by genetic engineering of tobacco, which is unable to accumulate GB (Yang et al. 2005). In this study, using transgenic tobacco, we investigated the role of GB in vivo in protecting photosynthesis from salt stress.

Materials and methods

Transformation of plants and growth conditions

The *BADH* gene, which had been isolated from spinach, was used to construct a vector plasmid that contained the promoter for 35S ribosomal RNA from cauliflower mosaic virus (CaMV35S), the sequence encoding the transit peptide of the small subunit of Rubisco of tobacco and the terminator of the gene for nopaline synthase (NOS) as described previously (Yang et al. 2005). *Nicotiana tabacum* (wild type K326) was transformed with the resultant plasmid by the standard *Agrobacterium*-mediated method and five independent lines of transgenic tobacco plants (L1, L2, L3, L4 and L5) were established as described previously. These transformed plants were used as the source of plant materials. Plants derived from untransformed seeds were designated wild-type plants, which did not contain GB.

The seeds of these transgenic and wild type plants were allowed to germinate, and plants were grown in vermiculite for 2 weeks and were then transplanted to plastic pots (20 cm in diameter and 15 cm in height) filled with sands and watered daily with half-strength Hoagland nutrient solution. The plants were grown in a greenhouse at 25 ± 1°C with PPFD of 300 μmol m^{-2} s^{-1}, a relative humidity of 75–80%, and a photoperiod of 14/10 h light/dark as described previously (Yang et al. 2005).

Salt stress treatments

The seedlings after growth for 2 months were subjected to salt treatments. Salt concentrations were stepped up in 25 mM d^{-1} increments until final concentrations (0, 75, 150 mM) were achieved. NaCl was dissolved in Hoagland nutrient solution and plants were watered daily to drip with approximate 0.5 l of salt solution. All measurements on the youngest leaves that were fully expanded were made 3 weeks after final treatment concentrations were reached, when plants had achieved a steady state.

Water status of plants

Leaf water potential was measured with a pressure chamber (Model 3000, Soil Moisture Equipment Co. USA) on the youngest and fully expanded leaves. Relative water

content was calculated as: (FW − DW)/(TW − DW) × 100, where FW is the fresh weight, TW is the turgid weight after rehydrating samples for 24 h by soaking the leaves with water, and DW is the dry weight after oven-drying samples at 85°C for 24 h.

Determination of sodium and chloride

Leaf samples were dried in an oven at 105°C for 15 min and then at 80°C to a constant weight. Then ca. 20 mg dried leaves were burnt into ashes in an oven at 500°C. The ashes were dissolved in HNO_3 solution and diluted with distilled water to 250 ml. Na^+ was measured by atomic absorption spectrometry (Hitachi Z-8000, Japan). Cl^- was determined by a chloride electrode (PXSJ-216, China).

Analysis of gas exchange

Measurements of net photosynthetic gas exchange were made on a fully expanded attached leave of tobacco seedlings using an open system (Ciras-1, PP systems, UK). CO_2 assimilation rate and stomatal conductance were made at a CO_2 concentration of 360 μl l^{-1} and at a temperature 25°C with a relative humidity 80% and saturating light (800 μmol m^{-2} s^{-1}).

Chlorophyll fluorescence

To investigate the effects of salt stress on in vivo electron transport rate under salt stress conditions, we estimated the maximal electron transport rate (Φ_{PSII}) using chlorophyll fluorescence by following the protocol of Mark Stitt who first used an oxygen electrode system (Stitt 1986). Φ_{PSII} was measured by transiently lowering the saturating light intensity to the compensation point to allow the dark reactions to use up the NADPH and ATP produced by the light reactions but not long enough for deactivation of the Calvin enzymes to occur. Under these special conditions, the measured Φ_{PSII} was determined by the amount of non-photochemical quenching (NPQ) that was present in the sample before the saturating light intensity was lowered. Thus, it should be pointed out that measurement of Φ_{PSII} in this study is different from normal measuring techniques for Φ_{PSII}.

Chlorophyll fluorescence was measured at room temperature with a portable fluorometer (PAM-2000, Walz, Germany) after the attached leaves had been dark-adapted for 15 min. The fluorometer was connected to a leaf-clip holder (2030-B, Walz) with a tri-furcated fiberoptic (2010-F, Walz) and to a computer with data acquisition software (DA-2000, Walz).

The minimal fluorescence level (F_o) with all PSII reaction centers open was measured by the measuring modulated light, which was sufficiently low (<0.1 μmol m^{-2} s^{-1}) not to induce any significant variable fluorescence. The maximal fluorescence level (F_m) with all PSII reaction centers closed were determined by a 0.8 s saturating pulse at 8,000 μmol m^{-2} s^{-1} in dark-adapted leaves. Then, the leaf was illuminated with saturating continuous background light (800 μmol m^{-2} s^{-1}) to come to steady state photosynthesis after about 8 min by following the steady-state value of fluorescence. The leaf was thereafter illuminated with the light (35 μmol m^{-2} s^{-1}), which was just above the compensation point for 30 s by decreasing saturating continuous background light and then the leaf was illuminated again with saturating light. Immediately, fluorescence value (F_t) was recorded and a second saturating pulse at 8,000 μmol m^{-2} s^{-1} was imposed to determine maximal fluorescence level (F'_m). The saturating background light was removed and the minimal fluorescence level (F'_o) was determined by illuminating the leaf with a 3 s pulse of far-red light.

Using above fluorescence parameters, we calculated: (1) the maximal efficiency of PSII photochemistry in the dark-adapted state, $F_v/F_m = (F_m - F_o)/F_m$, (2) the maximal PSII efficiency, $\Phi_{PSII} = (F'_m - F_t)/F'_m$, and (3) the efficiency of excitation capture by open PSII reaction centers, F'_v/F'_m, (4) the photochemical quenching coefficient, $q_P = (F'_m - F_t)/(F'_m - F'_o)$, (5) non-photochemical quenching, NPQ = $(F_m/F'_m) - 1$(Genty et al. 1989; van Kooten and Snel 1990).

Electron transport activities

After 3 weeks for salt stress treatment, the youngest fully expanded leaves were harvested and homogenized in a medium containing 0.4 M sucrose, 50 mM tricine (pH 7.6). The homogenate was filtrated through 16 layers of gauze and then the filtrate was centrifuged at 500*g* for 3 min to remove large debris. The supernatant was further centrifuged at 3,000*g* for 10 min. The pellet was washed twice by the buffer (50 mM tricine, 10 mM NaCl, 5 mM $MgCl_2$, pH 7.6) at 10,000 *g* for 10 min. The resulting washed pellet was thylakoid membranes and resuspended in the same buffer for measuring electron transport activities.

Electron transport activities in the thylakoid membranes were measured with a Clark-type oxygen electrode (Hansatech, King's Lynn, Norfolk, UK) suspended in the medium (0.4 M sucrose, 50 mM Tricine, 10 mM NaCl, 5 mM $MgCl_2$, pH 7.6) under white light with a saturating intensity (800 μmol m^{-2} s^{-1}). For measurement of whole-chain electron transport activity, 10 mM methylamine,

1 mM sodium azide, and 1 mM methyl viologen were added. PSI electron transport activity was measured with 10 mM methylamine, 10 μM 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU), 1 mM sodium azide, 500 μM 2,6-dichlorophenol indophenol (DCPIP), 2 mM sodium ascorbate, and 1 mM methyl viologen. PSII electron transport activity was determined using 1 mM phenyl-*p*-benzoquinone as electron acceptor.

GB extraction and quantification

The method developed by Naidu (1998) was followed. Leaf samples were ground in methanol:chloroform:water (12:5:1). The aqueous phase was fractioned by Dowex-1-OH^- and Dowex-50-H^+ ion-exchange chromatograph. The GB fraction was eluted with 6 M NH_4OH, dried under a stream of N_2 at 45°C and dissolved in 1 ml of distilled water. GB was analyzed by a HPLC system (Waters Corp., Milford, MA, USA). A Waters 600 Pump delivery System with Waters 996 PDA detector was equipped with a column of Hypersil 10SCX (4.6 × 250 mm, Dalian Elite Analytical Instruments Co., China). Data were analyzed with Millenium Chromatography Manager System Control software (Waters Corp.). The amount of GB in each sample was estimated from the refractive index by referring to standard GB solutions.

Enzyme activities and metabolite contents

The non-salt-stressed (control) and salt-stressed whole plants were irradiated with saturating light 800 μmol m^{-2} s^{-1} for 30 min at 25°C to promote activation of enzymes. After illumination, leaf tissues from non-salt-stressed and salt-stressed plants were harvested and immediately frozen in liquid nitrogen for analyses of enzyme activities and metabolite levels. For the assay of the enzymes, crude homogenates were prepared by grinding leaf tissues (3.2 cm^2) with 2 ml of extraction buffers (the extraction buffers were different for different enzymes) at 0–4°C in a prechilled mortar and pestle and the resulting solutions were centrifuged 1 min at 14,000*g*, 4°C. The resulting supernatants (1 ml) were further used for the assay of the activities of different enzymes in the different assay buffers (the assay buffers were different for different enzymes). During the assay of different enzymes, no further extra NaCl and GB were added into the assay buffers.

The effects of in vitro GB on the activities of the enzymes under salt stress conditions were carried by using broken chloroplasts after an osmotic shock from intact chloroplasts of wild type plants. The broken chloroplasts (2 ml) were incubated with exogenous GB (final concentration: 1.5 μmol mg^{-1} Chl) at different NaCl concentrations (final concentrations: 0, 20, and 40 μmol mg^{-1} Chl) for 30 min at 4°C. Then, the broken chloroplasts were centrifuged 1 min at 14,000*g*, 4°C. The resulting supernatants (1 ml) were used for the assay of the activities of different enzymes in the different assay buffers. During the assay of different enzymes, no further extra NaCl and GB were added into the assay buffers.

Total and initial ribulose 1,5-bisphosphate carboxylase/oxygenase (Rubisco) activities were determined as described previously (Feller et al. 1998). The activity of Rubisco activase was calculated as the relative ratio of initial to total Rubisco activities (Perchorowicz et al. 1981). The activity of plastid fructose-1,6-bisphosphatase (FBPase) was determined according to Schimkat et al. (1990). Fructose-1,6-bisphosphate aldolase (FBP aldolase) was measured as described previously (Haake et al. 1998). The activities of phosphoribulokinase (PRKase), phosphoglycerate kinase (PGA kinase), triose phosphate isomerase, ribulose-5-phosphate isomerase, sedoheptulose-1,7-bisphosphatase (SBPase), and transketolase were determined according to Leegood (1990). The contents of 3-phosphoglycerate (PGA), ribulose-1,5-bisphosphate (RuBP), and fructose 6 phosphate (F6P) were determined according to Schrader et al. (2004).

SDS-PAGE and immunological analyses

Samples were solubilized in the presence of 6 M urea and separated by SDS-PAGE (Laemmli 1970) using 15% (w/v) acrylamide gels with 6 M urea. For immunoblotting, polypeptides were electrophoretically transferred to a nitrocellulose membranes (Millipore, Saint-Quentin, France) and proteins were detected with antibodies raised against Rubisco activase (G. Chen, Shanghai Institute of Plant Physiology and Ecology), and Rubisco large subunit (L. Zhang, Institute of Botany, Chinese Academy of Sciences).

Isolation of chloroplasts

Intact chloroplasts were isolated by centrifugation on a Percoll density gradient according to the protocol of Mullet and Chua (1983). The percentage of intact chloroplasts was determined by measuring ferricyanide photoreduction before and after osmotic shock. Chlorophyll content was determined in 80% (v/v) acetone according to Porra et al. (1989). Protein content was determined by the dye-binding assay according to Bradford (1976).

Results

Effects of salt stress on the accumulation of sodium and chloride, water status, and growth of seedlings

Five independent lines of transgenic tobacco plants (L1, L2, L3, L4, and L5) were used in this study and there were no differences in the size or developmental stage of two-month-old wild type plants and transgenic plants (Yang et al. 2005). There were no differences in total soluble protein content of leaves either between wild type and transgenic plants or between control and NaCl-treated plants on a leaf area basis (data not shown). The GB content in youngest fully expanded leaves was about 3.2, 1.2, 2.9, 4.9, 0.4 μmol g^{-1} fresh weight in L1–L5 plants, respectively, after two-month growth and no GB was detected in wild type plants. Although betaine aldehyde dehydrogenase is specifically targeted to the chloroplast, not all GB was accumulated in the chloroplast. By comparing the contents of GB in the leaf and chloroplast, expressed on a chlorophyll basis and corrected for the percentage of broken chloroplasts present, the percentage of GB found in the chloroplast can be calculated. It was estimated that 61–85% of total leaf GB was localized in the chloroplast (Table 1).

After exposed to different salt concentrations for 3 weeks, salt treatment resulted in a significant accumulation of sodium and chloride. However, there were no significant differences in the levels of sodium and chloride between wild-type and transgenic L4 plants in both control and salt-stressed plants expressed either on the basis of leaf dry weight or chlorophyll. By comparing the contents of NaCl in the leaf and chloroplast expressed on a chlorophyll basis and corrected for the percentage of broken chloroplasts present, it was estimated that about 32% of total leaf NaCl was entered into the chloroplast (Table 2). We also determined the levels of sodium and chloride in other transgenic lines (L1, L2, L3, and L5) under salt stress. We observed that there were also no differences in the levels of sodium and chloride between wild-type plants and these transgenic lines and that about 32% of total leaf NaCl was entered into the chloroplast (data not shown). Salt stress also resulted in a significant decrease in water potential and relative water content in leaves in wild-type and transgenic plants but there were no significant differences in leaf water potential and relative water content between wild-type plants and transgenic plants (Table 3).

Figure 1 shows the effects of salt stress on the growth of wild type and transgenic plants. Salt stress decreased the growth in wild type and transgenic plants and such a decrease was greater in wild type plants than in transgenic plants with increasing salt concentration. The higher the accumulation of GB was, the higher the tolerance of the growth to salt stress was. Transgenic line 4 plants showed highest tolerance to salt stress. These results suggest that accumulation of GB in vivo enhances tolerance of the growth to salt stress in tobacco plants.

Effects of salt stress on CO_2 assimilation and stomatal conductance

Figure 2 shows the changes in CO_2 assimilation rate and stomatal conductance in wild type and five transgenic lines after exposed to salt stress for 3 weeks. CO_2 assimilation rate decreased significantly with increasing salt concentration in wild type plants and transgenic plants and such a decrease was much greater in wild type plants than in transgenic plants. Line 4 showed the highest tolerance of CO_2 assimilation rate to salt stress (Fig. 2a). Salt stress also resulted in a significant decrease in stomatal conductance in wild type plants and transgenic plants. However, there were no significant differences in stomatal conductance between wild type plants and transgenic plants (Fig. 2b). These results indicate that the increased tolerance of CO_2 assimilation

Table 1 Glycinebetaine (GB) levels in leaves and chloroplasts isolated from leaves of transgenic plants

Transgenic lines	GB in leaves (μmol g^{-1} fresh weight)	GB in leaves (μmol mg^{-1} Chl)	GB in isolated chloroplasts (μmol mg^{-1} Chl)	Intact chloroplasts (%)	GB in chloroplasts (%)
L1	3.22 ± 0.12	1.31 ± 0.09	0.96 ± 0.10	92.5 ± 4.0	78.2 ± 4.5
L2	1.23 ± 0.15	0.48 ± 0.04	0.31 ± 0.05	93.4 ± 5.2	68.8 ± 5.4
L3	2.91 ± 0.22	1.16 ± 0.12	0.82 ± 0.04	95.3 ± 3.1	75.2 ± 6.1
L4	4.92 ± 0.25	1.87 ± 0.08	1.49 ± 0.10	93.6 ± 3.2	85.1 ± 5.8
L5	0.44 ± 0.17	0.17 ± 0.07	0.10 ± 0.04	95.2 ± 3.1	60.8 ± 3.7

Values in the table are mean ± SE from four independent experiments. GB content in chloroplasts was corrected for the percentage of broken chloroplasts present. The percentage of GB found in the chloroplast was calculated by comparing leaf and chloroplast contents expressed on a chlorophyll basis

Table 2 Effects of salt stress on Na^+ and Cl^- levels in leaves and chloroplasts isolated from leaves of wild type and transgenic L4 plants

NaCl (mM)			Na^+ and Cl^- in leaves (μmol g^{-1} dw)	Na^+ and Cl^- in leaves (μmol mg^{-1} Chl)	Na^+ and Cl^- in isolated chloroplasts (μmol mg^{-1} Chl)	Intact chloroplasts (%)	Na^+ and Cl^- in chloroplasts (%)
0	Na^+	wt	5.6 ± 1.0	5.3 ± 0.2	1.5 ± 0.05	94.5 ± 1.6	31.2 ± 2.3
		L4	6.0 ± 2.0	5.5 ± 0.3	1.6 ± 0.07	94.6 ± 3.6	31.1 ± 2.4
	Cl^-	wt	8.0 ± 3.1	5.8 ± 0.2	1.7 ± 0.05	93.5 ± 1.1	32.2 ± 2.1
		L4	7.6 ± 3.6	5.3 ± 0.2	1.6 ± 0.05	94.4 ± 2.8	31.6 ± 2.6
75	Na^+	wt	45.1 ± 3.1	55.6 ± 2.3	17.3 ± 1.2	94.5 ± 2.6	32.5 ± 3.0
		L4	43.2 ± 3.7	54.2 ± 3.7	16.5 ± 1.5	92.4 ± 3.6	33.2 ± 2.1
	Cl^-	wt	53.1 ± 5.1	53.6 ± 3.3	16.3 ± 1.6	92.5 ± 2.1	31.4 ± 1.9
		L4	51.0 ± 3.1	55.2 ± 3.5	17.5 ± 1.1	93.4 ± 2.3	33.2 ± 2.0
150	Na^+	wt	120.3 ± 7.1	120.5 ± 5.6	38.3 ± 2.5	94.5 ± 2.6	32.9 ± 3.0
		L4	123.5 ± 11.1	124.6 ± 6.7	38.2 ± 2.6	92.4 ± 3.6	34.2 ± 2.5
	Cl^-	wt	124.1 ± 2.5	116.5 ± 7.2	36.9 ± 2.8	92.5 ± 3.1	33.7 ± 1.9
		L4	123.0 ± 8.0	122.6 ± 5.8	37.5 ± 3.1	91.4 ± 3.5	32.8 ± 2.4

Values in the table are mean ± SE from three independent experiments. Na^+ and Cl^- content in chloroplasts was corrected for the percentage of broken chloroplasts present. The percentage of Na^+ and Cl^- found in the chloroplast was calculated by comparing leaf and chloroplast contents expressed on a chlorophyll basis

rate in transgenic plants was not associated with the changes in stomatal conductance.

Effects of salt stress on function of PSII and PSI

In vitro studies with isolated thylakoid membranes demonstrate that salt stress showed no effects on the activities of PSII and PSI electron transport in wild type and transgenic plants (Table 3). We further examined the effects of salt stress on the maximal efficiency of PSII photochemistry (F_v/F_m), which is a direct measure of damage to PSII. Salt stress had no effects on F_v/F_m in both wild type and transgenic plants (Table 3). These results suggest that salt stress showed no damage to PSII in both wild type and transgenic plants.

In order to know the potential limitations of electron transport rate in vivo under salt stress, we followed Mark Stitt's protocol (Stitt 1986) and used chlorophyll fluorescence to compare maximum electron transport rate independent of the dark reactions by allowing the leaves to come to steady state photosynthesis under saturating light, decreasing the light to just above the compensation point for 30 s and then returning the leaves to saturating light. The measurement of the PSII efficiency (Φ_{PSII}) immediately after transition to saturating light can give a relative measurement of maximum electron transport rate in vivo. Figure 3a shows that (Φ_{PSII}) decreased significantly with increasing salt concentration in wild type and transgenic plants and such a decrease was much greater in wild type plants than in transgenic plants. Line 4 showed the highest resistance of Φ_{PSII} to salt stress. Concomitant to the measurement of Φ_{PSII}, we also measured the efficiency of excitation energy capture by open PSII reaction centers $\left(F'_v/F'_m\right)$, the coefficient of photochemical quenching (q_P), and the NPQ. The patterns of changes in F'_v/F'_m were similar to those of (Φ_{PSII}. F'_v/F'_m decreased significantly with increasing salt concentration in wild type and transgenic plants and such a decrease was much greater in wild type plants than in transgenic plants. Line 4 showed the highest resistance of F'_v/F'_m to salt stress (Fig. 3b). On the other hand, NPQ increased with increasing salt concentration in wild type plants and transgenic plants and such an increase was much greater in wild type plants than in transgenic plants (Fig. 3c). However, salt stress showed no effects on q_P in wild type and transgenic plants and also no significant differences in q_P between wild type plants and transgenic plants were observed (Table 3).

Effects of salt stress on the activities of the enzymes and metabolite levels

Since it has been shown that Rubisco activity is inhibited by salt stress (Brugnoli and Björkman 1992), we compared the effects of salt stress on the activity of Rubisco between wild type plants and transgenic plants. Figure 4 shows that salt stress resulted in a significant decrease in the total and initial Rubisco activities in both wild type plants and transgenic plants. However, the inhibition of Rubisco activity in wild type plants was much greater than that in transgenic plants. The activity of Rubisco in transgenic line 4 demonstrated the highest tolerance to salt stress. This inhibition of Rubisco activity was not due to the changes in the content of Rubisco, since western-blotting analysis revealed that there were no significant changes in the content of Rubisco during salt stress either in wild type plants or transgenic plants (Table 3).

Table 3 Effects of salt stress on water potential (MPa), relative water content (%), the activities of PSI and PSII electron transport (μmol O_2 mg^{-1} Chl h^{-1}), the maximal efficiency of PSII photochemistry (F_v/F_m), the coefficient of photochemical quenching (q_P), the content of Rubisco, Rubisco activation state, the content of Rubisco activase, the activities (μmol m^{-2} s^{-1}) of phosphoglycerate kinase, triose phosphate isomerase, ribulose-5-phosphate isomerase, transketolase activity, and SBPase of leaves in wild type and transgenic L4 plants

Parameters	NaCl (mM)	0	75	150
Water potential	wt	−0.35 ± 0.03	−0.78 ± 0.03	−1.42 ± 0.02
	L4	−0.35 ± 0.05	−0.86 ± 0.07	−1.40 ± 0.04
Relative water content	wt	97.2 ± 3.0	90.7 ± 2.5	80.1 ± 2.6
	L4	96.8 ± 3.6	90.0 ± 2.5	82.5 ± 3.0
PSI activity	wt	710 ± 43	732 ± 45	730 ± 32
	L4	732 ± 54	754 ± 34	762 ± 45
PSII activity	wt	321 ± 32	333 ± 31	324 ± 26
	L4	342 ± 26	324 ± 23	352 ± 19
F_v/F_m	wt	0.84 ± 0.01	0.84 ± 0.01	0.84 ± 0.01
	L4	0.84 ± 0.01	0.83 ± 0.01	0.84 ± 0.01
q_p	wt	0.82 ± 0.01	0.83 ± 0.02	0.84 ± 0.01
	L4	0.82 ± 0.02	0.83 ± 0.01	0.83 ± 0.02
Relative Rubisco content	wt	100.0 ± 6.0	99.6 ± 7.5	101.1 ± 6.6
	L4	99.6 ± 7.6	101.1 ± 8.5	102.2 ± 7.0
Rubisco activation state	wt	85.0 ± 5.1	86.0 ± 5.6	83.5 ± 6.1
	L4	83.4 ± 7.0	84.0 ± 5.1	85.2 ± 6.0
Relative Rubisco activase content	wt	100.0 ± 7.1	100.4 ± 5.6	103.2 ± 8.1
	L4	101.2 ± 7.3	102.3 ± 6.7	99.3 ± 6.7
Phosphoglycerate kinase activity	wt	192.1 ± 12.1	198.2 ± 11.0	192.1 ± 11.2
	L4	198.2 ± 10.2	192.1 ± 10.1	199.3 ± 10.2
Triose phosphate isomerase activity	wt	100.0 ± 7.1	100.4 ± 5.6	103.2 ± 8.1
	L4	101.2 ± 7.3	102.3 ± 6.7	104.3 ± 6.7
Ribulose-5-phosphate isomerase activity	wt	102.0 ± 6.6	107.4 ± 9.8	106.2 ± 9.7
	L4	105.2 ± 6.9	104.3 ± 7.2	104.2 ± 8.4
Transketolase activity	wt	12.0 ± 0.9	11.4 ± 0.5	11.8 ± 0.6
	L4	12.2 ± 0.4	10.2 ± 0.6	10.7 ± 0.6
SBPase activity	wt	7.0 ± 0.12	7.2 ± 0.10	7.4 ± 0.09
	L4	7.2 ± 0.14	6.9 ± 0.11	7.3 ± 0.10

Relative quantitation of Rubisco and Rubisco activase was calculated from western blotting by loading equal protein for electrophoresis. It should be noted that similar results were also observed when relative quantitation of Rubisco and Rubisco activase was calculated from western blotting by loading equal volume protein isolated from equal area leaf for electrophoresis. For easy comparison, the contents of Rubisco and Rubisco activase were expressed as relative percentage of wild type plants when grown at 0 mM NaCl conditions. It should be noted that although the parameters listed in this table were determined from the wild type and transgenic line 4 plants, similar results were also observed from other transgenic lines. The values are mean ± SE of three independent experiments

Since it has been shown that Rubisco activase plays a pivotal role in the regulation of Rubisco activity (Andrews et al. 1995; Eckhardt and Portis 1997), we investigated whether the decreased Rubisco activity under salt stress was due to the inhibition of the activity of Rubisco activase. Because there is the large amount of endogenous Rubisco in the leaves, it is difficult directly to measure the activity of Rubisco activase. Instead, activase activity is expressed by the ratio of the initial activity to the total activity of Rubisco, i.e., Rubisco activation state (Andrews et al. 1995; Eckhardt and Portis 1997). We examined the changes in Rubisco activation state in wild type plants and transgenic plants after exposure to salt stress for three weeks. Our results showed that salt stress had no effects on Rubisco activation state in both wild type and transgenic plants (Table 3). Western-blotting analysis further showed that there were also no significant changes in the content of activase during salt stress in wild type plants and transgenic plants. These results suggest that the decreased Rubisco activity under salt stress is not associated with the changes in Rubisco activase.

We also investigated the effects of salt stress on the activities of the other key enzymes involved in the Calvin Cycle, such as FBP aldolase, plastid FBPase, PRKase,

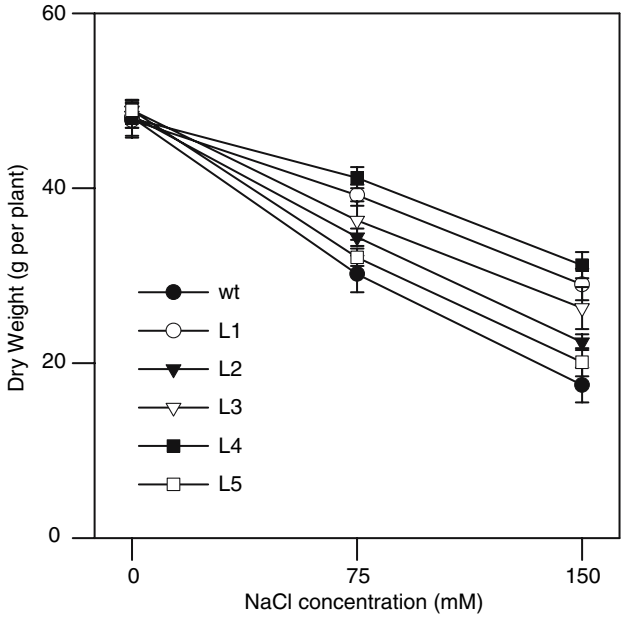

Fig. 1 Effects of salt stress on dry weight per plant in wild type and transgenic plants. The values are mean ± SE of three independent experiments

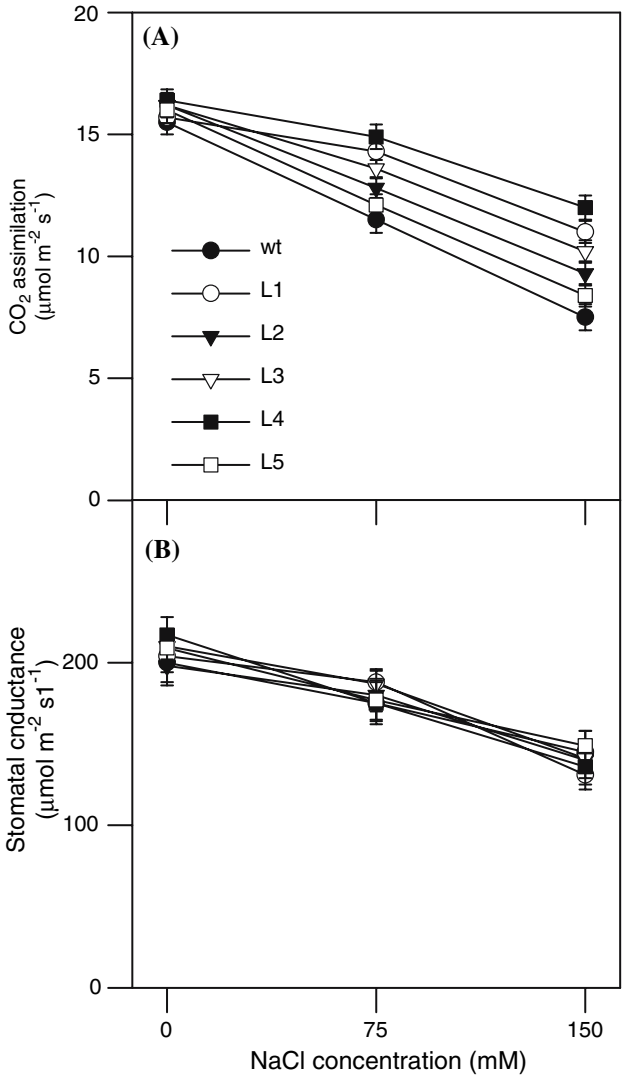

Fig. 2 Effects of salt stress on CO_2 assimilation rate (**a**) and stomatal conductance (**b**) of leaves in wild type and transgenic plants. The values are mean ± SE of three independent experiments

PGA kinase, triose phosphate isomerase, ribulose-5-phosphate isomerase, transketolase, and SBPase, in wild type plants and transgenic plants. Figure 5 shows that salt stress inhibited significantly the activities of FBPase, FBP aldolase, and PRKase in both wild type and transgenic plants but this inhibition was much greater in wild type plants than in transgenic plants. The activities of FBPase, FBP aldolase, and PRKase in line 4 demonstrated the highest tolerance to salt stress. These results demonstrate that the accumulation of GB in vivo protected Rubisco, FBPase, FBP aldolase, and PRKase against salt stress. However, our results demonstrated that there were no significant changes in the activities of PGA kinase, triose phosphate isomerase, ribulose-5-phosphate isomerase, transketolase, and SBPase in both wild type and transgenic plants under salt stress conditions (Table 3).

In addition, we also investigated the changes in the levels of RuBP, PGA, and F6P in wild type and transgenic plants under salt stress. Figure 6 shows that salt stress resulted in a decrease in the levels of RuBP, PGA, and F6P in both wild type and transgenic plants and such a decrease was greater in wild type plants than in transgenic plants. Transgenic line 4 had the highest levels of RuBP, PGA, and F6P among transgenic plants under salt stress conditions.

In order to investigate whether the decrease in the activities of Rubisco, FBPase, FBP aldolase, and PRKase was caused by a direct inhibition of the enzymes by NaCl carried over in the assay, we determined the levels of NaCl in the assay for the activities of the enzymes. Table 4 shows the concentrations of Na^+ and Cl^- in the assay for determining the activities of the enzymes isolated from leaves of wild type and transgenic L4 plants after treated with different NaCl concentrations for 3 weeks. Our results show that the levels of Na^+ and Cl^- in the assay increased significantly after treated with NaCl for 3 weeks. However, there were no big differences in the concentrations of Na^+ and Cl^- between wild type and transgenic plants either for control plants or NaCl-treated plants. Similar results were also observed in other transgenic lines (L1, L2, L3, and L5) (data not shown). By comparing the results of Table 4 to those of Table 2, we found out that concentrations of Na^+ and Cl^- in the assay were largely comparable to those in the chloroplast. These results suggest that NaCl was carried into the assay probably because the extract was fairly concentrated and that the decrease in the activities of

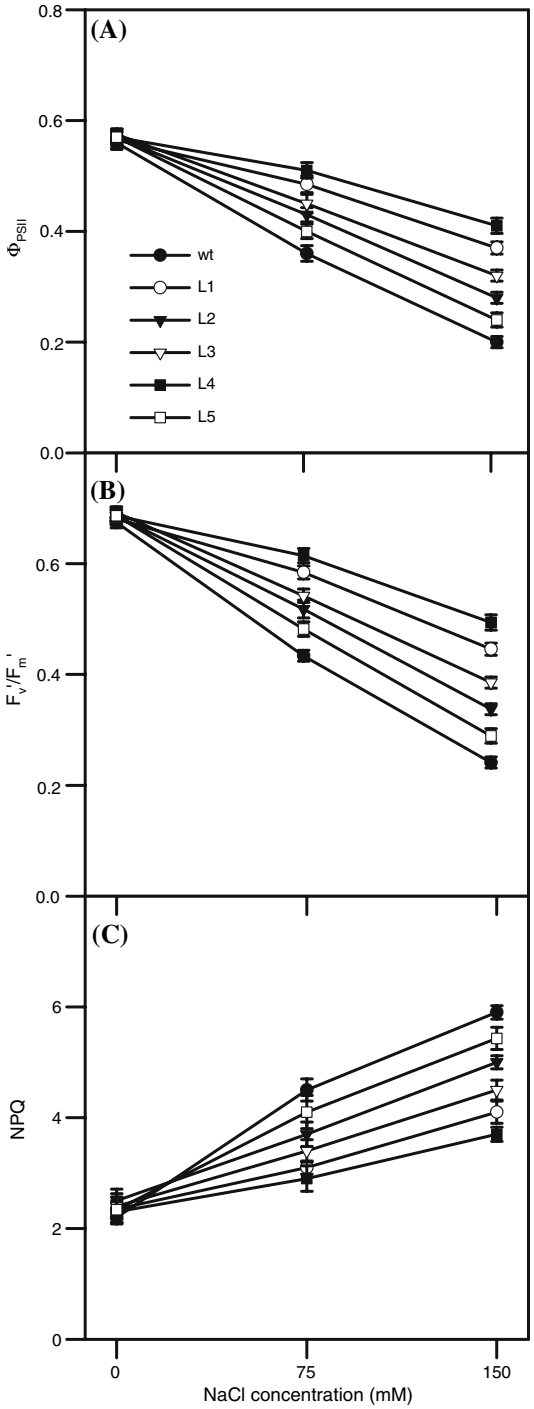

Fig. 3 Effects of salt stress on (**a**) the maximal PSII efficiency (Φ_{PSII}), (**b**) the efficiency of excitation energy capture by open PSII reaction centers $\left(F'_v/F'_m\right)$, and (**c**) non-photochemical quenching (NPQ) of leaves in wild type and transgenic plants. The values are mean ± SE of three independent experiments

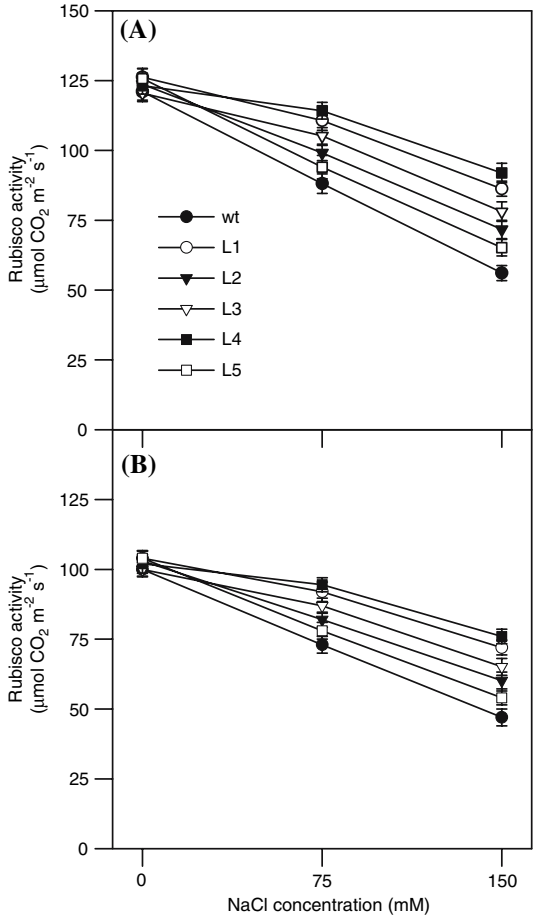

Fig. 4 Effects of salt stress on the total Rubisco activity (**a**) and the initial Rubisco activity (**b**) of leaves in wild type and transgenic plants. The values are mean ± SE of three independent experiments

Rubisco, FBPase, FBP aldolase, and PRKase may be caused by a direct inhibition of the enzymes by NaCl carried over in the assay.

In order to further investigate the role of GB in protecting Rubisco, FBPase, FBP aldolase, and PRKase against salt stress, we examined the effects of in vitro GB and NaCl on the activities of Rubisco, FBPase, FBP aldolase, and PRKase. The broken chloroplasts after an osmotic shock from intact chloroplasts of wild type plants were incubated with 1.5 μmol mg^{-1} Chl exogenous GB which was the concentration of GB in transgenic L4 plants (see Table 1) at different NaCl concentrations (0, 20, and 40 μmol mg^{-1} Chl) for 30 min. It should be noted that NaCl was added according to the concentrations of NaCl in the chloroplast (see Table 2). Figure 7 clearly shows that exogenous GB also significantly protected the activities of Rubisco, FBPase, FBP aldolase, and PRKase against salt stress.

Discussion

The results in this study demonstrate that the accumulation of GB in vivo in the chloroplast in tobacco plants by introducing the BADH gene for betaine aldehyde dehydrogenase from spinach (*Spinacia oleracea* L.) resulted in

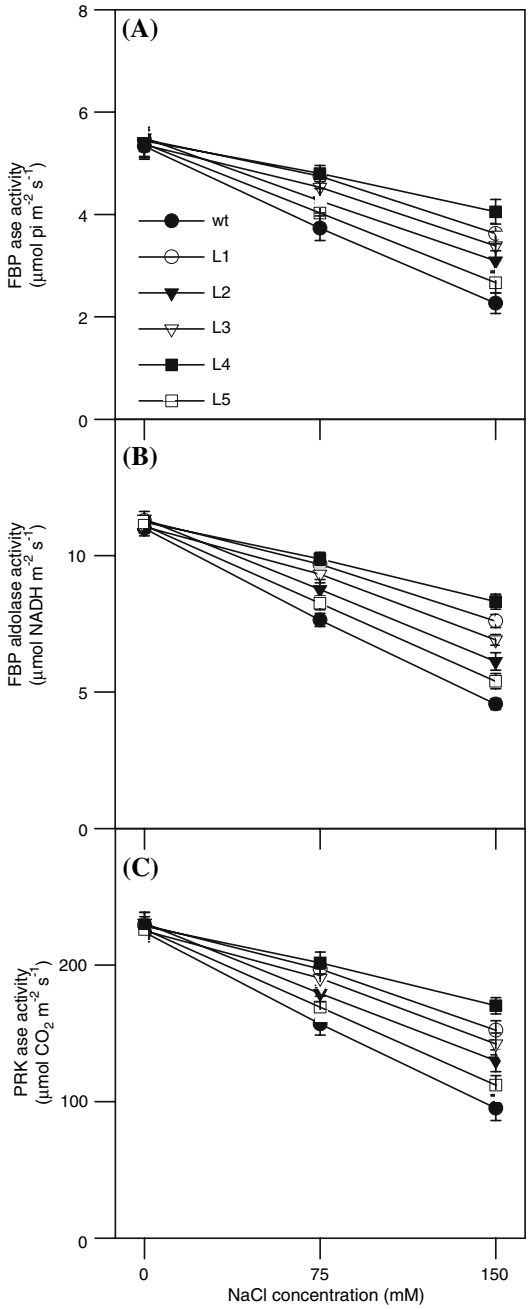

Fig. 5 Effects of salt stress on the activities of FBPase (**a**), FBP aldolase (**b**), and PRK (**c**) of leaves in wild type and transgenic plants. The values are mean ± SE of three independent experiments

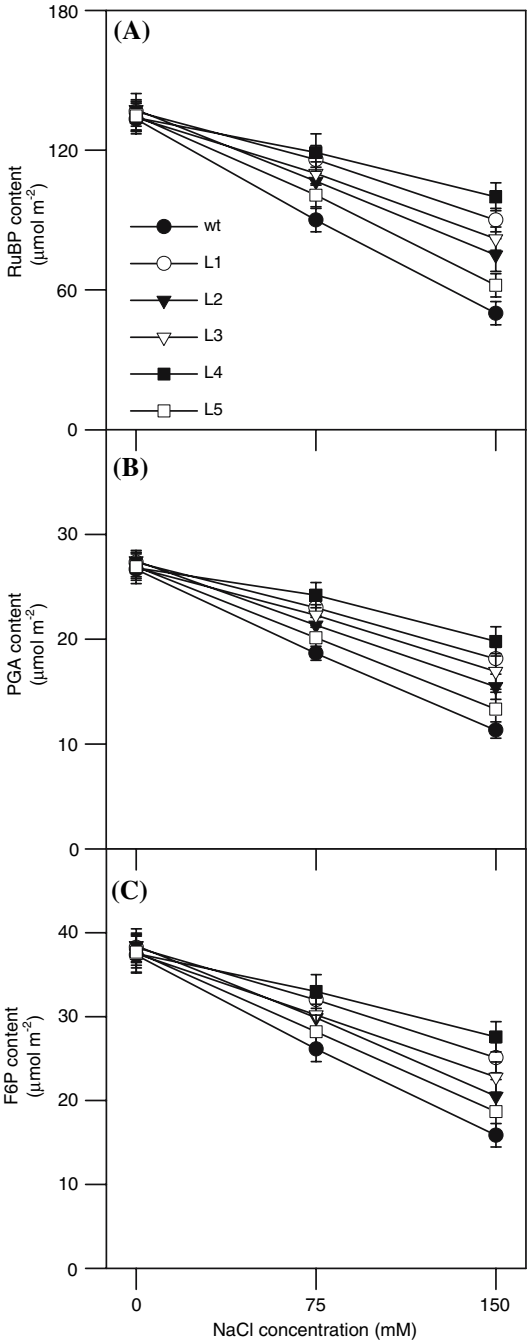

Fig. 6 Effects of salt stress on the contents of RuBP (**a**), PGA (**b**), and F6P (**c**) of leaves in wild type and transgenic plants. The values are mean ± SE of three independent experiments

increased tolerance of growth of young seedlings to salt stress. Using these transgenic tobacco plants, we investigated the possible physiological basis of GB in vivo for such an enhanced tolerance of growth to salt stress.

It has been suggested from a series of physiological studies that GB acts most likely by maintaining the osmotic balance between the intracellular and the extracellular environment under salt and drought stress. This maintaining occurs only when GB accumulates at high levels, e.g., 40 μmol g^{-1} fresh weight in certain specie under stress conditions (Rhode and Hanson 1993). The highest accumulation of GB in transgenic plants in this study was about 5 μmol g^{-1} fresh weight and such a low level should be insignificant in the context of osmotic regulation (Sakamoto and Murata 2001). However, the transgenic plants still showed significantly enhanced tolerance to salt stress (Fig. 1). The results in this study suggest that the increased

Table 4 Na^+ and Cl^- levels in the assay used for determining the activities of the enzymes isolated from leaves of wild type and transgenic L4 plants after treated with different NaCl concentrations for 3 weeks

Na^+ and Cl^- levels (μmol mg^{-1} Chl)	NaCl (mM)	0	75	150
Na^{+ac}	wt	1.8 ± 0.1	18.2 ± 2.4	39.2 ± 3.2
	L4	1.6 ± 0.3	20.2 ± 2.5	37.5 ± 3.9
Cl^-	wt	1.7 ± 0.3	19.6 ± 2.1	40.1 ± 3.8
	L4	1.5 ± 0.2	20.0 ± 2.8	39.5 ± 4.1

Values in the table are mean ± SE from three independent experiments

tolerance of growth to salt stress in transgenic plants may be associated with the other effects than osmoregulation (Sakamoto and Murata 2001).

Since the decline in growth observed in many plants under salt stress is often associated with a decrease in their photosynthetic capacity (Long and Baker 1986), we compared the effects of salt stress on CO_2 assimilation in both wild type and transgenic plants. The results in this study demonstrate that accumulation of GB in vivo led to increased tolerance of CO_2 assimilation to salt stress (Fig. 2a). Our results further show that although there was a significant decrease in stomatal conductance in salt-stressed wild type and transgenic plants, no significant differences were observed in the decreased stomatal conductance between wild type plants and transgenic plants (Fig. 2b). This result indicates that the increased tolerance of photosynthesis in transgenic plants to salt stress was not associated with stomatal response under salt stress conditions. No significant differences in stomatal conductance in wild type and transgenic plants can be explained by the fact that there were no significant differences in leaf water status between wild type plants and transgenic plants (Table 2).

Many studies have shown that salt stress inhibits in vitro PSII activity (Mishra et al. 1991; Belkhodja et al. 1994; Everard et al. 1994). Earlier studies using isolated thylakoid membranes have suggested a protective role of GB on PSII at high salt concentrations (Papageorgiou and Murata 1995). In order to investigate whether increased CO_2 assimilation in transgenic plants under salt stress is due to GB-induced-protection to PSII electron transport, we examined the differences in the activities of PSII electron transport between wild type and transgenic plants under salt stress. Our results showed that salt stress had no effects on PSII electron transport in vitro and the maximal efficiency of PSII photochemistry (F_v/F_m) (Table 3), suggesting that salt stress induced no damage to PSII electron transport. This is probably because salt stress in this study was performed under relatively moderate light

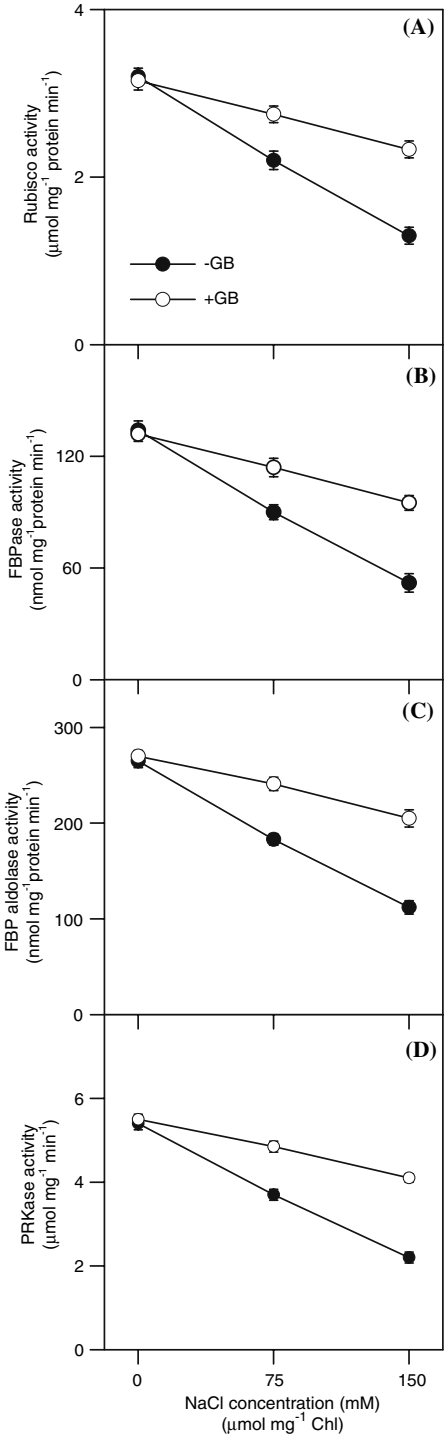

Fig. 7 Effects of in vitro GB on the activities of (**a**) Rubisco, (**b**) FBPase, (**c**) FBP aldolase, and (**d**) PRKase of chloroplasts isolated from wild type plants in the absence/presence of different NaCl concentrations. The values are mean ± SE of three independent experiments

intensity (300 μmol m^{-2} s^{-1}) under which condition photoinhibition was not induced. If wild type and transgenic plants were exposed to higher light intensity, it is possible

that photoinhibition may occur and there would be a decrease in F_v/F_m and in vitro PSII electron transport rate. Indeed, it has been shown that there is a decrease in F_v/F_m under salt stress condition when exposed to high light in transgenic tobacco and rice and the accumulation of GB shows a protective role in PSII under such conditions (Hayashi et al. 1997; Holmström et al. 2000).

However, in vivo studies using chlorophyll fluorescence demonstrated that salt stress resulted in a decrease in the maximum electron transport rate in vivo (Φ_{PSII}). Moreover, such a decrease was much greater in wild type plants than in transgenic plants (Fig. 3). How did salt stress show no damage to PSII electron transport but induce a decrease in the maximum electron transport rate in vivo in both wild type and transgenic plants? In this study, we observed no changes in F_v/F_m in salt-stressed wild type and transgenic plants (Table 3). No significant changes in F_v/F_m in salt-stressed wild type and transgenic plants indicate that the significant decrease in (Φ_{PSII}) can be the result of a down-regulation of PSII electron transport in vivo. Such a down-regulation was associated with a decrease in F'_v/F'_m, since $\Phi_{PSII} = q_P \times F'_v/F'_m$ (Genty et al. 1989) and there was no change in q_P in salt-stressed wild type and transgenic plants (Fig. 3, Table 3). The fact that there was a considerable decrease in F'_v/F'_m, but no decrease in F_v/F_m in salt-stressed wild type and transgenic plants suggests that the decreased F'_v/F'_m could be associated with an increase in energy dissipation in the PSII antennae (Horton et al. 1996; Gilmore 1997). Indeed, a significant increase in NPQ in salt-stressed wild type and transgenic plants was observed (Fig. 3c). NPQ has been closely associated with the onset of harmlessly dissipation of the excess energy present in the pigment bed of light harvesting complexes as heat (Horton et al. 1996; Gilmore 1997). Thus, the down-regulation of PSII electron transport in vivo in salt-stressed wild type and transgenic plants was associated with an increase in energy dissipation in the PSII antennae. In this study, we observed that there was a decrease in CO_2 assimilation in salt-stressed wild type and transgenic plants (Fig. 2). The decreased CO_2 assimilation would inevitably result in an excess of excitation energy, which would lead to photodamage to PSII if excess excitation energy could not be dissipated safely. Thus, the down-regulation of PSII electron transport in vivo through an increase in energy dissipation in the PSII can be considered to be a mechanism to down-regulate photosynthetic electron transport in vivo to match the lower demand for ATP and NADPH in the dark reactions of the Calvin cycle with decreased CO_2 assimilation rate in salt-stressed wild type and transgenic plants. Thus, the increase in NPQ was a response to the amount of excess light energy due to lower rates of photosynthesis rather than the changes in GB content. In addition, our results show that q_P was relatively maintained with high values under salt stress conditions (Table 3), indicating that the photochemical efficiency and rates of electron transport were essentially independent of constraints by the dark reactions. Clearly, the results in this study seem to suggest that the primary effect on photosynthesis induced by the accumulation of GB in vivo under salt stress conditions was not an effect of salt on electron transport but rather a direct effect on the activity of dark reaction enzymes.

In this study, we further investigated how the accumulation of GB in vivo affects the key enzymes in the Calvin cycle under salt stress conditions. Our results clearly demonstrate that the accumulation of GB in vivo protected Rubisco, FBPase, FBP aldolase, and PRKase against salt stress (Figs. 4, 5, 7). However, there were no significant changes in the activities of PGA kinase, triose phosphate isomerase, ribulose-5-phosphate isomerase, transketolase, and SBPase in both wild type and transgenic plants (Table 3). It has been reported that a variety of enzymes in higher plants can be inhibited by high salt concentrations (Flower et al. 1977). In vitro studies have shown that GB protects the activities of enzymes, such as Rubisco and malate dehydrogenase, from the inhibitory effects of high concentrations of NaCl and KCl (Pollard and Wyn Jones 1979; Incharoensakdi et al. 1986). These studies suggest that GB in vivo may play a role in protecting the activities of enzymes from salt stress. Indeed, the protective effects on enzymes by GB occur in vivo. Nomura et al. (1998) reported that the accumulation of GB by engineering *Synechococcus* with the *bet* operon from *E. coli* which encodes enzymes for the biosynthesis of GB substantially protected Rubisco from inactivation under salt stress condition. However, whether the accumulation of GB in higher-plant chloroplasts also protects Rubisco as well as other enzymes in the Calvin cycle under salt stress condition remains to be examined (Sakamoto and Murata 2002). To our knowledge, the results in this study for the first time demonstrate that the accumulation of GB in higher plants also protects Rubisco, FBPase, FBP aldolase, and PRKase against salt stress.

The results presented here strongly suggest a role for GB against salt-induced inactivation of some enzymes involved in the Calvin cycle, e.g. Rubisco, FBPase, FBP aldolase, and PRKase. Although the mechanism for this increased tolerance to salt stress in vivo is not clear, a possible role for GB can be tempted to be proposed. The molecular features of GB allow it to interact with both hydrophilic and hydrophobic domains of macromolecules, such as enzymes. It has been documented that, in vitro, GB stabilizes the structures and activities of enzymes against damaging effects of excessive salt, cold and heat (Gorham 1995). Our results show that under salt stress conditions, about 30% NaCl in the leaves was entered into the

chloroplast (Table 2). High concentrations of NaCl may result in direct inhibitory effects on the enzymes in the chloroplast. Previous in vitro studies have shown that GB protects Rubisco and malate dehydrogenase from the inhibitory effects of high concentrations of NaCl and KCl (Pollard and Wyn Jones 1979; Incharoensakdi et al. 1986). Our in vitro studies also show that high concentrations of NaCl inhibited the activities of Rubisco, FBPase, FBP aldolase, and PRKase but GB protects these enzymes against high concentrations of NaCl (Fig. 7). It has also been reported that GB promotes the association of small subunit with the octamer of large subunit of Rubisco in *Aphanothece halophytica* but does not interact with the active site of Rubisco (Incharoensakdi et al. 1986). In addition, it has been proposed that GB may act as a molecular chaperone in *Escherichia coli*, assisting in enzymatic refolding (Bourot et al. 2000). Taken together, we thus tentatively propose that the accumulation of GB in vivo stabilizes conformation of Rubisco, FBPase, FBP aldolase, and PRKase and maintains these enzymes in a functional state under salt stress by acting as a molecular chaperone.

Salt stress has become a major environmental factor that restricts growth and productivity of plants. Photosynthesis is sensitive to salt stress and is easily inhibited under salt stress condition. Although the genetic engineering of the synthesis of GB to tolerate abiotic stress appears promising (Sakamoto and Murata 2002), there are still no reports on the enhanced tolerance of photosynthesis to salt stress. Our study demonstrates the importance of transformation with the BADH gene for enhancing tolerance of growth and photosynthesis to salt stress. Thus, our results may benefit efforts to improve crop yields in saline, arid and semi-arid regions where plants suffer salt stress.

Acknowledgments This work was supported by the Frontier Project of the Knowledge Innovation Engineering of the Chinese Academy of Sciences (grants number: KSCX2-YW-N-042) and the Program of 100 Distinguished Young Scientists of Chinese Academy of Sciences to C. Lu.

References

Abadía A, Belkohodja R, Morales F, Abadía J (1999) Effects of salinity on the photosynthetic pigment composition of barley (*Hordeum vulgare* L.) growth under a triple-line-source sprinkler system in the field. J Plant Physiol 154:392–400

Alia, Hayashi H, Chen THH, Murata N (1998a) Transformation with a gene for choline oxidase enhances the cold tolerance of *Arabidopsis* during germination and early growth. Plant Cell Environ 21:232–239

Alia, Hayashi H, Sakamoto A, Murata N (1998b) Enhancement of the tolerance of *Arabidopsis* to high temperatures by genetic engineering of the synthesis of glycinebetaine. Plant J 16: 155–161

Andrews TJ, Hudson GS, Mate CJ, von Caemmerer S, Evans JR, Avridsson YBC (1995) Rubisco, consequences of altering its expression and activation in transgenic plants. J Exp Bot 46:1293–1300

Belkhodja R, Morales F, Abadia A, Gomez-Aparisi J, Abadia J (1994) Chlorophyll fluorescence as a possible tool for salinity tolerance screening in barley (*Hordeum vulgare* L.). Plant Physiol 104:667–673

Bourot S, Sire O, Trautwetter A, Touze T, Wu LF, Blanco C, Bernard T (2000) Glycinebetaine-assisted protein folding in a lysA mutant of *Escherichia* coli. J Biol Chem 275:1050–1056

Boyer JS (1982) Plant productivity and environment. Science 218:443–448

Bradford MM (1976) A rapid and sensitive method for the quantification of microgram quantities of protein using the principal of protein-dye binding. Anal Biochem 72:248–254

Brugnoli E, Björkman O (1992) Growth of cotton under continuous salinity stress: influence on allocation pattern, stomatal and non-stomatal components of photosynthesis and dissipation of excess light energy. Planta 187:335–345

Downton WJ, Grant WJR, Robinson SP (1985) Photosynthetic and stomatal response of spinach leaves to salt stress. Plant Physiol 77:85–88

Eckhardt NA, Portis AR Jr (1997) Heat denaturation profiles of ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) and Rubisco activase and the inability of Rubisco activase to restore activity of heat-denaturated Rubisco. Plant Physiol 113:243–248

Epstein E, Norlyn JD, Rush DW, Kingsbury RW, Kelly DB, Cunningham GA, Wrona AF (1980) Saline culture of crops: a genetic approach. Science 210:399–404

Everard JD, Gucci R, Kann SC, Flore JA, Loescher WH (1994) Gas exchange and carbon partitioning in the leaves of celery (*Apium graveolens* L.) at various levels of root zone salinity. Plant Physiol 106:281–292

Feller U, Crafts-Brandner SJ, Salvucci E (1998) Moderately high temperatures inhibit ribulose-1,5-bisphosphate carboxylase/oxygenase activase-mediated activation of Rubisco. Plant Physiol 116:539–546

Flower TJ, Troke PF, Yeo AR (1977) The mechanism of salt tolerance in halophytes. Annu Rev Plant Physiol 28:89–121

Genty B, Briantais JM, Baker NR (1989) The relationship between the quantum yield of photosynthetic electron transport and quenching of chlorophyll fluorescence. Biochim Biophys Acta 99:87–92

Gilmore AM (1997) Mechanistic aspects of xanthophyll cycle-dependent photoprotection in higher plant chloroplasts and leaves. Physiol Plant 99:197–209

Gorham J (1995) Betaines in higher plants-biosynthesis and role in stress metabolism. In: Wallsgrove RM (ed) Amino acids and their derivatives in higher plants. Cambridge University Press, Cambridge, pp 171–203

Haake V, Zrenner R, Sonnewald U, Stitt M (1998) A moderate decrease of plastid aldolase activity inhibits photosynthesis, alters the levels of sugars and starch, and inhibits growth of potato plants. Plant J 14:147–157

Hayashi H, Alia, Mustardy L, Deshnium P, Ida M, Murata N (1997) Transformation of *Arabidopsis thaliana* with the coda gene for choline oxidase: accumulation of glycinebetaine and enhanced tolerance to salt and cold stress. Plant J 12:133–142

Holmström K-O, Somersalo S, Mandal A, Palva ET, Welin B (2000) Improved tolerance to salinity and low temperature in transgenic tobacco producing glycine betaine. J Exp Bot 51:177–185

Horton P, Ruban AV, Walters RG (1996) Regulation of light harvesting in green plants. Annu Rev Plant Physiol Plant Mol Biol 47:655–684

Incharoensakdi A, Takabe T, Akazawa T (1986) Effect of betaine on enzyme activity and subunit interaction of ribulose 1,5-bisphosphate carboxylase/oxygenase from *Aphanothece halophytica*. Plant Physiol 81:1044–1049

Laemmli UK (1970) Cleavage of structural proteins during the assembly of head of bacteriophage T4. Nature 227:680–685

Leegood RC (1990) Enzymes of the Calvin cycle. In: Lea PJ (ed) Methods in plant biochemistry, vol 3. Academic Press, London, pp 16–45

Long SP, Baker NR (1986) Saline terrestrial environments. In: Baker NR, Long SP (eds) Photosynthesis in contrasting environments. Elsevier, Amsterdam, pp 63–102

Lu C, Zhang J (1998) Thermostability of photosystem II is increased in salt-stressed sorghum. Aust J Plant Physiol 25:317–324

Lu C, Qiu N, Lu Q, Wang B, Kuang T (2002) Does salt stress lead to increased susceptibility of photosystem II to photoinhibition and changes in photosynthetic pigment composition in halophyte *Suaeda salsa* grown outdoors? Plant Sci 163:1063–1068

Lu C, Jiang G, Wang B, Kuang T. (2003a) Photosystem II photochemistry and photosynthetic pigment composition in salt-adapted halophyte *Artimisia anethifolia* grown under outdoor conditions. J Plant Physiol 160:403–408

Lu C, Qiu N, Wang B, Zhang J (2003b) Salinity treatment shows no effects on photosystem II photochemistry but increases the resistance of photosystem II to heat stress in halophyte *Suaeda sal*sa. J Exp Bot 54:851–860

Mishra SK, Subrahmanyam D, Singhal GS (1991) Interactionship between salt and light stress on the primary process of photosynthesis. J Plant Physiol 138:92–96

Morales F, Abadia A, Gomez-Aparis J, Abadia J (1992) Effects of combined NaCl and $CaCl_2$ salinity on photosynthetic parameters of barley grown in nutrient solution. Physiol Plant 86:419–426

Mullet JE, Chua NH (1983) In vitro reconstitution of synthesis, uptake, and assembly of cytoplasmically synthesized chloroplast proteins. Meth Enzymol 97:502–509

Nomura M, Hibino T, Takabe T, Sugyama T, Yokota A, Miyake H, Takabe T (1998) Transgenically produced glycinebetaine protects ribulose 1,5-bisphosphate carboxylase/oxygenase from inactivation in *Synechococcus* sp. PCC7942 under salt stress. Plant Cell Physiol 39:425–432

Papageorgiou GC, Murata N (1995) The unusually strong stabilizing effects of glycine betaine on the structure and function of the oxygen-evolving photosystem II complex. Photosynth Res 44:243–252

Park E-J, Jeknić Z, Sakamoto A, DeNomal J, Yuwansiri R, Murata N, Chen THH (2004) Genetic engineering of glycinebetaine synthesis in tomato protects seeds, plants, and flowers from chilling damage. Plant J 40:474–487

Perchorowicz JT, Raynes DA, Jensen RG (1981) Light limitation of photosynthesis and activation of ribulose bisphosphate carboxylase in wheat seedlings. Proc Natl Acad Sci USA 78:2985–2989

Pollard A, Wyn Jones RG (1979) Enzyme activities in concentrated solutions of glycinebetaine and other solutes. Planta 144: 291–298

Porra RJ, Thompson WA, Kriedemann PE (1989) Determination of accurate extinction coefficients and simultaneous equations for assaying chlorophyll *a* and *b* extracted with four different solvents: verification of the concentration of chlorophyll standards by atomic absorption spectroscopy. Biochim Biophys Acta 975:384–394

Rhodes D, Hanson AD (1993) Quaternary ammonium and tertiary sulfonium compounds in higher plants. Annu Rev Plant Physiol Plant Mol Biol 44:357–384

Sakamoto A, Alia, Murata N (1998) Metabolic engineering of rice leading to biosynthesis of glycinebetaine and tolerance to salt and cold. Plant Mol Biol 38:1011–1019

Sakamoto A, Murata N (2001) The use of bacterial choline oxidase, a glycine betaine-synthesizing enzyme, to create stress-resistant transgenic plants. Plant Physiol 125:180–188

Sakamoto A, Murata N (2002) The role of glycine betaine in the protection of plants from stress: clues from transgenic plants. Plant Cell Environ 25:163–171

Sakamoto A, Valverde R, Alia, Chen THH, Murata N (2000) Transformation of *Arabidopsis* with the coda gene for choline oxidase enhances freezing tolerance of plants. Plant J 22: 449–453

Saneoka H, Nagasaka C, Hahn DT, Yang WJ, Premachandra GS, Joly RJ, Rhodes D (1995) Salt tolerance of glycinebetaine-deficient and containing maize lines. Plant Physiol 107:631–638

Schimakat D, Heineke D, Heldt HW (1990) Regulation of sedoheptulose-1,7-bisphosphatase by sedohepulose-7-phosphate and glycerate, and fructose-1,6-bisphosphatase by glycerate in spinach chloroplasts. Planta 181:97–103

Schrader SM, Wise RR, Wacholtz WE, Ort DR, Sharkey TD (2004) Thylakoid membrane responses to moderately high leaf temperature in *Pima* cotton. Plant Cell Environ 27:725–735

Su J, Hirji R, Zhang L, He C., Selvaraj G., Wu R (2006) Evaluation of the stress-inducible production of choline oxidase in transgenic rice as a strategy for producing the stress-protectant glycine betaine. J Exp Bot 57:1129–1135

Stitt M (1986) Limitation of photosynthesis by carbon metabolism. I. Evidence for excess electron transport capacity in leaves carrying out photosynthesis in saturating light and CO_2. Plant Physiol 81:1115–1122

Szabolcs L (1989) Salt-affected soils. CRC Press, Inc., Boca Raton

Tanji KK (1990) Nature and extent of agricultural salinity. In: Tanji KK (ed) Agricultural salinity assessment and management. American Society of Civil Engineers, New York, pp 1–17

van Kooten O, Snel JFH (1990) The use of chlorophyll fluorescence nomenclature in plant stress physiology. Photosynth Res 25: 147–150

Yang X, Liang Z, Lu C (2005) Genetic engineering of the biosynthesis of glycinebetaine enhances photosynthesis against high temperature stress in transgenic tobacco plants. Plant Physiol 138:2299–2309