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Diffusional conductance to CO_2 is the key limitation to photosynthesis in salt-stressed leaves of rice (*Oryza sativa*)

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Salinity significantly limits leaf photosynthesis but the factors causing the limitation in salt-stressed leaves remain unclear. In the present work, photosynthetic and biochemical traits were investigated in four rice genotypes under two NaCl concentration (0 and 150 m*M*) to assess the stomatal, mesophyll and biochemical contributions to reduced photosynthetic rate (*A*) in salt-stressed leaves. Our results indicated that salinity led to a decrease in *A*, leaf osmotic potential, electron transport rate and CO_2 concentrations in the chloroplasts (C_c) of rice leaves. Decreased *A* in salt-stressed leaves was mainly attributable to low C_c, which was determined by stomatal and mesophyll conductance. The increased stomatal limitation was mainly related to the low leaf osmotic potential caused by soil salinity. However, the increased mesophyll limitation in salt-stressed leaves was related to both osmotic stress and ion stress. These findings highlight the importance of considering mesophyll conductance when developing salinity-tolerant rice cultivars.

Introduction

Soil salinity is a global problem that limits crops production worldwide. Rice (*Oryza sativa* L.) is one of the most important cereal crops; however, it has been reported to be very sensitive to salt stress, and it has been listed as the most salt-sensitive cereal crop (Negrão et al. 2011, Munns et al. 2016). Salinity reduces rice yield, partially by restraining biomass accumulation, which is associated with a decreasing rate of photosynthesis (Moradi and Ismail 2007, Wankhade et al. 2013). A considerable effort has been made, in recent decades, to understand the negative effect of salinity on photosynthesis, but it has yet to be fully understood. According to the Farquhar model (Farquhar et al. 1980), leaf photosynthesis in C_3 plants is limited by the capacity of Rubisco to consume RuBP (Rubisco-limited photosynthesis), by the capacity of electron transport and Calvin cycle enzymes to regenerate RuBP (RuBP regeneration-limited photosynthesis) or by the capacity of starch and sucrose synthesis to consume triose phosphates and to regenerate inorganic phosphate for photophosphorylation (Pi regeneration-limited photosynthesis). In general, the Rubisco capacity to consume RuBP is the predominant

Abbreviations – α, leaf light absorptance efficiency; β, the distribution of electrons between PSI and PSII; Γ^*, CO_2 compensation point in the absence of respiration; Φ_{PSII}, quantum efficiency of photosystem II; *A*, photosynthetic rate; CA, carbonic anhydrase; C_c, CO_2 concentration at carboxylation sites; CE, apparent Rubisco activity; Chl, total chlorophyll content; C_i, intercellular CO_2 concentration; ETR, electron transport rate; F_0, initial fluorescence of photosystem II in darkness; F_m, maximum fluorescence of photosystem II; F_v, maximum variable fluorescence of photosystem II; F_v/F_m, maximum quantum efficiency of photosystem II; g_m, mesophyll conductance; g_s, stomatal conductance; J_{max}, maximum electron transport rate; K, leaf K content; LMA, leaf mass per area; LYP9, Liangyoupei 9; N, leaf N content; P, leaf P content; PPFD, photosynthetic photon flux density; OP, osmotic potential; Protein, leaf total soluble protein content; qN, non-chemical quenching efficiency; R_d, day respiration; R_{dark}, dark respiration; Rubisco, Rubisco content; SY63, Shanyou 63; TXZ25, Texianzhan 25; V_{cmax}, maximum carboxylation rate; VPD, vapor pressure deficit.

limitation on photosynthesis at low chloroplasts CO_2 concentration (C_c) and Rubisco activity; RuBP regeneration limiting related to photosystem electron transport rate (ETR) and the activity of relative enzymes; and Pi regeneration limits photosynthesis under very high C_c. The C_c is mainly determined by stomatal conductance (g_s), mesophyll conductance (g_m) as well as the Rubisco carboxylation capacity (i.e. V_{cmax}). Due to the complex responses of leaves to salinity, there is debate over whether the decreased photosynthetic rate (A) in salt-stressed leaves is primarily limited by g_s, g_m, ETR, the activity of relative enzymes (i.e. Rubisco) or a combination of several of these factors (Flexas et al. 2012, Flexas et al. 2016).

A large number of previous studies have described the stomatal limitations in salt-stressed leaves (Delfine et al. 1998, Delfine et al. 1999, Centritto et al. 2003, Moradi and Ismail 2007, Chaves et al. 2011, Chen et al. 2015), because the low leaf water potential introduced by low osmotic potential (termed the 'osmotic effect') could cause stomatal closure, while Khan et al. (2015) reported that the decreasing A in salt-stressed chickpea leaves was predominately caused by photosystem II damage rather than by g_s. However, although the g_m has rarely been investigated in previous studies, there is no consistent conclusion about mesophyll limitations in salt-stressed leaves. Several previous studies observed that both g_s and g_m are the primary factors limiting A (Delfine et al. 1998, Centritto et al. 2003, Sade et al. 2014). In contrast, other studies have shown that the limitation of g_m on A in salt-stressed leaves can be ignored in *Cucumis sativus* (Chen et al. 2015) and *Hordeum vulgare* (Perez-Lopez et al. 2012). These results indicated that the limitations of g_m on photosynthesis in salt-stressed leaves might be species dependent. Although the g_s response to salinity in rice has been investigated by many researchers (Moradi and Ismail 2007, Negrão et al. 2011, Wankhade et al. 2013), no previous studies, to our knowledge, has investigated the response of g_m to salinity in rice, which is one of the most salinity sensitive species.

Salinity may also directly inhibit A due to the uptake and the accumulation of sodium and chloride in mesophyll tissues (termed as 'ionic effects'). As the first step of Calvin–Benson cycle and the most abundant protein in C_3 plants, the content and activity of Rubisco has been suggested as one of the limiting factors involved in reducing A under salinity (Delfine et al. 1998, James et al. 2002, James et al. 2006, Yamane et al. 2012). In contrast, Centritto et al. (2003) suggested that the biochemical capacity is not affected by salinity.

The reduction of ETR in salt-stressed leaves that is often associated with decreases in the actual quantum yield of PSII (Φ_{PSII}) and maximal efficiency of PSII photochemistry (F_v/F_m) was observed in some species (Moradi and Ismail 2007, Koyro et al. 2013) but not in others (James et al. 2002, Koyro et al. 2013). Similarly, the responses of ETR, Φ_{PSII} and F_v/F_m to salinity were genotype dependent in rice (Moradi and Ismail 2007, Wankhade et al. 2013). The reduction in Φ_{PSII} in some species/genotypes may be due to the salt-induced regulation of energy transduction from the antennae to the reaction centers to prevent photosystem energy surpluses. It was also demonstrated by increased NPQ, an indicator of the excess radiant energy dissipation to heat in the PSII antenna complexes (Murchie and Lawson 2013) under salt-stressed leaves. Moreover, Stepien and Johnson (2009) demonstrated that plastid terminal oxidase acts as an alternative electron sink in *Halophyte thellungiella* a salt-tolerant species. Here, we hypothesized that the balance between PSII photochemical activity and the electron requirement for photosynthesis might be broken when CO_2 concentration in chloroplasts (C_c) decreased due to the reduction of g_s and g_m in salinity-sensitive species/genotypes, and this leads to over-excitation and, subsequently, photoinhibition.

In this study, we measured leaf gas exchange and biochemical traits in the model monocot species *Oryza sativa* to reveal the limiting factors of photosynthesis under salinity by using limitation analysis (Grassi and Magnani 2005, Buckley and Diaz-Espejo 2014). The aims of this study as follows: (1) to quantify the limitations of g_s, g_m and biochemical factors on A in salt-stressed leaves and (2) to test the hypothesis that the decreased A in rice is related to photoinhibition under salt stress.

Materials and methods

Plant materials and growth conditions

Rice seeds of four genotypes with different salt tolerances (Xiong et al., unpublished data), Liangyoupei 9 (LYP9), N22, Shanyou 63 (SY63) and Texianzhan 25 (TXZ25) were germinated and grown in a nursery for 3 weeks, in a growth chamber (Model GR48, Conviron, Controlled Environments Limited, Winnipeg, MB, Canada). In the chamber, the air temperature was set to 28/22°C (day/night), with a relative humidity of 70% and photosynthetic photon flux density (PPFD) of 600 µmol m^{-2} s^{-1} with a 12/12 h light/dark regime. The plants were then transplanted into 11-l plastic pots containing 10 kg of soil with three plants per pot in the same chamber. Before transplanting, 7.0 g of compound fertilizer ($N:P_2O_5:K_2O = 16:16:16\%$, Batian Ecological Engineering Limited, Shenzhen, China) was mixed into the soil per pot, and 30 days after transplanting 1.3 g of urea was top-dressed per pot. For each genotype, 10 pots were

grown and randomly arranged. To avoid water stress, at least a 2-cm water layer was maintained. Seven weeks after transplanting, half of the pots of each genotype were irrigated with 1 l of 150 m*M* NaCl solution every 2 days for 1 week. All of the measurements were performed in fully expanded young leaves.

Gas exchange and chlorophyll fluorescence measurements

Gas exchange was measured, in a growth chamber between 8:30 and 16:00, on the newly and fully expanded leaves of the three plants in each treatment. Gas exchange was measured using a Licor-6400 portable photosynthesis system equipped with a Li-6400-40 chamber (LI-COR Inc., Lincoln, NE). In the leaf chamber, the PPFD was maintained at 1200 μmol m^{-2} s^{-1}, a leaf-to-air vapor pressure deficit (VPD) at 1.5–2.0 kPa, and a CO_2 concentration adjusted to 400 μmol m^{-2} s^{-1} with a CO_2 mixer. The block temperature during measurements was maintained at 28°C. After equilibration to a steady state (usually more than 20 min after clamping the leaf), the gas exchange parameters, steady-state fluorescence (F_s) and maximum fluorescence (F_m') were recorded. The actual photochemical efficiency of photosystem II (Φ_{PSII}) was calculated as follows:

$$\Phi_{PSII} = \frac{\left(F'_m - F_s\right)}{F'_m}$$

The electron transport rates (ETR) were computed as follows:

$$ETR = \Phi_{PSII} \times PPFD \times \alpha \times \beta$$

where, α is the leaf absorbance, and β represents the distribution of electrons between PSI and PSII.

Five days after the NaCl treatment, the light response curves were performed under low O_2 concentration (<2%) to estimate α and β. The gas exchange system was switched to low O_2 concentration (<2%) by injecting pure N_2. Simultaneous measurements of light response curves and chlorophyll fluorescence were then performed. During the measurements, the chamber conditions were the same as those described above, except that PPFD was controlled across a gradient of 2000, 1500, 1200, 1000, 800, 600, 400, 200, 100, 0 and 1200 μmol m^{-2} s^{-1}. After reaching a steady state, the parameters of gas exchange and chlorophyll fluorescence were simultaneously recorded. The slope of the relationship between Φ_{PSII} and $4\Phi_{CO2}$ (the quantum efficiency of CO_2 uptake) is considered to be the value of $\alpha \times \beta$ (Valentini et al. 1995). There was no difference in $\alpha \times \beta$ values between the control and the salt-stressed leaves (Fig. S1A), thus the average value of all the genotypes were used in the current study.

The mesophyll conductance of CO_2 (g_m) was calculated based on the variable *J* method described in (Harley et al. 1992). In this method, the CO_2 concentration in the chloroplast (C_c) was calculated as follows:

$$C_c = \frac{\Gamma^* \left(ETR + 8\left(A + R_d\right)\right)}{ETR - 4\left(A + R_d\right)}$$

where, Γ^* represents the CO_2 compensation point in the absence of respiration and R_d is the day respiration, which was assumed to be half of the dark respiration rate (R_{dark}). Γ^* is related to the Rubisco-specific factor ($S_{C/O}$), which is relatively conserved under a given temperature condition. In the present study, rice $S_{C/O}$ at 28°C was obtained from Hermida-Carrera et al. (2016). Then, g_m was calculated as follows:

$$g_m = \frac{A}{C_i - C_c}$$

where, C_i represents the intercellular CO_2 concentration.

After 7 days of salt treatment, the CO_2 response curves ($A-C_i$ curves) were measured with two Licor-6400 portable photosynthesis system equipped with a Li-6400-40 chamber in 3 days. The CO_2R was set at 400, 300, 200, 150, 100, 50, 0, 400, 600, 800, 1000, 1500, 2000 and 400 μmol mol^{-1}, the PPFD was set as 1200 μmol m^{-2} s^{-1} with a 10:90 blue:red light, the flow rate at 150 μmol s^{-1} and the relative humidity at 65 (±5)%. When the stomatal conductance was stable (less than 5% variation during 10 min), the automatic program was run. For each step, a 4–5 min wait was carried out. The dead rice leaves (obtained by heating the leaves until no variable chlorophyll fluorescence was observed) were used to estimate the leakage effects of the chamber under different CO_2 concentrations (Flexas et al. 2007, Xiong et al. 2015b). In the current study, the sum of the photorespiration and mitochondrial respiration in the light (R_L) was calculated by extrapolating the $A-C_i$ curve to $C_i = 0$ (Escalona et al. 1999, Flexas et al. 2002).

Seven days after NaCl treatment, the g_m was calculated with two methods: Harley's method (Harley et al. 1992) and Ethier's method (Ethier and Livingston 2004). The method of Ethier and Livingston (2004) uses only gas exchange measurements, by adjusting the non-linear model of Farquhar et al. (1980) to extract the g_m. In the present study, some NaCl treatment leaves did not reach satisfactory results by using Ethier's method. Therefore, we used the values obtained by Harley's method to compare with other parameters in the manuscript, while a good correlation was obtained between the two estimates of g_m considering the data averaged per treatments ($R^2 = 0.86$; $P < 0.001$; Fig. S1B).

Dark respiration (R_{dark}) was measured by Li-Cor 6400 after $A-C_i$ curves were performed. Before the R_{dark} was measured, rice plants were acclimatized to darkness for at least 2 h. In the Li-Cor leaf chamber, the ambient CO_2 concentration was adjusted to 400 µmol mol^{-1} using a CO_2 mixture, the block temperature was maintained at 28°C, the PPFD was 0 µmol m^{-2} s^{-1}, the leaf-to-air VPD was between 1.1 and 1.5 kPa and the flow rate was 100 µmol s^{-1}. After the leaf reached a steady state, usually after 10 min, gas exchange parameters were recorded.

Photosynthetic limitation analysis

Limitation analysis is a helpful tool to quantify the stress effects of changes in various factors on A (Grassi and Magnani 2005, Buckley and Diaz-Espejo 2014), and it has been widely used in recent years (Flexas et al. 2009, Galle et al. 2009, Galle et al. 2011, Chen et al. 2015, Tosens et al. 2015). Relative photosynthetic limitations including stomatal (l_s), mesophyll (l_s) and biochemical (l_b) relative limitations were calculated according to Grassi and Magnani (2005).

$$l_s = \frac{g_t/g_s \cdot \partial A/\partial C_c}{g_t + \partial A/\partial C_c}$$

$$l_m = \frac{g_t/g_m \cdot \partial A/\partial C_c}{g_t + \partial A/\partial C_c}$$

$$l_b = \frac{g_t}{g_t + \partial A/\partial C_c}$$

To assess the effects of salinity on changes in photosynthetic limitations in each genotype and treatment duration, the relative limitations were linked to overall changes in A:

$$\frac{dA}{A} = LS + LM + LB = \frac{dg_s}{g_s} l_s + \frac{dg_m}{g_m} l_m + \frac{dV_{cmax}}{V_{cmax}} l_b$$

where LS, LM and LB are the reduction fractional limitation in A caused by reduction in stomatal conductance, mesophyll conductance and biochemistry, respectively. In the current study, the photosynthetic parameters of control were defined as the references.

Chlorophyll fluorescence

Chlorophyll fluorescence parameters were measured at pre-dawn to investigate the F_v/F_m. A portable pulse amplitude modulation fluorescence instrument (PAM 2000, Walz, Effeltrich, Germany) was used. A measuring light of approximately 0.5 µmol m^{-2} s^{-1} was set to a frequency of 600 Hz to determine the background fluorescence signal (F_o) as well as the maximum fluorescence (F_m), and the F_v was calculated as $F_v = F_m - F_o$.

Determination of N, P, Na and K content

After 7-days salt treatment, the fully expanded young leaves were sampled after taking a picture for leaf area estimation, and were dried under 80°C to a constant weight. The dry samples were digested by the micro-Kjeldahl method (Xiong et al. 2015c). The N and P concentrations were measured with a discrete wet chemistry analyzer (SmartChem 200, AMS-Westco, Rome, Italy). The Na and K concentrations were measured by an atom absorption spectrometer (PinAAcle 900T, Perkin Elmer, Waltham, MA). The leaf area was measured by using ImageJ software (National institute of Health, Bethesda, MD).

Determination of the total soluble protein, Rubisco and chlorophyll content

Leaf samples were harvested in the morning of the seventh-day after NaCl treatment, and immersed in liquid nitrogen. The samples were stored at −80°C until the soluble protein and Rubisco concentration were measured. The frozen leaf sample was ground in liquid nitrogen and homogenized in ice in an extraction buffer containing 50 m*M* Tris–HCl buffer (pH 8.0), 5 mmol *β*-mercaptoethanol, and 12.5% glycerol (v/v). After centrifuging, the supernatant fluid was used as a total soluble protein as well as for a Rubisco content analysis (Xiong et al. 2015b, Xiong et al. 2015c). The Rubisco samples were loaded onto sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) containing a 12.5% (w/v) polyacrylamide gel. After electrophoresis (DYY-11, Beijing Liuyi Instrument Factory), the gels were washed with deionized water several times and then dyed in 0.25% commassie blue staining solution for 9 h and decolorized until the background was colorless. Then, the Rubisco was transferred into a 5-ml cuvette with 1.5 ml of formamide and washed in a 50°C water bath at room temperature for 8 h. The washed solutions were measured at 595 nm (Infinite M200, Tecan U.S. Inc) using the background glue as a blank, and bovine serum albumin as the standard protein.

Osmotic potential measurements

The fully expanded young leaves were sampled in the morning of the seventh-day after NaCl treatment. The leaf samples were immersed in liquid nitrogen and then stored at −80°C until measured. The leaf osmotic potential was measured by using a vapor pressure osmometer (VAPRO 5520, Wescor Inc., Logan, UT).

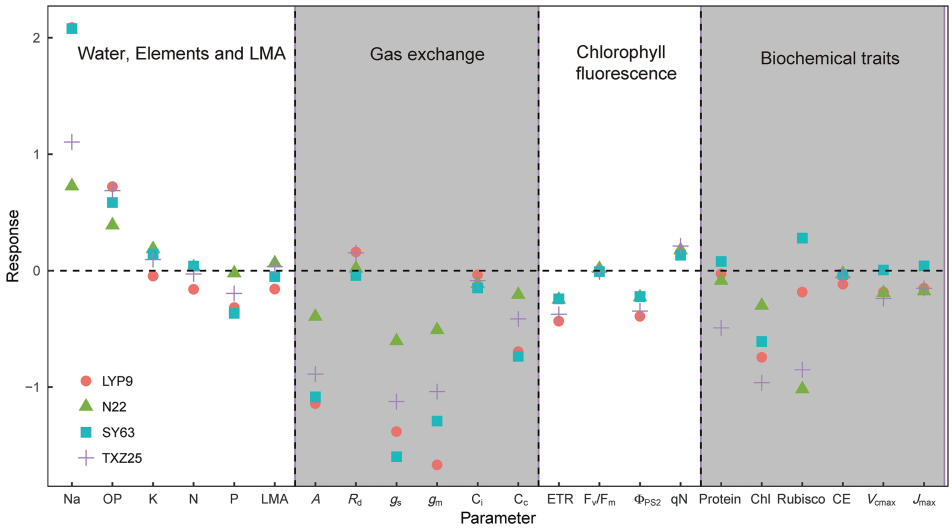

Fig. 1. Response of variation to 7-days salinity in four rice genotypes. The responses were calculated by $\ln(X_T/X_{CK})$, where the X_T and X_{CK} represent the mean values of the parameter under NaCl treatment and control, respectively.

Statistical analysis

ANOVA with a post hoc Tukey's HSD test was used to test the differences and interactions in the measured traits among genotypes and treatments. Regression analyses were performed with mean values to test the correlations between parameters. Regressions were fitted with linear model, except in Fig. 5, which is fitted by a power model ($y = ax^b$). Regression lines were shown for $P < 0.05$. All of the analyses were performed in R version 3.3.1 (https://cran.r-project.org).

Results

We observed substantial variations in the responses of chemical composition, photosynthetic traits, chlorophyll fluorescence as well as the osmotic potential of salinity in rice (Fig. 1). The salinity responses of those traits also varied with genotype; overall, N22 was more tolerant of salinity than the other three genotypes.

Effects of salinity on leaf biochemical parameters

Overall, the leaf Na^+, P and K content in salt-stressed rice increased significantly after 7 days of NaCl treatments, while a substantial genetic variation was observed (Table 1; Fig. 1). There was no difference in the LMA and leaf N content between the control and salinity treatment, despite a significant variation among genotypes. Across all four genotypes, salt stress decreased the total leaf solution protein and Rubisco content, whereas

Table 1. Effects of salinity on leaf mass per area and leaf biochemical traits in four rice genotypes. N, nitrogen content; P, phosphorus content; K, potassium content; Na, sodium content; protein, soluble protein content; Rubisco, Rubisco content; T, treatment and G, genotype. ns, no significant; $*P < 0.05$; $**P < 0.01$; and $***P < 0.001$.

Genotype	Treatment	LMA ($g\,m^{-2}$)	N ($g\,m^{-2}$)	P ($mg\,m^{-2}$)	K ($g\,m^{-2}$)	Na ($mg\,m^{-2}$)	Protein ($g\,m^{-2}$)	Rubisco ($g\,m^{-2}$)
LYP9	CK	60.6 ± 4.0	1.41 ± 0.12	99.0 ± 4.4	1.14 ± 0.09	5.7 ± 1.2	4.41 ± 0.05	1.82 ± 0.64
	NaCl	51.8 ± 6.4	1.20 ± 0.11	72.1 ± 8.3	1.08 ± 0.18	46.1 ± 17.0	4.30 ± 0.28	1.51 ± 0.46
N22	CK	37.7 ± 1.2	1.09 ± 0.06	66.7 ± 4.0	0.57 ± 0.02	3.7 ± 1.3	3.41 ± 0.21	1.65 ± 0.26
	NaCl	40.2 ± 2.3	1.12 ± 0.03	65.2 ± 1.5	0.69 ± 0.03	7.6 ± 2.2	3.13 ± 0.20	0.6 ± 0.10
SY63	CK	53.6 ± 2.9	1.44 ± 0.11	112.9 ± 9.1	0.72 ± 0.07	5.5 ± 1.4	3.48 ± 0.44	1.13 ± 0.32
	NaCl	50.7 ± 4.6	1.49 ± 0.15	78.3 ± 8.0	0.83 ± 0.08	43.8 ± 25.1	3.76 ± 0.18	1.49 ± 0.40
TXZ25	CK	40.1 ± 3.7	1.29 ± 0.12	90.3 ± 9.7	0.55 ± 0.07	6.5 ± 1.1	4.37 ± 0.42	1.55 ± 0.47
	NaCl	41.6 ± 3.3	1.25 ± 0.09	74.2 ± 8.5	0.61 ± 0.06	19.6 ± 4.0	2.67 ± 0.20	0.66 ± 0.21
Significance	T	ns	ns	*	*	**	*	***
	G	*	*	*	*	*	ns	ns
	T × G	ns	ns	*	*	**	*	***

Table 2. Effects of salinity on leaf physiological traits in four rice genotypes. A, light-saturated photosynthetic rate; R_{dark}, dark respiration; C_i, intercellular CO_2 concentration; C_c, CO_2 concentration at chloroplasts, V_{cmax}, maximum carboxylation rate; J_{max}, maximum electron transport rate; and OP, osmotic potential. ns, no significant; $*P<0.05$; $**P<0.01$ and $***P<0.001$.

Genotype	Treatment	A (μmol m^{-2} s^{-1})	R_{dark} (μmol m^{-2} s^{-1})	C_i (μmol mol^{-1})	C_c (μmol mol^{-1})	V_{cmax} (μmol m^{-2} s^{-1})	J_{max} (μmol m^{-2} s^{-1})	CE (mol m^{-2} s^{-1})	OP (MPa)
LYP9	CK	11.58 ± 2.98	0.672 ± 0.010	302 ± 26	189 ± 21	114.2 ± 31.2	123.7 ± 21.3	0.11 ± 0.05	−0.98 ± 0.12
	NaCl	3.34 ± 0.67	0.790 ± 0.048	291 ± 20	93 ± 10	95.1 ± 3.0	106.4 ± 14.3	0.12 ± 0.01	−2.02 ± 0.08
N22	CK	8.53 ± 2.25	0.500 ± 0.017	283 ± 23	131 ± 32	104.5 ± 2.8	110.5 ± 6.9	0.14 ± 0.03	−1.30 ± 0.18
	NaCl	5.58 ± 1.54	0.510 ± 0.016	253 ± 31	111 ± 12	86.3 ± 8.2	92.7 ± 14.0	0.13 ± 0.01	−1.92 ± 0.20
SY63	CK	11.18 ± 0.87	0.778 ± 0.027	291 ± 9	189 ± 39	132.1 ± 2.4	135.7 ± 20.4	0.15 ± 0.02	−1.15 ± 0.05
	NaCl	3.42 ± 1.44	0.747 ± 0.015	251 ± 34	84 ± 6	132.9 ± 22.9	141.4 ± 8.0	0.15 ± 0.04	−2.06 ± 0.19
TXZ25	CK	9.65 ± 0.95	0.574 ± 0.030	265 ± 8	122 ± 24	144.7 ± 1.4	148.3 ± 20.5	0.16 ± 0.02	−0.94 ± 0.08
	NaCl	3.65 ± 0.19	0.669 ± 0.041	243 ± 28	83 ± 4	114.0 ± 6.5	127.4 ± 17.5	0.15 ± 0.01	−1.88 ± 0.09
Significance	T	***	ns	*	**	*	*	ns	***
	G	*	ns	ns	ns	ns	ns	ns	ns
	T × G	**	ns	ns	**	*	*	ns	ns

both the total leaf solution protein and Rubisco content increased in SY63.

Gas exchange and osmotic potential

A, g_s, g_m and ETR in salt-stressed rice leaves declined rapidly after starting the NaCl treatment (Fig. S2). A decreased by approximately 30% in the salt-stressed leaves of SY63 and TXZ25, but no response in N22 on the first day after NaCl treatment. After 3 days of NaCl treatment, the A in salt-stressed leaves of all the four genotypes decreased. A similar response pattern was found in g_s, g_m and ETR (Fig. S2). After 7 days of NaCl treatment, the biggest decline of A was in the salt-stressed leaves of LYP9 (72%) and the mildest decline occurred in N22 (38%) (Fig. S2). The gas exchange and osmotic potential parameters after 7 days of NaCl treatment are shown in Table 2. Overall, substantial variation in A of rice leaves was found among genotypes as well as in the NaCl treatments. Similar to A, both g_s and g_m decreased significantly in the salt-stressed leaves of LYP9, SY63 and TXZ25, but not in N22 (Fig. 2). Across the genotypes and NaCl treatments, A was tightly correlated with g_s ($R^2 = 0.91$; $P < 0.001$) and g_m ($R^2 = 0.98$; $P < 0.001$). There was no significant difference of R_{dark} and CE among genotypes and NaCl treatments. While no genetic variation in C_i, C_c, V_{cmax}, J_{max} and osmotic potential were found, salinity significantly decreased those parameters (Fig. 3; Table 2).

Both ETR and ΦPSII trended lower in salt-stressed leaves than in the control (Fig. 4), although only LYP9 and TXZ25 showed statistical significance (Fig. 4). However, F_v/F_m showed no difference between the control and salinity in all of the estimated genotypes (Fig. 4B). In contrast, qN exhibited an increasing tendency in salt-stressed leaves, while only TXZ25 exhibited a significant increase. The ratio of ETR/A varied widely with varying C_c across four genotypes and two NaCl treatments (Fig. 5); however, the ratio of ETR/$(A + R_L)$ exhibited constant with varying C_c. When, C_c was lower than 100 μmol mol^{-1}, the ratio of ETR/A increased fast with decreasing C_c.

Limitation analysis

The impact of 7-days of salinity treatment on the relative stomatal (l_s), mesophyll (l_m) and biochemical (l_b) limitations are shown in Fig. S4. Under normal condition (control) the A of the estimated rice genotypes was mainly limited by l_b. However, in salt-stressed leaves, both l_s and l_m increased in all of the genotypes except the l_m in N22. In Fig. 6, the contributions of three relative limitations to decrease A are shown. In salt-stressed leaves, LS (averaging 25%) and LM (averaging 30%) increased dramatically in all four genotypes; however, the LB was relatively small , except in N22.

Although both the leaf osmotic potential and Na^+ content varied greatly among genotypes and NaCl treatments (Fig. S5, Table 2), the linear relationships between l_s and the leaf osmotic potential ($R^2 = 0.48$; $P = 0.033$) were found across genotypes and NaCl treatments, but not between l_s and the leaf Na^+ content (Fig. 7). Moreover, the negative correlations between the Na^+ content and g_s as well as transpiration rate (E) were found (Fig. S6). Unlike l_s, l_m linearly correlated with the leaf osmotic potential ($R^2 = 0.86$; $P < 0.001$) and leaf Na^+ content ($R^2 = 0.52$; $P = 0.026$).

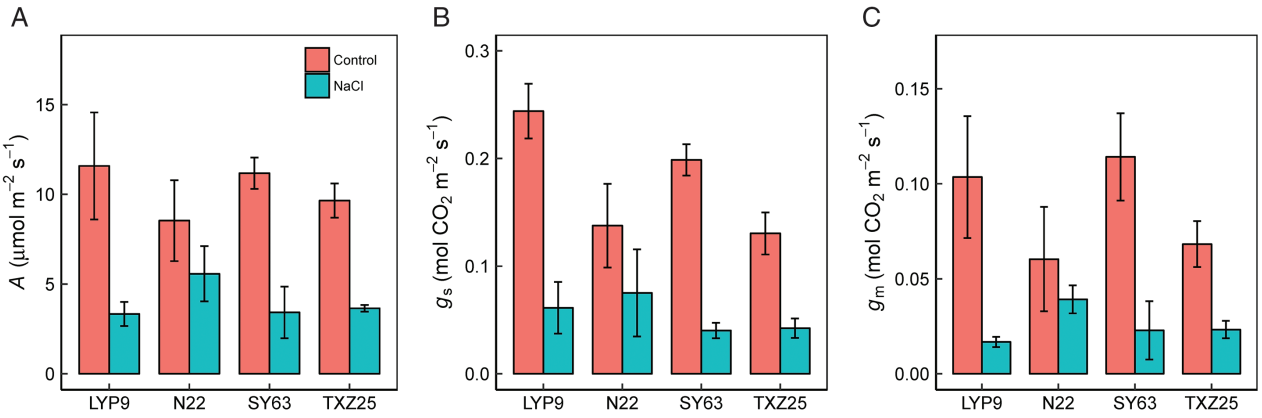

Fig. 2. Effects of 7-days salt treatment on (A) Light saturated photosynthetic rate (*A*), (B) stomatal conductance (g_s) and (C) mesophyll conductance (g_m) of four rice genotypes. Bars represent the mean ± SE of at least three replicates. ns, no significant; $*P < 0.05$; $**P < 0.01$ and $***P < 0.001$.

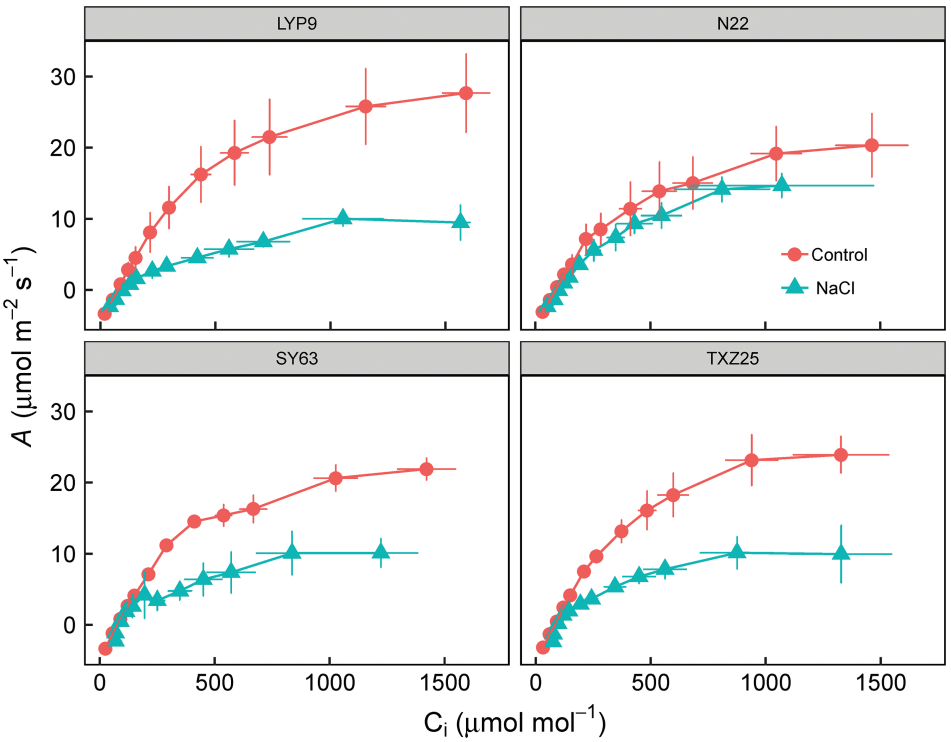

Fig. 3. Responses of light-saturated photosynthetic rate (*A*) to intercellular CO_2 concentration (C_i) in four rice genotypes. Each point represents the mean ± SE of at least three replicates.

Discussion

What determines the CO_2 assimilation rate of salt-stressed leaves in rice?

In the present study, we showed that the leaf physiological and biochemical traits of rice were dramatically affected by soil salinity (Tables 1, 2; Figs 1–3). After 7 days of NaCl treatment, *A* decreased significantly in LYP9, SY63 and TXZ25 but not in N22. Generally, it is assumed that stomatal closure is the first response to salinity due to osmotic stress (Delfine et al. 1998, Delfine et al. 1999, Centritto et al. 2003, Moradi and Ismail 2007, Chaves et al. 2011, Chen et al. 2015). However, we observed that g_s, g_m and ETR decreased dramatically in some of the genotypes one day after NaCl treatment, and multiple leaf parameters involving biochemical and physiological traits were affected in almost all of the genotypes after 7 days of treatment (Fig. 1).

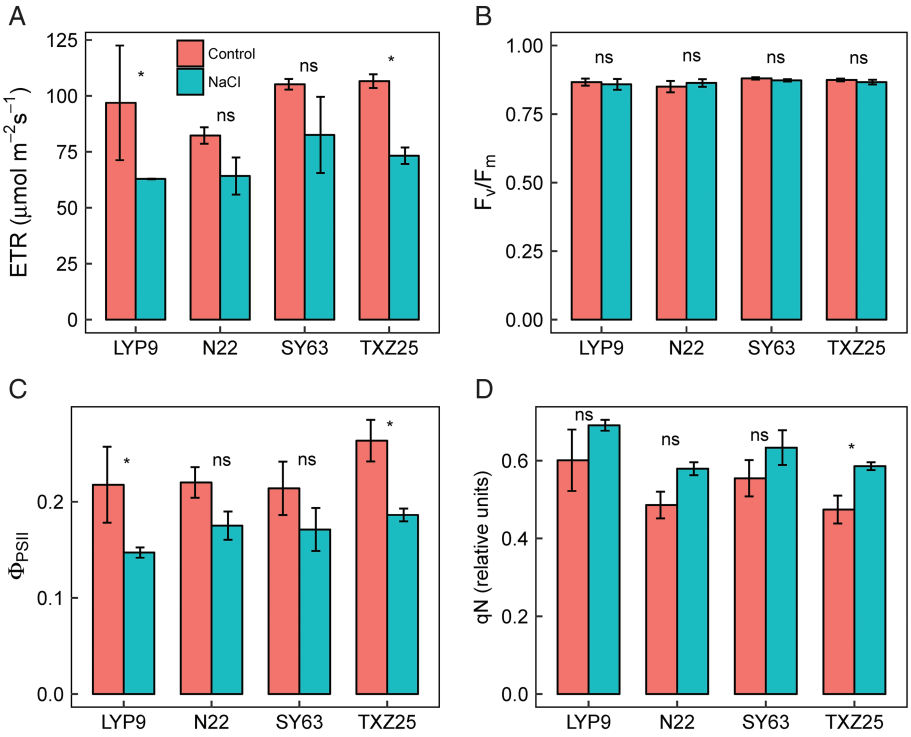

Fig. 4. Effects of 7-days salt treatment on (A) electron transport rate (ETR), (B) the maximum quantum efficiency (F_v/F_m), (C) actual quantum efficiency (Φ_{PSII}), and (D) non-photochemical quenching coefficient (qN) of four rice genotypes. Bars represent the mean ± SE of at least three replicates. ns, not significant; $*P < 0.05$; $**P < 0.01$ and $***P < 0.001$.

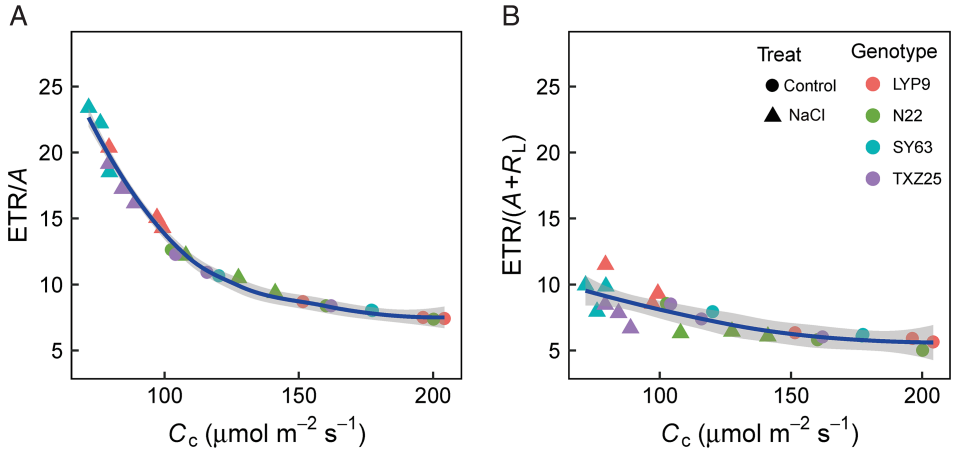

Fig. 5. Ratio of electron transport rate (ETR) to (A) light-saturated photosynthetic rate (A) and to (B) gross CO_2 assimilation accounting for day respiration ($A + R_L$) vs. CO_2 concentration in chloroplasts (C_c).

To quantify the stomatal, mesophyll and biochemical limitations on A in salt-stressed rice leaves, the limitation analysis approach was used here. The results highlighted that CO_2 diffusion conductance from the atmosphere to the sites of carboxylation (g_s and g_m) played a key role in limiting A under salt stress (Fig. 6; Fig. S3), whereas biochemical factors played an important role in limiting A in rice under normal conditions (Fig. S3C). In contrast to the previous studies of *C. sativus* (Chen et al. 2015) and *H. vulgare* (Perez-Lopez et al. 2012), LM contributed largely to reducing A in salt-stressed rice leaves. In fact, g_m was not affected by salinity in *H. vulgare*, and only a slight change was observed in *C. sativus*; however, g_m decreased more than 50% in all

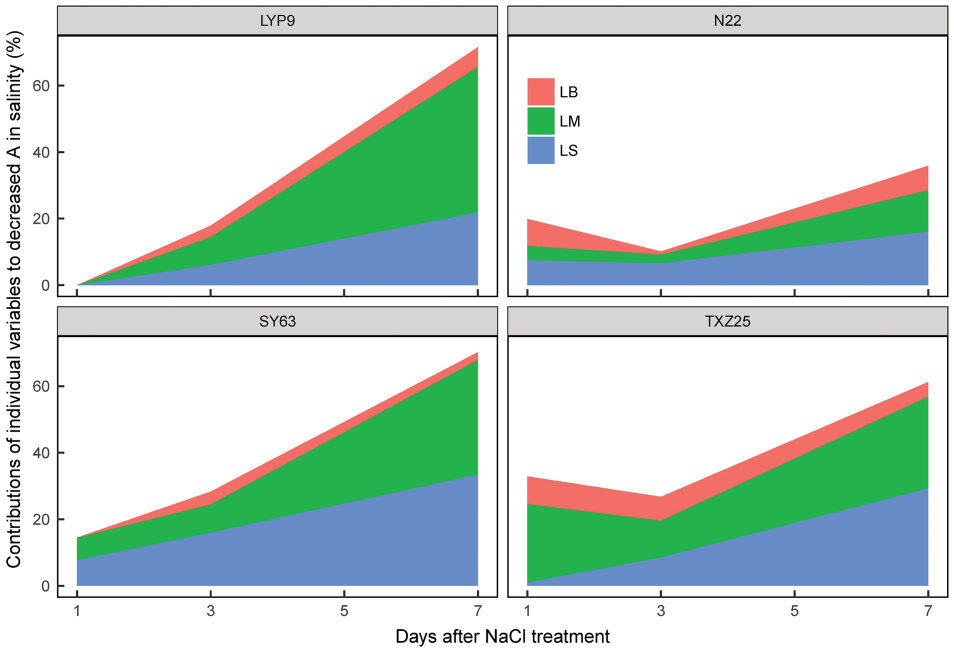

Fig. 6. Contributions of stomatal conductance limitation (LS), mesophyll conductance limitation (LM) and biochemical limitation (LB) to decreases in light-saturated photosynthetic rate (*A*) of four rice genotypes.

the estimated rice genotypes, except N22 in the current study (Fig. 2). Indeed, the decline in *A* that occurred in the salt-stressed rice leaves was closely correlated with the low g_s and g_m (Fig. S2). The contributions of LB to reducing *A* in salt-stressed rice leaves were relatively small (Fig. 6), in disagreement with two studies on *H. vulgare* (Perez-Lopez et al. 2012) and *C. sativus* (Chen et al. 2015) under salt stress. This might be explained by a lower C_c in the current study than in the other studies. In the current study, the C_c in the salt-stressed leaves was typically lower than 100 µmol mol^{-1}, except in N22 (Table 2); however, in the study of Perez-Lopez et al. (2012) the C_c was higher than 140 µmol mol^{-1}. In fact, when the C_c is relatively higher (i.e. under normal conditions) biochemical factors were the predominat photosynthetic limiting factors in rice (Fig. S4).

Our results indicate that the influences of salt stress on protein and Rubisco contents varied greatly between genotypes (Table 1). In fact, the degradation of Rubisco – the most abundant protein – in the process of forming chloroplast protrusions in salt-stressed rice has been observed in a previous study (Yamane et al. 2012). Moreover, the response of the Rubisco content to salt stress was observed to be dependent on salt treatment duration (Delfine et al. 1998). Therefore, the strong decrease in Rubisco content in TXZ25 and N22 might indicate a fast degradation in those genotypes. Although the Rubisco content decreased in N22 and TXZ25, the decline in V_{cmax} in salt-stressed leaves was relatively small, which supported the hypothesis that Rubisco also plays a role as a storage protein in C_3 plant and is a major source of nitrogen for remobilization (Sage et al. 1987, Masclaux-Daubresse et al. 2010). More importantly, CE was not affected by salt stress in rice, which also supports the idea that biochemical traits may not be the key factor causing decrease in *A* in salt-stressed leaves (Table 2). Overall, the results indicate that the reduction of *A* in salt-stressed rice leaves was mainly related to the low g_s and g_m under salt stress.

Salinity effects on CO_2 diffusion

The g_s was determined by both stomatal anatomy (i.e. size and density) and opening status under given ambient air conditions (Xiong et al. 2017). While we did not investigate stomatal anatomical traits, it is unlikely that the stomatal size and density of the fully expand leaves can change fast enough to explain the decline in g_s by salinity over the very short time of the present study. Many previous studies (Delfine et al. 1998, Delfine et al. 1999, Centritto et al. 2003, Moradi and Ismail 2007, Chaves et al. 2011, Chen et al. 2015) have reported that osmotic stress caused by salinity can decrease the leaf osmotic/water potential, and then provoke stomatal close. Moreover, ionic stress due to the high leaf-Na^+ content has been suggested as another factor provoking

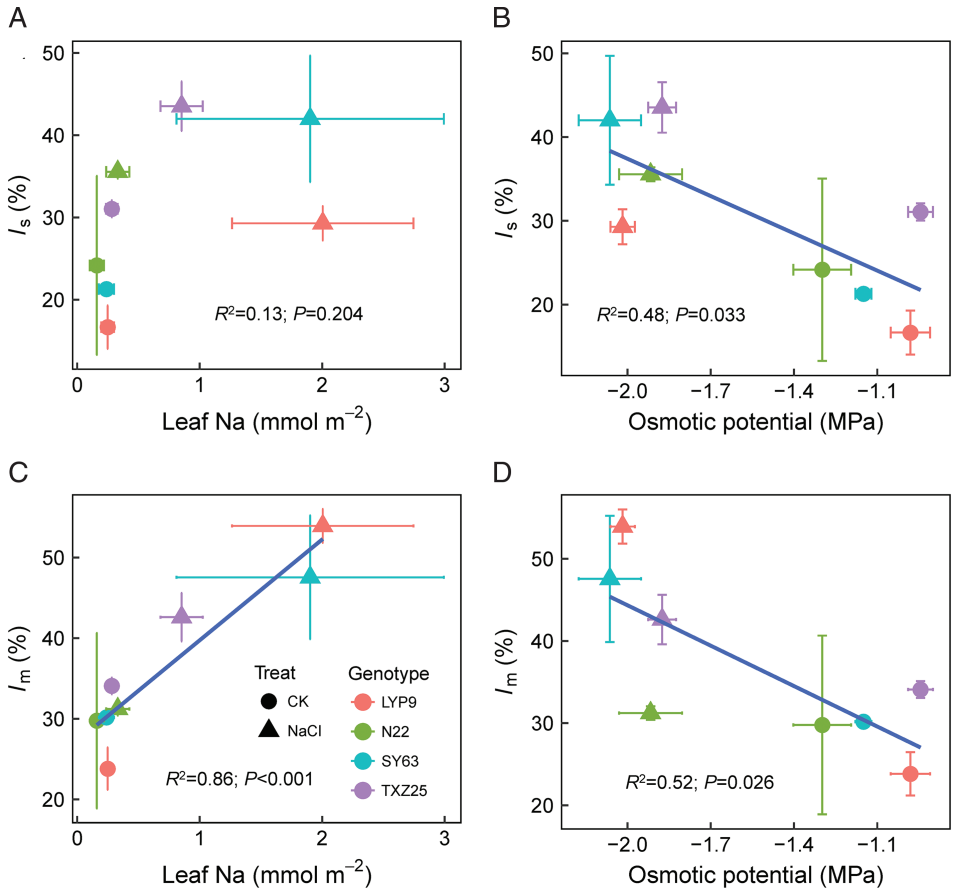

Fig. 7. Effects of leaf Na content and osmotic potential on stomatal limitation (l_s) (A, B) and mesophyll limitation in four rice genotypes. Each point represents the mean ± SE of at least three replicates.

stomatal closure in *Aster tripolium* (Perera et al. 1994). The regressive analysis showed that stomatal limitation correlated with leaf osmotic potential but not with leaf Na^+ content in rice (Fig. 7). The results suggest that salinity induced low g_s is mainly related to osmotic stress rather than ion stress in rice.

In general, g_m is related to leaf anatomical and biochemical traits under a given measurement condition (Evans et al. 2009, Tomas et al. 2013, Xiong et al. 2017). Previous studies have shown that long-term salinity significantly influences the leaf anatomical traits (Delfine et al. 1998, Wankhade et al. 2013); however, short-term salt-stress as in this study, causing leaf anatomical variation has rarely been estimated. Generally, the cell wall thickness (T_{cw}) and the chloroplast surface area facing the intercellular airspace per unit leaf area (S_c) are the two most important parameters related to g_m (Evans et al. 2009, Tomas et al. 2013, Xiong et al. 2017). The changes in T_{cw} are one of the potential reasons for the decline in g_m under salt stress. This is because osmotic stress usually introduced changes in the bulk elastic modulus, which relates to the alternation of biochemical composition and/or the thickness of the cell wall (Flexas and Diaz-Espejo 2014). The S_c is related to the mesophyll cell shape and the chloroplast shape as well as the light-dependent chloroplast arrangement (movement) inside the cells. Chloroplast movement is believed to alleviate photodamage to photosystems under stress conditions and rapid rearrangement of chloroplasts can profoundly impact S_c (Tholen et al. 2008, Xiong et al. 2015a). Moreover, previous studies have shown that mesophyll and chloroplast shape can be dramatically affected by short-term dehydration (Scoffoni et al. 2016, Scoffoni et al. 2017), which indicates that the low S_c caused by osmotic stress might be one of the reasons for the low g_m in salt-stressed leaves. Indeed, the linear correlation between l_m and leaf osmotic potential was observed in the present study (Fig. 7). The effects of biochemical traits on g_m in salinity have been suggested as being related to the functions of AQPs on membranes and carbonic anhydrase (CA) in cytosol and chloroplast

stroma (Pongsomboon et al. 2009, Gao et al. 2010, Hu et al. 2012).

Interestingly, the relationship ($R^2 = 0.86$; $P < 0.001$) between l_m and the leaf-Na^+ content across rice genotypes and NaCl treatments was closer than the relationship ($R^2 = 0.52$; $P = 0.026$) between l_m and the osmotic potential. Moreover, although the osmotic potential in the salt-stressed leaves of N22 decreased significantly, the leaf-Na^+ content did not increase (Fig. S5). Surprisingly, the g_m and l_m in the salt-stressed leaves of N22 did not change. These results suggest that the decreased g_m in the salt-stressed rice leaves would be more related to the accumulation of Na^+ (ion effect). However, the mechanisms of ion impacts on g_m are unclear. One possibility is that AQPs regulate the Na^+ absorption/distribution and CO_2 diffusion across membranes under salinity. Indeed, the positive effects of AQPs on g_m have been demonstrated by a large number of previous studies (Yang et al. 2000, Hanba et al. 2004, Flexas et al. 2006, Galmés et al. 2007, Uehlein et al. 2012, Mori et al. 2014). Recently, Gao et al. (2010) reported that overexpressing an AQP gene from wheat caused the transgenic Arabidopsis to have lower Na^+ levels than the wild type plants under salt stress. Otherwise, the Cl^- concentration which often correlates linearly with Na^+ concentration in salt-stressed leaves (Chen et al. 2015), might be another important ions that affects g_m (Tavakkoli et al. 2011). Further studies providing insights into the impacts of salinity on mesophyll anatomy, AQPs and CA, and thus g_m, are necessary.

Salinity effects on leaf photochemistry and photorespiration

When both g_s and g_m decreased significantly, the salinity could be expected to reduce C_c in the leaves. We observed a strong decrease in C_c in salt-stressed rice leaves except in N22. When A is limited by a low C_c, it has been suggested that an imbalance occurs between PSII photochemical activity and the electron requirement for photosynthesis; consequently, photoinhibition results (James et al. 2006, Zhang et al. 2010, Perez-Lopez et al. 2012, Munns et al. 2016). While qN increased slightly in salt-stressed leaves, F_v/F_m was unaffected (Fig. 4), which suggests that permanent photoinhibition was not the key factor in decreasing A, as already observed in previous studies. Although the reduction in ETR and Φ_{PSII} was observed in rice plant (Fig. 4), as reported in many species by previous studies (Moradi and Ismail 2007, Stepien and Johnson 2009), the decrease in A was far more serious than ETR and Φ_{PSII} (Fig. 1). Indeed, when C_c was lower than 100 μmol mol^{-1}, the ETR/A increased very fast with decreasing C_c in rice (Fig. 5A). The results indicate that alternative sinks (i.e. photorespiration and Mehler reaction) for electrons replaced photosynthesis. However, ETR/($A + R_L$) was almost constant with changing C_c in rice (Fig. 5B), although a slight increase was found at a very low C_c. Our results suggest that most of the thylakoid electron transport in rice leaves was used for the carboxylation plus oxygenation of Rubisco, and alternative sinks for electrons such as the Mehler reaction (photoinhibition), were very low under salinity conditions. Interestingly, similar conclusions were drawn by Flexas et al. (2002) in grapevines under field drought conditions. In fact, the high O_2/CO_2 ratio inside chloroplasts that were caused by low C_c led to higher photorespiration in salt-stressed leaves.

Conclusions

The present study clearly validated that the A in rice leaves is predominately limited by low C_c rather than biochemical factors (i.e. Rubisco activity) under salt stress. Low osmotic potential introduced by salinity caused a strong increase in stomatal limitation and mesophyll limitation, and the accumulation of ions enhanced the mesophyll limitation. The results of the present study suggest that photoinhibition does not seriously increase in salt-stressed leaves. Furthermore, N22 exhibited higher salt tolerance than the other three genotypes, because N22 can maintain a lower tissues Na^+ concentrations and higher osmotic potential than other genotypes under the same soil salt concentrations.

Author contributions

D. X. planned and designed the research; X. W. and W. W. performed the experiments; D. X. and X. W. analyzed the data; X. W., D. X., J. H. and S. P. wrote the manuscript.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Fig. S1. (A) Relationship between photochemical efficiency of photosystem II (Φ_{PSII}) and Φ_{CO2} [(An + Rd)/PPFD] in rice leaves and (B) The relationship between mesophyll diffusion conductance (g_m) measured with Harley's method and with Ethier's method.

Fig. S2. Changes of light-saturated photosynthetic rate (*A*), electron transport rate (ETR), stomatal conductance (g_s) and mesophyll conductance (g_m) to NaCl treatment time.

Fig. S3. Correlations of light-saturated photosynthetic rate (*A*) and (A) stomatal conductance (g_s) and (B) mesophyll conductance (g_m) across four rice genotypes.

Fig. S4. Effects of salinity on photosynthetic limitations of four rice genotypes.

Fig. S5. Impacts of salinity on (A) leaf osmotic potential and (B) leaf Na content in rice.

Fig. S6. Impacts of leaf Na content on (A) stomatal conductance to CO_2 (g_s) and (B) transpiration rate (*E*) across four rice genotypes.

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