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Photosynthetic response of salt-tolerant and sensitive soybean varieties

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Abstract

The physiological response of two soybean varieties to salt stress was examined. The results showed that salt stress induced a significantly ($p<0.01$) lower decrease of the net photosynthetic rate (P_N) in salt-tolerant S111-9 than in salt-sensitive *Glycine max.* P_N decrease was positively related to the decrease of stomatal conductance (g_s) and intercellular CO_2 concentration (C_i) in S111-9, while with g_s in *G. max*. a threshold of relative water content (RWC) was found, above which a slight decrease in RWC lead to a sharp reduction in g_s. The photochemical quenching (q_P), the efficiency of open PSII centers (Φ_{PSII}) and the Rubisco activity (RA) significantly decreased with increasing salinity level in *G. max*. The maximum PSII quantum yield (F_v/F_m) decreased significantly under the highest NaCl in both varieties. The higher reduction of RA in *G. max* was attributed to Rubisco content, which was mainly regulated at LSU expression level rather than at *rbc*L transcript level. These findings led us to conclude that the salt-induced reduction in P_N was mainly due g_s and RA for S111-9 and *G. max*, respectively.

Additional key words: chlorophyll fluorescence; NaCl; photosynthesis; Rubisco.

Introduction

Salinity is one of the major abiotic stress factors decreasing crop yield all over the world. Though great efforts have been made in understanding the mechanism of salt tolerance (Centritto *et al.* 2003; Moradi and Ismail 2007), there are still debates on the relative contribution of stomatal factor or non-stomatal factors to the photosynthetic decline caused by salt stress. Burman *et al.* (2003) underline that salinity reduced photosynthesis primarily by the decrease of stomatal conductance (g_s) and Wilson *et al.* (2006) and Yang and Lu (2005) by the decrease of CO_2 diffusion to the chloroplast. Other studies, however, proved that non-stomatal factors decreased photosynthesis, such as the depression in PSII activity, electron transport and photophosphorylation activity (Everard *et al.* 1994).

Soybean (*Glycine max*) is one of the most important crops in some countries, such as China (Chen *et al.* 2006) and Brazil (Ribeiro *et al.* 2008). Using somatic hybridization method, the stable somatic descendents S111-9 between salt-tolerant *Glycine cyrtoloba* and salt-sensitive *G. max* were selected. S111-9 was characterized by a higher salt stress tolerance and it shows a higher photosynthetic rate than *G. max* under salt stress (Yang *et al.* 2007). Nevertheless, it is not clear if the photosynthetic response depends on stomatal or non-stomatal factors. Aragäo *et al.* (2005) found that Vita3 had a lower RA than Vita5 under salt stress, while the former grew better than the latter.

The aim of this study was to analyse differences in gas exchange, chlorophyll (Chl) fluorescence parameters

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Abbreviations: Chl – chlorophyll; C_i – intercellular CO_2 concentration; ETR – electron transport rate; F_o – initial chlorophyll fluorescence; F_m – maximal chlorophyll fluorescence; F_m' – maximal chlorophyll fluorescence under light; F_v – variable fluorescence; F_v' – variable fluorescence under light; F_v/F_m – maximum PSII quantum yield; F_v'/F_m' – the quantum yield of open PSII centers under irradiation; g_s – stomatal conductance; LSU – large subunit of Rubisco; PFD – photon flux density; P_N – net photosynthetic rate; PSII – photosystem II; q_P – photochemical quenching; RA – Rubisco activity; RWC – relative water content; Φ_{PSII} – efficiency of open PSII centers.

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between these two different salt tolerant soybeans in response to salinity stress. Moreover, we would like to verify whether the genotypic differences in photosynthetic response to salinity were related to gene and protein expression of Rubisco.

Materials and methods

Plant materials and growth conditions: The salt-sensitive soybean *G. max* Melrose (cv. Melrose) and S111-9, the stable somatic hybrid descendants of the wild salt-tolerant *G. cyrtoloba* (series number ACC547) (Yang *et al.* 2007) were considered. About 100 seeds of each type were germinated and cultivated in Zijingang campus of Zhejiang University (Lu *et al.* 2008). When the sixth leaves grew out, the well grown plants were selected and transferred to the complete culture solution (5 mM $Ca(NO_3)_2$, 1.75 mM K_2SO_4, 0.25 mM KCl, 1.25 mM $MgSO_4$., 25 mM H_3BO_3, 1.5 mM Mn SO_4, 1.5 mM $ZnSO_4$, 0.5 mM $CuSO_4$, 0.25 mM KH_2PO_4, 25 μM $(NH_4)_6Mo_7O_{24}$, 20 μM $FeSO_4$, and 20 μM EDTA-Na_2H_2O, Lu *et al.* 2008) with 0, 50, 100, and 150 mM NaCl and cultivated for 5 d. Eight plants were used for each treatment. Then the fourth fully expanded leaves (about 32 leaflets) were used for the experiments.

For RNA extraction, well grown plants with two fully expanded leaves were selected and transferred to the complete solution with 0, 50, 100, and 150 mM NaCl for 5 d. Eight plants were used for each treatment. Then the second fully expanded leaves were sampled, wrapped 0.3 g for each group with silver paper and then immediately frozen in liquid nitrogen and stored at –80 ºC till RNA extraction.

Chl: Small fragments of leaf were extracted with 80 % acetone in the dark for 48 h at 25 ºC in order to guarantee the complete extraction of Chl from leaf. The concentration of Chl was determined spectrophotometrically according to Porra *et al.* (1989).

Leaf gas exchange and Chl fluorescence were conducted simultaneously according to Jin *et al.* (2008) using a portable photosynthesis system (*LI-6400; LICOR*, Lincoln, USA) with an integrated fluorescence fluorometer (*LI-6400–40* leaf chamber fluorometer) at 25 ºC set in the leaf chamber. All measurements were carried out at a photon flux density (PFD) of 1500 μmol m^{-2} s^{-1}, with a constant airflow rate of 500 μmol s^{-1}. The cuvette CO_2 concentration was set at about 400 μmol(CO_2) mol^{-1}(air). The initial chlorophyll fluorescence (F_o) and maximal chlorophyll fluorescence (F_m) were measured after dark-adaptation for overnight. The variable fluorescence (F_v) was calculated as (F_m–F_o). The efficiency of open PSII centers [$\Phi_{PSII} = (F_m'–F_s)/ F_m'$], the quantum yield of open PSII centers under irradiation $(F_m'–F_o')/F_m'$, and the photochemical quenching [$q_P = (F_m'–F_s)/(F_m'–F_o')$] were calculated from the measured parameters (Genty *et al.* 1989). A series of reference CO_2 concentration for CO_2 response curves was set as 400, 200, 150, 100, 50, 400, 400, 600, 800, 1 000, 1 200 μmol mol^{-1} with the *LI-COR* CO_2 injection system at a PFD of 1500 μmol m^{-2} s^{-1}.

Leaf RWC: Five leaflets used for gas exchange were sampled. After measuring the fresh mass (FM), leaves were left in distilled water for 24 h at 4 °C in darkness to obtain the mass at full turgor (TM). Leaf dry mass (DM) was obtained after drying the samples in an oven at 80 °C for 48 h. RWC was calculated according to Flexas *et al.* (2006) as: RWC [%] = (FM–DM)×100/(TM–DM).

Rubisco: Fresh leaf samples (0.1 g) used for gas exchange measurements were rapidly ground to a fine powder in a mortar previously chilled with liquid nitrogen and insoluble polyvinylpolypyrrolidone (PVP), then were homogenized with 2 ml ice-cold extraction medium containing 50 mM Tris-HCl (pH 7.5), 1 mM EDTA, 10 mM $MgCl_2$, 12 % (v/v) glycerol, 1 % (w/v) PVP-40 (soluble PVP) and 0.1 % (v/v) β-mercaptoethanol. After centrifugation (15 000×*g*, 15 min, 0–4 °C), RA in the supernatant was assayed immediately according to Jin *et al.* (2006).

SDS-PAGE was performed using 10 % (m/v) acrylamide, 0.5 % bisacrylamide on the miniprotein three cell system (*Bio-Rad*) as described by Laemmli *et al.* (1970). Before loading, the extracted Rubisco samples were solubilized in SDS loading buffer (63 mM Tris–HCl, 0.2 M β-mercaptoethanol, 2 % (w/v) SDS, 10 % (v/v) gycerol, 0.025 % (w/v) bromophenol blue, pH 6.8) and boiled for 5 min. Insolubilized materials were removed by centrifugation at 8 000 × *g* for 8 min. For immunoblotting the proteins were transferred to a polyvinylidene difluoride membrane (*Millipore*, Bedford, MA) (Lu *et al.* 2008). The membranes were then blocked with fat-free milk, incubated with mice monoclonal antibody raised against purified LSU from rice prepared in our lab. Then the blots were incubated with alkaline phosphatase-conjugated secondary antibody [AP-goat anti-mice IgG (H+L) conjugate] and visualized using *NBT-BCI*P color developing kit (*Boehringer* Mannheim, Germany).

Total RNA was extracted from 0.3 g leaf using RNAiso Reagent (*TaKaRa*, Japan) and reverse-transcribed with oligo (dT) using reverse transcriptase (*Invitrogen*, Carlsbad, CA, USA). cDNA was then amplified by PCR. Specific primers for *rbc*L and tubulin sequence amplification were designed according to the function of their conserved sequences: 5’-ATT TGC GAA TCC CTA

CTG CT -3’ and 5’-GTG TCC TAA AGT TCC TCC GC-3’ for *rbc*L: 5'- CTG GGA ACT CTA CTG CCT CG -3' and 5'- GAC TGC GGT TGA AAC CTG TG -3' for tubulin, giving a product of 484 bp. Amplification was achieved under the following conditions: 3 min at 94 °C; 35 cycles of denaturation at 94 °C for 30 s, annealing at 55 °C (*rbc*L) for 30 s or 50 °C (tubulin) for 45 s, and elongation at 72 °C for 45 s (*rbc*L) or 1 min (tubulin). The final extension step was for 7 min at 72 °C. The RT-PCR products were analyzed by agarose gel electrophoresis.

Statistical methods: Statistical treatment of the data was performed by One-way *ANOVA* method. Differences between means were established using a *Duncan* test ($p<0.05$). For these analyses *SPSS 10.0* software (*SPSS*, Chicago, IL, USA) was used.

Results

Chl content decreased with increasing NaCl concentration in both varieties (decreased by 17.6 % and 13.6 % under 150 mM NaCl in *G. max* and S111-9, respectively) (Fig. 1*A*) There were no significant changes in the ratios of Chl *a*/*b* in both the varieties (Fig. 1*B*).

Gas exchange and Chl fluorescence: Salt stress significantly reduced P_N, g_s and RWC in both the varieties (Fig. 1*C,E,F*). P_N and g_s reduction were 98 and 86 %, respectively under 150 mM NaCl in *G. max*, and 41 and 75 % in S111-9, respectively. RWC was 87 % in S111-9 and 59 % in *G. max* at high salinity. C_i increased in *G. max* under 150 mM NaCl, while it kept on decreasing with increasing NaCl concentration in S111-9 (Fig. 1*D*). P_N was positively correlated to *gs* in both the varieties, moreover, P_N was positively correlated to C_i in S111-9 ($p<0.001$), but negatively in *G. max* ($p<0.01$) (Fig. 2). Exponential regression curves existed between RWC and g_s for both the varieties (Fig. 3), however, the similar degree in RWC reduction induced g_s reduction much greater in S111-9 than in *G. max*.

F_v/F_m decreased by 1.84 and 0.86 % for *G. max* and S111-9, respectively ($p<0.05$) (Fig. 4*A*). F_v'/F_m' remained relative constant in *G. max*, while it decreased in S111-9

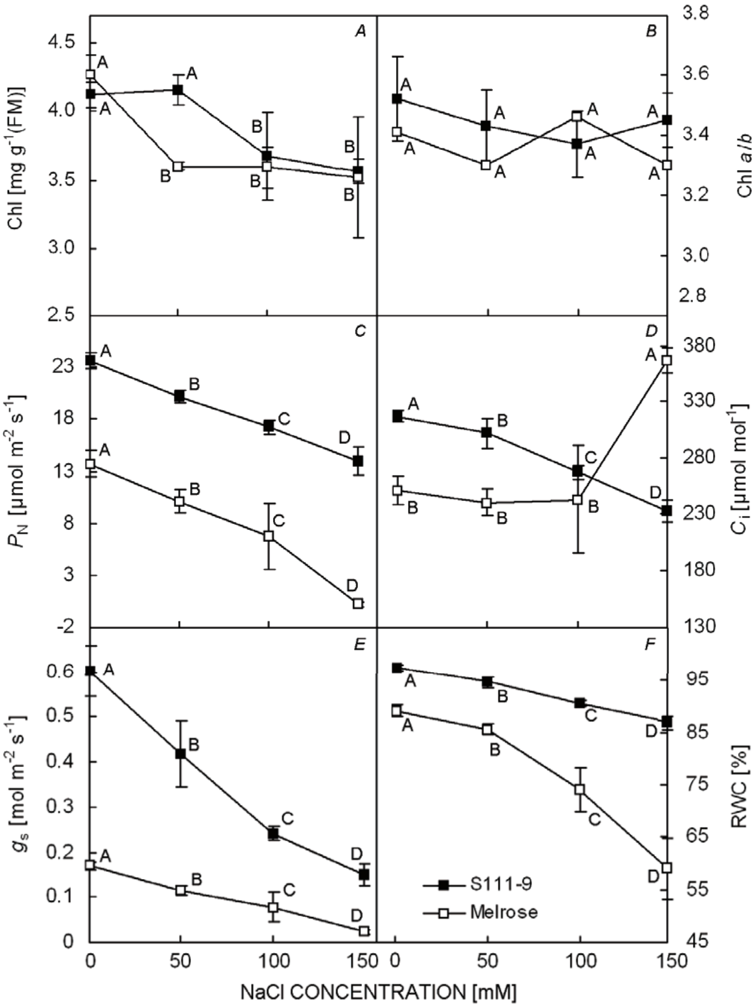

Fig. 1. Effects of NaCl stress on total chlorophyll (Chl) content (*A*), ratio of Chl *a*/*b* (*B*), net photosynthetic rate (P_N) (*C*), intercellular CO_2 concentration (C_i) (*D*), stomatal conductance (g_s) (*E*) and relative water content (RWC) (*F*) in leaves of S111-9 (■) and Melrose (□) for 5 days. Data are the means with ±SD ($n=4$). Mean values designated with different letters are significantly different ($p<0.05$) among different NaCl concentrations in the same variety.

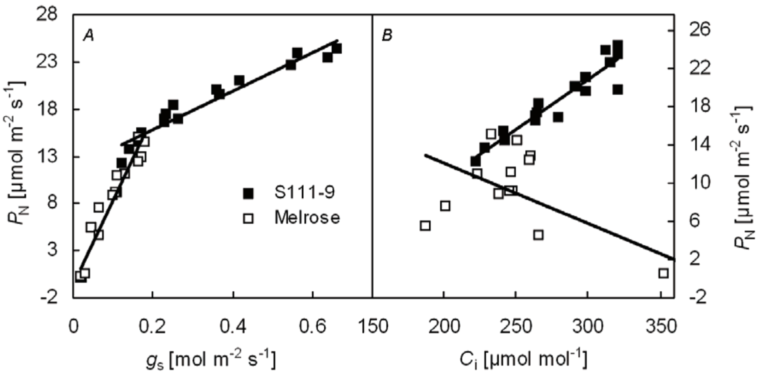

Fig. 2. Correlation analysis between net photosynthetic rate (P_N) and stomatal conductance (g_s) (*A*) or P_N and intercellular CO_2 concentrations (C_i) (*B*) in the S111-9 (■) and Melrose (□).

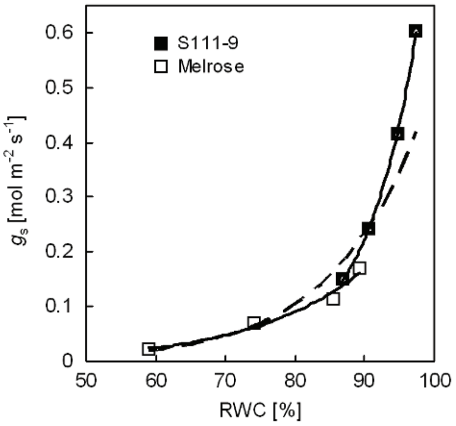

Fig. 3. Relationship between relative water content (RWC) and stomatal conductance (g_s). Both solid lines represent the regression curve for both varieties – S111-9(■) and Melrose(□), respectively, while the dotted line represents the regression curve for both varieties together.

under salt stress (Fig. 4*C*). Both the Φ_{PSII} and q_P decreased with increasing NaCl concentration in both the varieties (Fig. 4*B, D*), while the reduction was more rapid in *G. max* compared to that in S111-9.

RA, LSU expression and *rbc*L transcription: Since the initial slope was thought to be related to the carboxylation efficiency (Bota *et al.* 2004), P_N-C_i curve and its corresponding initial slope in response to NaCl treatments were made to illustrate the effect of salt stresson carbon metabolism (Fig. 5). Obviously, NaCl at moderate concentrations (0 to 100 mM) had little effect on the initial slope in S111-9 (Fig. 5*A,C*), while this initial slope decreased evidently in *G. max* with salt concentration increased (Fig. 5*B,D*), implying that the carboxylation efficiency was inhibited much greater in *G. max* than in S111-9.

Salt stress reduced both total and initial RA significantly in *G. ma*x under 150 mM NaCl, however, no significant difference in both parameters were found between control and treatments in S111-9 (Fig. 6). Similarly, LSU protein decreased with increasing NaCl concentration in *G. max* (Fig. 7*A*), but not in S111-9 (Fig. 7*B*). Furthermore, salt-induced decrease in LSU expression was coupled with the decrease in *rbc*L transcript level in *G. max*, (Fig. 7*B*), while in S111-9, difference in *rbc*L transcript level between control and treatments were not significant (Fig. 7*B*).

Discussion

Previous studies have shown that S111-9 could maintain higher photosynthetic rate and less necrotic lesions than *G. max* after stressed by 50 mM NaCl (Yang *et al.* 2007). According to Kao *et al.* (2006) and Moradi *et al.* (2007), the relatively higher salt-tolerant species would have less reduced P_N, our results proved that salt induced less reduction in Chl content and P_N (Fig. 1*A,C*) conferred S111-9 higher salt-tolerance. Moreover, P_N reduction is in relationship with g_s and C_i in S111-9, while it is in relationship only with g_s in *G. max* (Fig. 2), suggesting that C_i is not the limiting factor for photosynthetic reduction in *G. max* under salt stress. All these results indicated that the stomatal limitation may be the limiting factor for P_N reduction in S111-9, but not in *G. max*.

Water deficit is one of the main effects of salt stress on plant (Munns *et al.* 2006). Higher salt-tolerant plants should have more efficient way to cope with the reduction in RWC. Our results showed that RWC decreased with increasing salinity and the reduction in RWC coupled with the decrease in g_s in both soybean varieties (Fig. 3). However, clear difference could be found between two varieties: in S111-9, salt-induced decrease in RWC could induce a sharp reduction in g_s, while in *G. max*, the response of g_s to the reduction of RWC is not so proportional as is in S111-9. According to Moradi *et al.* (2007) and Centritto *et al.* (2003), the large reduction of g_s is considered as a major way to decrease water loss from the leaves and could be considered as an adaptive character to salt tolerance. So it could be concluded that salt-induced RWC reduction in S111-9 would trigger its stomatal closure efficiently, and the latter could be reflected by the sharp reduction in g_s (Fig. 1*E*). All these enable S111-9 to maintain higher RWC even under 150 mM NaCl. But these positive mechanisms are not so

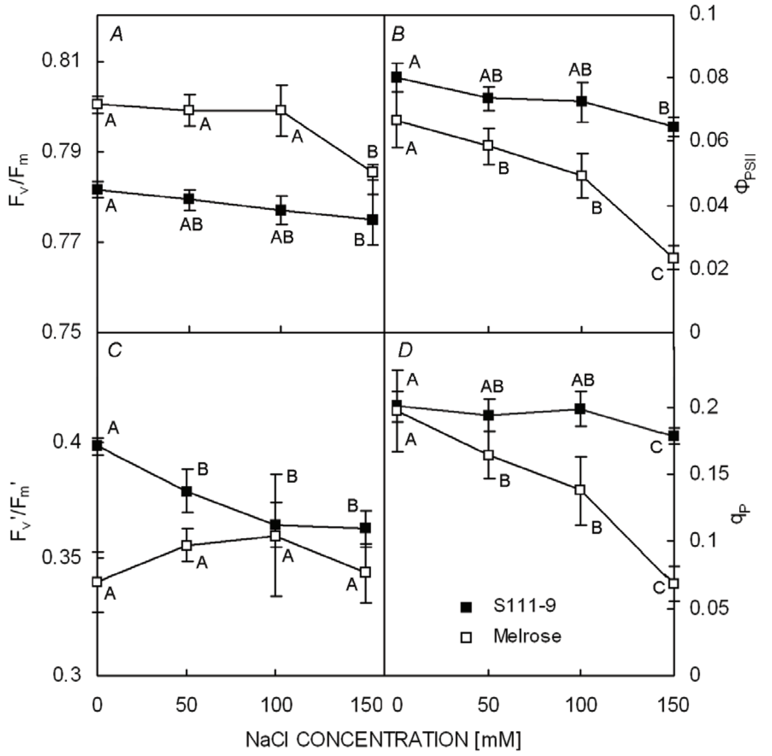

Fig. 4. Effects of NaCl stress on chlorophyll fluorescence parameters in the fourth fully expanded leaves of S111-9 (■) and Melrose (□) *A*: maximal photochemical efficiency (F_v/F_m); *B*: PS2 trapping efficiency (F_v'/F_m'); *C*: efficiency of open PSII centers (Φ_{PSII}); *D*: relative electron transport rate (ETR). Data are presented as means ± SD (*n*= 4). Mean values designated with different letters are significantly different ($p<0.05$) among different NaCl concentrations in the same variety.

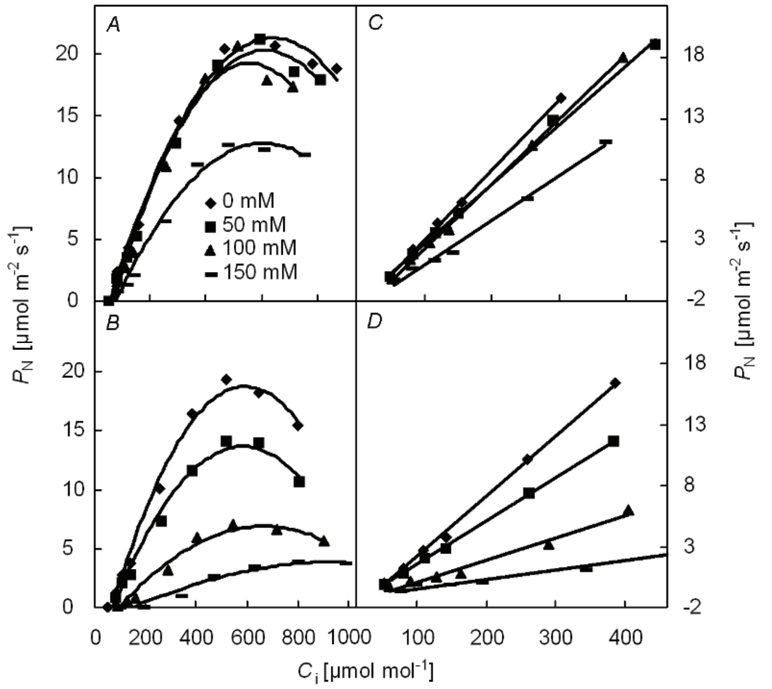

Fig. 5. Regression curves between P_N and C_i of S111-9 (*A*) and Melrose (*B*), and the initial slopes of the response of P_N to C_i in leaves of S111-9 (*C*) and Melrose (*D*) under different NaCl concentrations.

efficient in *G. max* as that in S111-9, which partially well account for their difference in salt tolerance.

Chl fluorescence is a rapid and non-intrusive tool used to screen varieties for salinity tolerance (Maxwell and Johnson 2000; Baker and Rosenqvist 2004). Similar to those reported by Netondo *et al.* (2004), F_v/F_m is not significantly different between the control and the treatment at moderate NaCl content in both varieties, suggesting that the PSII structural integrity is not significantly impaired. q_P reflects the redox state of PSII and the balance between energy supply and utilization (Ou *et al.* 2003). We found that salt-induced variations of q_p and Φ_{PSII} followed a pattern similar to those of P_N, showing a greater reduction in *G. max* than in S111-9. The reduction in q_P may imply that Q_A, the primary acceptor of PSII, is overreduced. The overreduction in Q_A

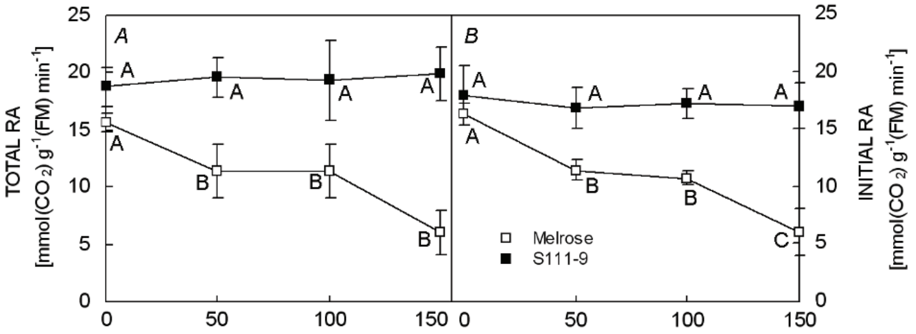

Fig. 6. The change in total (*A*) and initial (*B*) Rubisco activities (RA) in leaves of S111-9 S111-9 (■) and Melrose (□) with different NaCl concentration. Data are presented as with ±SD (*n*=4). Mean values designated with different letters are significantly different (p<0.05) among different NaCl concentrations in the same variety.

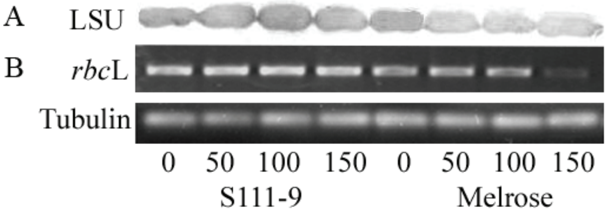

Fig. 7. Changes in LSU content analyzed using protein gel blot methods (*A*), and *rbc*L transcript levels expressed through RT-PCR analysis (*B*) in the leaves of S111-9 and Melrose stressed by different NaCl concentration. Tubulin was used as an inner standard.

reflects the saturation of photosynthetic electron trans–port, which would reflect the reduction in carbon assimilation. Also the strong reduction of Φ_{PSII} observed in *G. max* may indicate the onset of these carbon metabolism limitations (Loreto *et al.* 2003).

It has been proposed that the activity of key photosynthetic enzyme, Rubisco, is strongly correlated with the decline in photosynthesis during stress conditions (Dionisio-Sese and Tobita 2000; Flexas *et al.* 2006; Parry *et al.* 2002). The initial slope of P_N to C_i reflects the leaf carboxylation efficiency and correlates with RA (von Caemmerer *et al.* 1994). The significant decrease in initial slope of P_N to C_i, as well as in initial RA under salt stress indicated that Rubisco may be a limiting factor for P_N in *G. max*, whereas in S111-9 both the initial slope and RA hardly changed under moderate NaCl concen–tration (0 to 100 mM), suggesting that the Rubisco is not the main reason for salt induced reduction in P_N. According to Parry *et al.* (2002) and Tezara *et al.* (2002), our results further confirmed that the relatively higher P_N in S111-9 under salt stress is closely related to its higher RA. The depression of RA always occurres when the cell water content decreases to a certain degree (Parry *et al.* 2002). So the larger decrease of RWC in *G. max* might be responsible for its larger reduction in RA.

We conclude that under salt stress condition, the reduced RA in *G. max* impaired the carbon metabolism, leading to the overaccumulation of ATP and NADPH, then reducing the photosynthetic electron transport, resulting in the overreduction of Q_A, all which lead to the reduction of q_P and the Φ_{PSII} (Melis 1999).

Environmental factors are known to influence gene expression. We hypothesized that the reduction of RA after salt stress may be related to the expression of Rubisco. The results underline that high salinity concentration reduces the LSU content in *G. max*. Because the translational arrest of LSU was tightly coordinated with the cessation of Rubisco assembly (Cohen *et al.* 2005) and the decreased Rubisco content always leads to the decreased RA (Parry *et al.* 2002; Zhou *et al.* 2006), we conclude that the salt-induced greater reduction of RA in *G. max* is mainly due to the decreased Rubisco content, which is reflected in the reduction of LSU content. Moreover, the LSU content is not always accompanied by the *rbc*L transcript except at high NaCl level in *G. max*, suggesting that the translational arrest of the LSU most likely occurs at post transcriptional level, maybe at a postinitiation stage as reported by Cohen *et al.* (2005).

In summary, P_N decreases progressively with the increasing salt concentration in both salt-tolerant and sensitive soybean varieties, and the less reduction in S111-9 adjusts RWC more efficiently than *G. max*. Furthermore, the P_N reduction in S111-9 is mainly attributed to the stomatal factor, while to RA in *G. max*. And the salt-induced reduction in RA is closely related to the reduction in LSU content which is mainly regulated at post transcriptional level.

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