Stomatal and Metabolic Limitations to Photosynthesis Resulting from NaCl Stress in Perennial Ryegrass Genotypes Differing in Salt Tolerance

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ABSTRACT. Plants possess abiotic stress responses that alter photosynthetic metabolism under salinity stress. The objective of this study was to identify the stomatal and metabolic changes associated with photosynthetic responses to NaCl stress in perennial ryegrass (*Lolium perenne*). Five-week-old seedlings of two perennial ryegrass genotypes, PI 516605 (salt-sensitive) and BARLP 4317 (salt-tolerant), were subjected to 0 and 250 mM NaCl for 8 days. The salt tolerance in perennial ryegrass was significantly associated with leaf relative water content (RWC) and photosynthetic capacity through the maintenance of greater metabolic activities under prolonged salt stress. BARLP 4317 maintained greater turf quality, RWC, and stomatal limitations but a lower level of lipid peroxidation [malondialdehyde (MDA)] and intercellular CO₂ concentration than PI 516605 at 8 days after treatment (DAT). Ribulose-1, 5-bisphosphate carboxylase:oxygenase (Rubisco) activity and activation state, transcriptional level of *rbcL* gene, and expression level of Rubisco large subunit (LSU) declined in stressed perennial ryegrass but were higher in salt-tolerant genotype at 8 DAT. Furthermore, photosynthetic rate increased linearly with increasing Rubisco activity, Rubisco activation state, and RWC in both genotypes. The same linear relationship was found between RWC and Rubisco activity. However, MDA content decreased linearly with increasing RWC in both genotypes. Salinity-induced inhibition of photosynthesis in perennial ryegrass was mainly the result of stomatal limitation during early salt stress and metabolic limitation associated with the inhibition of RWC, activity of Rubisco, expression level of *rbcL* gene, and LSU under a prolonged period of severe salinity.

Salinity limits plant growth and crop yield through disordering physiological and biochemical process of plants (Zhu, 2001). Salinity has a profound impact on photosynthetic ability attributed to a decrease in leaf water content, cell membrane lipid peroxidation, stomatal limitation, and metabolism disorders (Niu et al., 1995; Schachtman et al., 1991). However, there are still debates on the contribution of stomatal and metabolic limitations to the photosynthetic decline caused by salt stress.

Salinity results in an imbalance of plant leaf water and dehydration at the cellular level (Hernández et al., 1999). The loss of leaf water provokes stomatal closure, restricts CO₂ diffusion into the leaves, and leads to a decrease in net photosynthetic rate (Hernández and Almansa, 2002). Previous studies also reported that the decrease in CO₂ diffusion from the intercellular spaces to the chloroplast was a main cause of the limitation of CO₂ assimilation (Sánchez-Blanco et al., 1991). With prolonged salinity stress, decreased photosynthetic rate contributed to

non-stomatal limitation such as the decrease of the enzyme activity and the expression of photosynthetic proteins and gene (Soussi et al., 1998; Xia et al., 2009). However, these studies in various plant species are mainly in annual crops.

Ribulose-1, 5-bisphosphate carboxylase: oxygenase (Rubisco), a 4.8 to 5.9 GDa complex, is a chloroplast-specific photosynthetic protein, which consists of eight large subunits (≈50 to 55 kDa each) and eight small subunits [SSU (12 to 15 kDa each)] and accounts for 50% of the total soluble leaf protein in C₃ plants (Blair and Ellis, 1973). Activation of Rubisco is highly related to CO₂ assimilation and regulated by cell metabolites such as ions, Rubisco activase, adenosine-5'-triphosphate (ATP)/adenosine diphosphate, and phosphate (Portis, 2003; Zhang et al., 2002). Previous studies reported that salinity affected the activity of Rubisco in many plants including soybean [*Glycine soja* (Lu et al., 2009)], spinach [*Spinacia oleracea* (Delfine et al., 1998, 1999)], egyptian pea [*Sesbania sesban* (Sivakumar et al., 2000)], chickpea [*Cicer arietinum* (Soussi et al., 1998)], maize [*Zea mays* (Khodary, 2004)], grapevine [*Vitis vinifera* (Cramer et al., 2007)], and tobacco [*Nicotiana tabacum* (Yang et al., 2008)]. Khodary (2004) observed a lower level of Rubisco activity with increasing NaCl concentration in maize and thought that Rubisco

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activity limited leaf photosynthetic capacity in the NaCl-treated plants. Similar results have been obtained for Egyptian pea and tobacco (Sivakumar et al., 2000; Yang et al., 2008). However, some studies have demonstrated that Rubisco activity was unaffected or even increased by the short-term salinity stress but was suppressed only under long-term or severe salinity such as spinach at 20 DAT, maize at 12 DAT, and chickpea at 7 DAT (Cramer et al., 2007; Delfine et al., 1998, 1999; Soussi et al., 1998). In addition, Lu et al. (2009) showed that the changes of Rubisco activity varied in soybean cultivars with different salt tolerance. Cramer et al. (2007) reported that Rubisco activity was greater in grapevine subjected to greater than 75 mM of salinity for 12 d. Increasing evidence indicated that the enzyme activity of Rubisco was regulated by the expression of photosynthetic proteins and gene (Cheng et al., 1998). However, there is limited information on the regulation of expression of photosynthetic proteins and gene in perennial ryegrass in response to salinity stress, particularly the differential expression patterns between salt-tolerant and salt-sensitive perennial ryegrass genotypes.

Perennial ryegrass is widely used forage and cool-season turfgrass in the United States, Europe, Japan, Australia, and New Zealand because of its rapid establishment rate (Xiong et al., 2007). Salinity survival is a major strategy for perennial grass species, and therefore the effects of salinity stress on germination and growth (Nizam, 2011), chlorophyll content (Liu et al., 2008), the antioxidant system (Hu et al., 2011), and transgenic effect (Wu et al., 2005) of perennial ryegrass have been investigated. However, there is limited information on how changes in different photosynthetic components (stomatal and metabolic) are involved in perennial grass adaptation to salt stress. The objectives of this study were to examine stomatal and/or metabolic components involved in plant adaptation to salt stress in two genotypes of perennial ryegrass differing in salt tolerance.

Materials and Methods

PLANT MATERIAL AND TREATMENTS. Seeds of two perennial ryegrass genotypes, salt-tolerant BARLP 4317 (origin the United States) and salt-sensitive PI 516605 (origin Morocco), were sowed in plastic cups (10 cm diameter and 15 cm deep) filled with sand. The seedlings were fertilized twice weekly with half-strength Hoagland's solution (Hoagland and Arnon, 1950) until dripping. Five-week-old seedlings were rinsed thoroughly with distilled water and transferred into 300-mL Erlenmeyer flasks filled with ≈ 290 mL half-strength Hoagland's solution with $0.1 \mu\text{mol CaO}_2$, which provided oxygen. The nutrient solutions were completely replaced each week. The flasks were wrapped with aluminum foil to prevent potential growth of algae and the bottlenecks were closed with a proper amount of absorbent paper. All flasks were kept in a controlled walk-in growth room with daily temperature of $21 \pm 0.5^\circ\text{C}$, photosynthetically active radiation at $300 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ at the canopy level, and a 14-h photoperiod. The plant-flask system was weighed at 48-h intervals to determine transpiration rate (T_r) before starting salt treatment based on the method described by Hu et al. (2011). Plants with similar T_r were selected for each replicate of the NaCl treatments. Plants were allowed to establish a canopy and root system and grow in the mentioned conditions for 4 weeks before the NaCl treatments were applied.

Perennial ryegrass was subjected to two salinity levels (0 and 250 mM NaCl) in each flask by adding NaCl to the half-strength Hoagland nutrition. Each treatment maintained the final concentration for 8 d. The same amount of corresponding treatment medium was added into each plant-flask system every 2 d during the experimental period for making up the loss of water resulting from evaporation. Photosynthetic gas exchange was determined and the leaf samples for physiological, gene, and protein expression analysis were harvested at 0, 4, and 8 DAT, respectively.

MEASUREMENTS. Turf quality was evaluated visually based on turfgrass color, density, and uniformity using a 0 to 9 scale where 0 = brown or dead turf; 6.0 = minimum acceptable quality; 7.0 = very good quality; and 9.0 = optimum greenness, uniformity, and density (Turgeon, 2002).

Leaf relative water content was determined using the method described by Barrs and Weatherley (1962). Fully expanded leaves [≈ 0.15 g fresh weight (FW)] were immediately soaked in the tubes with distilled water and kept at 4°C for 24 h. Then, leaf samples were taken out, dried by blotting off surface water with paper towels, and turgid weight (TW) weighed. Samples were then dried in an oven at 80°C to a constant weight (DW). Leaf RWC was calculated using the following formula: $\text{RWC}(\%) = (\text{FW} - \text{DW}) / (\text{TW} - \text{DW}) \times 100$.

MDA content was analyzed by the thiobarbituric acid reaction according to Heath and Packer (1968). The reaction mixture contained 1.0 mL enzyme and 2.0 mL extract reaction solution (20% w/v trichloroacetic acid and 0.5% w/v thiobarbituric acid). The mixture was heated in a water bath at 95°C for 30 min, then cooled quickly to room temperature in an ice-water bath, and centrifuged at $21,913 \text{ g}$ for 20 min. Absorbance of the supernatant was examined at 532 and 600 nm by spectrophotometer (ultraviolet-2600; UNICO Instruments, Shanghai, China). The subtraction of absorbance between 532 and 600 nm was calculated. The amount of MDA was calculated based on adjusted absorbance and extinction coefficient of $155 \text{ mmol}^{-1}\cdot\text{cm}^{-1}$.

Photosynthetic gas exchange was measured with a portable gas-exchange system (LI-6400XT; LI-COR, Lincoln, NE) under a controlled photosynthetic photon flux of $600 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ at 0, 4, and 8 DAT. Net photosynthetic rate (P_n), stomatal conductance (g_s), and intercellular CO_2 concentration (C_i) were measured with four fully expanded leaves (second from the top) from three subsamples in each pot. Values of stomatal limitation (L_s) were calculated as the formula: $L_s = 1 - C_i/C_a$ (where C_a is the CO_2 concentration in the air) (Farquhar and Sharkey, 1982).

Ribulose-1, 5-bisphosphate carboxylase/oxygenase (Rubisco, electrical conductivity 4.1.1.39) extraction and activity were assessed based on the procedure reported by Chen and Cheng (2004) with some modifications. A total of 0.2 g frozen leaves was ground in liquid nitrogen with a pre-cool mortar and homogenized in 3 mL extraction buffer containing 50 mM Hepes-NaOH (pH 7.5), 10 mM dithiothreitol, 10 mM MgCl_2 , 2 mM EDTA, 1.0% (v/v) Triton X-100 (Amresco, Boise, ID), 1.0% (v/v) bovine serum albumin (Prospec, East Brunswick, U.K.), 10% (v/v) glycerol, and 1.5% (w/v) insoluble polyvinyl polypyrrolidone (Sinopharm Chemical Reagent, Ningbo, China). The extract was homogenized for 30 s and then centrifuged at $21,913 \text{ g}$ for 6 min at 4°C . The supernatant was used for the initial and total (fully activated) Rubisco activity assay.

Initial and total Rubisco activity was assayed at 25 °C coupled RuBP carboxylation (Sigma, St. Louis, MO) to nicotinamide-adenine dinucleotide [NADH (Sigma)] oxidation according to Lilley and Walker (1974). NADH oxidation was monitored at 340 nm in a spectrophotometer (ultraviolet-2600). To determine initial Rubisco activity, a 50-μL sample of extract and 50 μL of 10 mM RuBP were immediately added to a semimicrocuvette containing 900 μL of an assay solution and then mixed well. The change of absorbance at 340 nm was immediately recorded every 5 s for 2 min. To determine total Rubisco activity, a sample extract was added to 900 μL of assay solution to allow activation of all Rubisco at 25 °C; 50 μL of 10 mM RuBP was added 15 min later. The assay solution for both initial and total Rubisco activity measurements contained 100 mM bicine (pH 8.0 at 25 °C), 25 mM KHCO₃, 20 mM MgCl₂, 3.5 mM ATP (Sigma), 5 mM phosphocreatine (Sigma), 5 units glyceraldehyde-3-phosphate dehydrogenase (Sigma), 5 units 3-phospho-glyceric phosphokinase (Sigma), 17.5 units creatine phosphokinase (Sigma), and 0.25 mM NADH. Leaf Rubisco activity was calculated using the following formula:

$$\begin{aligned} \text{Rubisco activity} (\mu\text{mol} \cdot \text{s}^{-1} \cdot \text{g}^{-1} \text{FW}) &= (A_{340}/\text{min}) \\ &\times (\text{extraction volume/sample volume}) \\ &\times (0.00134 \mu\text{mol}^{-1} \cdot \text{s}^{-1}) \times (1 \cdot \text{g}^{-1}) \end{aligned}$$

Rubisco activation state was calculated as the ratio of initial activity to total activity.

PROTEIN EXTRACTION. To assay soluble leaf protein content, leaves were collected at 0, 4, and 8 DAT and placed immediately in liquid nitrogen and stored in a refrigerator at -80 °C until analyzed. For extraction of total leaf soluble protein, a 0.5-g leaf sample was powdered with a pre-cooled mortar and pestle in liquid nitrogen, put into a 50-mL microtube, homogenized with a mixer, incubated with 4 mL of ice-cold phosphate buffer (150 mM, pH 7.0), and then centrifuged at 21,913 *g_n* at 4 °C for 30 min following the procedure described by Li et al. (1996). The supernatant containing soluble protein was collected in a 2-mL test tube. A 30-μL supernatant was mixed with 3 mL of Bradford dye solution. Then, the absorbance of the solution was measured at 595 nm after 2 min and before 1 h using a spectrophotometer (ultraviolet-2600). The amount of soluble protein in the solution was calculated using a protein standard curve as described by Bradford (1976).

SDS-PAGE. Soluble protein extracts were mixed with sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer [60 mM Tris-HCl (pH 6.8), 25% glycerol (v/v), 2% SDS (w/v), 5% b-mercaptoethanol (v/v), 0.1% bromophenol blue (w/v)] with a ratio of 4:1, heated at 95 °C for 5 min, and then subjected to electrophoresis according to the method of Hu et al. (2009). Proteins samples (30 μg/lane) were separated using a 5% stacking gel and 12% running gel by discontinuous SDS-PAGE with an electrophoresis unit (JY-SCZ-2; June Yi Oriental, Beijing, China). Gels were stained at room temperature for least 2 h by Colloidal Coomassie Blue G-250 (Sinopharm Chemical Reagent, Ningbo, China) and then destained by deionized water (Neuhoff et al., 1988).

RT-PCR ANALYSIS. Total RNA was extracted from the leaf tissues using Trizol reagent (Invitrogen, Paisley, U.K.) according to the user manual. RNA samples were treated with RNase-free DNaseI to remove any contaminating genomic DNA. The

concentration and quality of RNA preparations were determined by read absorbance at 260 and 280 nm and checked on a gel electrophoresis in 1.5% agarose gels with 1 μL RNA (0.5 μg·μL⁻¹).

For reverse transcription-polymerase chain reaction (RT-PCR), 2 μg purified RNA was reverse-transcribed with oligo (dT)₁₈ primer using a cDNA synthesis kit (Fermentas, Burlington, Ontario, Canada) according to the manufacturer's instructions. The first cDNA template was diluted 6-fold and kept at -20 °C for RT-PCR amplification analysis. The following primers were used to amplify the *rbcL* gene encoding the large subunits of Rubisco: 5'-TGG ATY TAT TYG ARG AAG GTT C-3' and 5'-TCG CAT GTA CCT GCA GTA GC-3'. *YT521-B* gene was used to confirm equal template loading. The PCR reactions were performed in a thermal cycler (Uno II; Biometra, Göttingen, Germany) programmed as follows: 3 min at 95 °C for initial denaturation, 38 cycles of 10 s at 94 °C, 20 s at 55 °C, and 20 s at 72 °C followed by 5 min at 72 °C. Amplified products were analyzed by 1.5% (w/v) agarose gel at 100 V for 40 min in 1 × TE buffer (10 mM Tris, 1 mM EDTA) and stained in ethidium bromide (0.5 μg·mL⁻¹). The band intensity was visualized and photographed by the Gel Doc XR system (Bio-Rad, Hercules, CA).

EXPERIMENTAL DESIGN AND STATISTICAL ANALYSIS. The experiment was arranged in a completely randomized block design with four replications. Plants of each perennial ryegrass genotype were exposed to salt stress in four flasks (four replicates). Treatment effects were determined by analysis of variance using the general linear model procedure of SAS (Version 9.0 for Windows; SAS Institute, Cary, NC). No interaction difference was observed between genotypes and salt levels. Significantly different means were separated by the Fisher's least significant difference test (*P* = 0.05).

Results

TURF QUALITY, LEAF RWC AND MDA CONTENT. Turf quality, leaf RWC, and MDA content were similar for two untreated perennial ryegrass genotypes. Salt stress reduced turf quality in both genotypes as measured at 4 and 8 DAT. BARLP 4317 maintained better visual quality relative to PI 516605 under salt stress (Table 1). Salinity stress caused a significant decrease in RWC of both genotypes when compared with the control plants. The decrease in RWC was 2.5% and 9.7% greater in PI 516605 vs. in BARLP 4317 at 4 and 8 DAT, respectively. Salt stress increased MDA content in both genotypes (Table 1). MDA content increased by 55.6% and 98.1% in BARLP 4317 when subjected to salt stress at 4 and 8 DAT, respectively, and 64.9% and 137.8% for PI 516605. BARLP 4317 maintained lower MDA content relative to PI 516605 under salt stress.

LIMITATIONS TO PHOTOSYNTHESIS. Salinity stress led to a substantial reduction in *P_n* and *g_s* for both genotypes at 4 and 8 DAT. BARLP 4317 exhibited 63.8% and 75.7% decrease of leaf *P_n* at 4 and 8 DAT, respectively, compared with 76.4% and 87.0% of PI 516605. BARLP 4317 had higher *P_n* relative to PI 516605 at 8 DAT (Table 2). *g_s* decreased by 84.3% and 78.6% in BARLP 4317 when subjected to salt stress at 4 and 8 DAT, respectively, and 86.3% and 82.5% in PI 516605. However, there was no significant difference in *g_s* between the two genotypes at 8 DAT.

Salt stress induced 38.2% and 21.8% decrease of leaf *C_i* for BARLP 4317 and PI 516605 at 4 DAT, respectively, when

Table 1. Effects of salt stress on turf quality, relative water content, and malondialdehyde (MDA) content in leaves of two perennial ryegrass genotypes (salt-tolerant BARLP 4317 and salt-sensitive PI 516605) 4 and 8 d after treatment began.

Treatments	Time after treatment began (d)		
	0	4	8
-----Turf quality (0–9 scale) ^z -----			
Untreated BARLP 4317	8.5 a A ^y	8.4 a A	8.4 a A
Untreated PI 516605	8.4 a A	8.2 a A	8.3 a A
Salted BARLP 4317	8.3 a A	7.0 b B	5.0 b C
Salted PI 516605	8.3 a A	6.0 c B	3.8 c C
----- Relative water content (%) -----			
Untreated BARLP 4317	95.1 a A	96.7 a A	96.3 a A
Untreated PI 516605	93.7 a A	94.7 a A	95.1 a A
Salted BARLP 4317	96.6 a A	78.6 b B	71.9 b B
Salted PI 516605	93.4 a A	76.0 b B	62.2 c C
-- MDA content (μmol·g ⁻¹ fresh weight)--			
Untreated BARLP 4317	5.46 a A	5.38 b A	5.16 c A
Untreated PI 516605	5.81 a A	5.20 b A	5.59 c A
Salted BARLP 4317	5.36 a BC	8.34 a B	10.62 b A
Salted PI 516605	5.61 a C	9.25 a B	13.33 a A

^zTurf quality was evaluated visually based on turfgrass color, density, and uniformity on a 1 to 9 scale where 6.0 = minimum acceptable quality; 0 = brown or dead turf; 7.0 = very good quality; and 9.0 = optimum greenness, uniformity, and density.

^yData are expressed as means of four replicates (n = 4). Means followed by the same upper case letter in the same row are not significantly different; means followed by the same lower case letter in the same column are not significantly by Fisher's least significant difference test at $P = 0.05$.

compared with the control. With prolonged salt stress, C_i increased for both genotypes. C_i was 33.4% greater in BARLP 4317 at 4 DAT vs. 8 DAT. PI 516605 had 16.0% higher in C_i at 4 DAT than 8 DAT. There was no difference in C_i between salt-treated plants and untreated plants at 8 DAT. At 4 d in treated plants, greater L_s was observed as a response to salt application in both perennial ryegrass genotypes and 118.2% and 107.6% increase for BARLP 4317 and PI 516605, respectively, when compared with the control. No difference in L_s was observed between salt-treated plants and untreated plants at 8 DAT.

Salt stress reduced Rubisco activity and activation state in both genotypes. Rubisco activity and activation state were greater in BARLP 4317 relative to PI 516605 under salt stress (Fig. 1). The Rubisco activity was 31.6% and 51.7% lower for BARLP 4317 at 4 and 8 DAT, respectively, when compared with the control, and 55.9% and 72.0% lower for PI 516605 (Fig. 1A). Salt stress inhibited Rubisco activation state by 26.4% and 34.7% for BARLP 4317 compared with the untreated regimes at 4 and 8 DAT, respectively, and 39.4% and 62.8% for PI 516605. Salt-treated BARLP 4317 exhibited a higher level of Rubisco activity and activation state than PI 516605 at 8 DAT.

RELATIONSHIPS BETWEEN PHOTOSYNTHETIC TRAITS AND PHYSIOLOGICAL TRAITS. Pooling together controls and salt-stressed leaves, both genotypes exhibited the same relationships between photosynthetic traits and physiological trait. P_n increased linearly with increasing leaf Rubisco activity for both genotypes (BARLP 4317, $r^2 = 0.656$, $P < 0.001$; PI 516605, $r^2 = 0.914$, $P < 0.001$) but curvilinearly related to Rubisco activation state for both genotypes (BARLP 4317, $r^2 = 0.905$, $P < 0.001$; PI 516605, $r^2 = 0.927$, $P < 0.001$) (Fig. 2). On a leaf FW basis,

Table 2. Effects of salt stress on photosynthetic rate (P_n), stomatal conductance (g_s), intercellular CO_2 concentration (C_i), and stomatal limitation (L_s) in leaves of two perennial ryegrass genotypes (salt-tolerant BARLP 4317 and salt-sensitive PI 516605) 4 and 8 d after treatment began.

Treatments	Time after treatment began (d)		
	0	4	8
----- P_n [CO_2 (μmol·m ⁻² ·s ⁻¹)]-----			
Untreated BARLP 4317	11.21 a A ^z	10.23 a A	10.24 a A
Untreated PI 516605	11.12 a A	10.94 a A	10.08 a A
Salted BARLP 4317	11.19 a A	4.04 b B	2.72 b C
Salted PI 516605	11.45 a A	2.70 b B	1.49 c C
----- g_s (mmol·m ⁻² ·s ⁻¹) -----			
Untreated BARLP 4317	0.27 a A	0.30 a A	0.19 a A
Untreated PI 516605	0.32 a A	0.39 a A	0.26 a A
Salted BARLP 4317	0.22 a A	0.03 b C	0.05 b B
Salted PI 516605	0.32 a A	0.04 b C	0.06 b B
----- C_i (μmol·mol ⁻¹) -----			
Untreated BARLP 4317	347.0 a A	325.5 a A	285.7 ab A
Untreated PI 516605	353.2 a A	337.0 a A	302.5 ab A
Salted BARLP 4317	325.1 a A	200.8 c B	267.9 b AB
Salted PI 516605	357.6 a A	279.6 b B	324.3 a AB
----- L_s -----			
Untreated BARLP 4317	0.18 a A	0.17 c A	0.25 ab A
Untreated PI 516605	0.18 a A	0.15 c A	0.22 b A
Salted BARLP 4317	0.23 a B	0.50 a A	0.33 a B
Salted PI 516605	0.16 a B	0.34 b A	0.14 b B

^zData are expressed as means of four replicates (n = 4). Means followed by the same upper case letter in the same row are not significantly different; means followed by the same lower case letter in the same column are not significantly by Fisher's least significant difference test at $P = 0.05$.

P_n (BARLP 4317, $r^2 = 0.888$, $P < 0.001$; PI 516605, $r^2 = 0.914$, $P < 0.001$), Rubisco activity (BARLP 4317, $r^2 = 0.700$, $P < 0.001$; PI 516605, $r^2 = 0.903$, $P < 0.001$), and Rubisco activation state (BARLP 4317, $r^2 = 0.716$, $P < 0.001$; PI 516605, $r^2 = 0.917$, $P < 0.001$) all increased linearly with increasing leaf RWC. In contrast, MDA content (BARLP 4317, $r^2 = 0.702$, $P < 0.001$; PI 516605, $r^2 = 0.427$, $P < 0.001$) were strongly and negatively correlated with leaf RWC (Fig. 3).

GENE EXPRESSION AND PROTEIN CONTENT. The down-regulation of *rbcL* expression could be just found in PI 516605 at 4 DAT compared with the untreated plants (Fig. 4A). The salt-treated perennial ryegrass had a lower expression of *rbcL* at 8 DAT compared with the untreated regimes regardless of the genotypes. In stressed plants, a higher expression level of *rbcL* was observed in BARLP 4317 relative to PI 516605 at the same treatment time and a more dramatic change in expression was found between BARLP 4317 and PI 516605 at a longer salt-stressed time.

No difference in SSU abundance was observed between the salt-treated regimes and the untreated regimes at 4 and 8 DAT for both genotypes. However, salt stress resulted in a lower level of LSU abundance for both genotypes at 4 and 8 DAT. BARLP 4317 had a higher level of LSU abundance vs. in PI 516605 at 4 and 8 DAT (Fig. 4B).

Discussion

In this study, we investigated the effects of salinity stress on leaf stomatal behavior, water status and photosynthetic enzyme

activity, protein, and gene expression in two genotypes of perennial ryegrass differing in salt tolerance. The toxicity to plant growth, gas exchange, and Rubisco activity was as expected for perennial ryegrass grown up to 8 d under salt stress. However, the leaf stomatal limitations and non-stomatal limitations to photosynthetic response varied with salt stress duration and genotypes. Data in this study showed that salt-tolerant BARLP 4317 had a higher level of turf quality and RWC but lower MDA than salt-sensitive PI 516605 when exposed to salinity stress. The results agreed with our earlier

study in which toxicity to plant growth and cell damage varied in different perennial ryegrass genotypes and salt-sensitive genotype had greater MDA (Hu et al., 2012).

Osmotic stress was one of toxic effects of NaCl on plant growth resulting from higher apoplastic levels of Na^+ accumulated in cells (Niu et al., 1995). Salinity induces a suite of metabolic changes similar to those caused by water stress reducing the ability of plants to take up water (Munns, 2002). Data in this study exhibited a decrease in leaf RWC for both genotypes subjected to salinity stress as compared with the control, suggesting that salt stress led to drought stress because of limited water absorbed. However, RWC was greater for salt-tolerant BARLP 4317 vs. salt-sensitive PI 516605 under salinity stress, suggesting that salt-tolerant BARLP 4317 had a greater ability to absorb and hold water. These results were consistent with earlier results obtained by Chakraborty et al. (2012) and Sairam and Srivastava (2002), who reported a relatively higher capacity of water uptake in salt-tolerant genotypes of wheat (*Triticum aestivum*) and mustard (*Brassica juncea*) than that in salt-sensitive ones under salinity stress. However, Munns et al. (2006) reported that there was little difference between wheat genotypes in effect of salinity on RWC when exposed to salt stress for 4 weeks.

MDA is an aldehydic end product in cell membrane lipid peroxidation and is generally used as an indicator of cell damages level induced by salinity stress (Hu et al., 2011). Maintaining a lower level of MDA content is considered one of the mechanisms for plants to survive salinity (Stevens et al., 2006). This study indicated that salt-sensitive PI 516605 had more leaf MDA relative to salt-tolerant BARLP 4317 in response to salinity stress. When subjected to salinity stress, salt-sensitive germplasm tended to accumulate more MDA than salt-tolerant germplasm reported in sugar beet [*Beta vulgaris* (Bor et al., 2003)], tobacco (Duan et al., 2009), tomato [*Solanum lycopersicum* (Szepesi et al., 2009)], barley [*Hordeum vulgare* (Hafsi et al., 2010)], and rosemary [*Rosmarinus officinalis* (Tounekti et al., 2011)]. These research results suggested that salt-tolerant germplasm might involve better protection against cell oxidative damage.

Maintaining healthy grass under salinity may depend on the photosynthetic ability to produce enough carbohydrate. Salt stress decreased 63.8% and 75.7% of P_n for BARLP 4317 at 4 and 8 DAT, respectively, when compared with the control and 76.4% and 87.0% for PI 516605. Lower P_n was attributed to rapid stomatal closure at 4 DAT, because the lower P_n coincided with the dramatic decline in C_i and g_s but an increase of L_s . These results were in agreement with previous studies on salt-stressed mangrove tree [*Avicennia germinans* (Suárez and Medina, 2006)] and soybean (Kao et al., 2003). However, both C_i and g_s increased rapidly along with a decrease of both P_n and L_s at 8 DAT for both genotypes, suggesting that lower photosynthetic rate induced by salinity stress in perennial ryegrass was the result of non-stomatal factors.

Rubisco is a key enzyme in the process of photosynthesis (Portis, 2003). Salinity stress induced a dramatic decrease in the activity of Rubisco for salt-sensitive soybean 'Melrose'. However, salinity stress had no effects on both total and initial activity of Rubisco

Fig. 1. Effects of salt stress on Rubisco activity (A) and Rubisco activation state (B) in salt-tolerant BARLP 4317 and salt-sensitive PI 516605 perennial ryegrass seedlings. Plants were not stressed (ck) or were salt-stressed (salt) for 4 or 8 d. Vertical bars represent least significant difference values ($P = 0.05$) indicating significant differences of treatment comparisons at a given day.

Fig. 2. Correlations of net photosynthetic rate (P_n), Rubisco activity, and activation state in leaves of two perennial ryegrass genotypes (BARLP 4317 and PI 516605) in response to salt stress.

Fig. 3. Correlations of net photosynthetic rate (P_n), Rubisco activity and activation state, malondialdehyde (MDA), and relative water content (RWC) in leaves of two perennial ryegrass genotypes (BARLP 4317 and PI 516605) in response to salt stress.

Fig. 4. Reverse transcription-polymerase chain reaction analyses of *rbcL* gene in the leaves of two perennial ryegrass genotypes (BARLP 4317 and PI 516605) at 0, 4, and 8 d of salinity stress. *Yt521-B* gene was used as the reference gene for cDNA normalization (A). The sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) profiles of Rubisco small subunit (SSU) and Rubisco large subunit (LSU) from leaves of both genotypes under salt stress (B). Plants were not stressed (CK) or were salt-stressed (Salt) for 0, 4, or 8 d.

enzyme in salt-tolerant soybean ‘S111-9’ at 150 mM NaCl (Lu et al., 2009). This study indicated that salt-stressed perennial ryegrass had a lower level of both Rubisco activity and activation state compared with the control level and a greater reduction with a prolonged period of salinity stress, suggesting that the Rubisco may be one key non-stomatal factor to inhibit photosynthesis in perennial ryegrass.

SDS-PAGE analysis in this study indicated that the expression of LSU was rapidly down-regulated for both genotypes as salt stress progressed. However, SSU remained unchanged in both genotypes during the entire 8-d experimental period. The difference in SSU and LSU might be the result of the damage extent in the different organelle for synthesis of SSU (nuclear-encoded) and LSU (chloroplast-encoded) (Daley and Whelan, 2005). It had reported that degradation of thylakoid membranes of chloroplast in LSU syn-

thesis occurred as a result of salt-induced oxidative stress (Yamane et al., 2003, 2004). In addition, results in this study indicated that the transcript levels of *rbcL* genes (coding for the LSU) were significantly lower than the control levels at 8 DAT for both genotypes, which might have contributed to LSU degradation. Thus, the greater decrease in Rubisco activity and activation state and expression of LSU and *rbcL* in salt-stressed leaves of both genotypes compared with controls can be attributed to a greater decrease in the P_n .

P_n increased linearly with increasing leaf Rubisco activity and curvilinearly related to Rubisco activation state, which was consistent with the hypothesis that Rubisco, playing one key role in the photosynthesis unit, was one primary non-stomatal factor to inhibit photosynthesis in perennial ryegrass. A similar relationship between P_n and Rubisco activity in response to abiotic stress has been reported for boron deficiency and boron excess in pummelo [*Citrus grandis* (Han et al., 2009)] and nitrogen excess in apple [*Malus ×domestica* (Cheng and Fuchigami, 2000)]. Both Rubisco activity and activation state were positively and linearly related to leaf RWC. However, MDA content was negatively and linearly correlated with leaf RWC, suggesting that the loss of water induced by salt stress damaged the leaf cells and inhibited the activity of Rubisco. In addition, there was a significant correlation between P_n and RWC after pooling together controls and salt-stressed leaves, suggesting that photosynthesis was limited by declining RWC (Tezara et al., 2003). In general, this is also a clear indication that the capacity of holding water and Rubisco activity in leaf may be the main limitation factor of photosynthesis for salt-tolerant as well as -sensitive perennial ryegrass genotypes.

In summary, salt-induced inhibition of photosynthesis in perennial ryegrass was mainly the result of a metabolic limitation associated with the reduction of RWC, activity of Rubisco, expression level of *rbcL* gene and LSU degradation under a prolonged period of severe salinity, and a stomatal

factor associated with inhibition of photosynthesis during early salt stress. Maintaining greater leaf water content, activity of Rubisco, transcriptional level of *rbcL* gene, and expression level of LSU may contribute to superior salt resistance in salt-tolerant BARLP 4317. These metabolic traits can be used as potential and practical selection criteria for determining salt-tolerant perennial ryegrass genotypes.

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