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BRIEF COMMUNICATION

Reduced nitrogen allocation to expanding leaf blades suppresses ribulose-1,5-bisphosphate carboxylase/oxygenase synthesis and leads to photosynthetic acclimation to elevated CO_2 in rice

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Abstract

Net photosynthetic rate (P_N) measured at elevated CO_2 concentration (C_e), ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco), and nitrogen (N) content in rice leaves decreased significantly after exposure to long term C_e. The reduction in P_N, Rubisco, and leaf N at C_e was similar for the last fully expanded leaf blade (LFELB) and expanding leaf blade (ELB). Spatial leaf N content in the ELB was highest in the zone of cell division, sharply declined as cell expansion progressed and gradually increased with cell maturation. Maximum reduction in spatial leaf N and Rubisco content was found at C_e only within cell expansion and maturation zones. The spatial leaf N content correlated well with the amount of Rubisco synthesized during leaf expansion, suggesting that N deposition into the expanding leaf blade may be the key for Rubisco synthesis and possibly photosynthetic acclimation to C_e.

Additional key words: elevated CO_2; photosynthesis; ribulose-1,5-bisphosphate carboxylase/oxygenase; rice; spatial nitrogen deposition.

Net photosynthetic rate (P_N) acclimation to elevated CO_2 concentration (C_e) is characterized by lower leaf CO_2 assimilation rates associated with a reduction in the concentrations of Rubisco and leaf N (Nakano *et al.* 1997, Stitt and Krapp 1999). The underlying mechanism for the reduction in leaf Rubisco content by C_e is still not resolved. Rubisco content is the rate-limiting factor for P_N at the current atmospheric CO_2 concentration (C_a) (Farquhar *et al*. 1980) and accounts for 15–35 % of total leaf N in C_3 plants (Evans 1989, Makino and Osmond 1991). The concentration of Rubisco in leaf blades is determined by the balance between protein synthesis and degradation. Rubisco concentration rapidly increases during leaf expansion, reaching a maximum when the leaf is fully expanded (Imai *et al.* 2008, Suzuki *et al.* 2001). Suppression of Rubisco synthesis at C_e is suggested to be mediated through the sugar sensing mechanism (Moore *et al.* 1999, Pandurangam *et al.* 2006). However, a negative relationship between accumulation of soluble sugar and Rubisco synthesis is not always reported (Ludewig *et al.* 1998, Nakano *et al.* 2000). We tested the hypothesis that N deposition in the growing leaf blade during leaf expansion is linked to Rubisco synthesis and then P_N acclimation to C_e. Rice is a good model system to test this hypothesis because leaf growth, protein and pigment synthesis, and nutrient deposition is unidirectional (Gastal and Nelson 1994, Schäufele and Schnyder 2001, Seneweera and Conroy 2005).

Rice (*Oryza sativa* L.) plants were grown hydroponically in environmentally controlled growth chambers at 1,000 μmol photons m^{-2} s^{-1} (in the 400–700 nm range), day/night cycle of 12/12h and day/night temperature of 25/20°C respectively. Vapour pressure deficit (VPD) was maintained at 1.5–1.9 kPa throughout the experiment. The seedlings were transferred to pots containing a nutrient solution as described by (Mae and Ohira 1981). N was supplied at a concentration of 2.0 mM (1.0 mM NH_4NO_3). Plants were grown for 70 days at CO_2 concentrations of

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Abbreviations: C_a – ambient CO_2 concentration; C_e – elevated CO_2 concentration; DAP – days after planting; DM – dry mass; ELB – expanding leaf blade; FM – fresh mass; LER – leaf blade elongation rates; LFELB – last fully expanded leaf blade; N – nitrogen; P_N – net photosynthetic rate; PPFD – photosynthetic photon flux density; Rubisco – ribulose 1,5-bisphosphate carboxylase/oxygenase.
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either 390 (C_a) or 1,000 μmol(CO_2) mol^{-1} (C_e). At this stage, $leaf_{13}$ was at the expanding stage (ELB) while $leaf_{12}$ was fully expanded (LFELB). Leaf gas-exchange measurements were carried out using a portable photosynthesis system (*LI-6400, LI-COR*, Lincoln, USA) as described by Seneweera *et al.* (2002). Leaf chamber CO_2 concentration was maintained by the *LI-COR*-CO_2 injection system, and an irradiance of 1,700 μmol(quantum) m^{-2} s^{-1} was supplied by the in-built LED lamp (red/blue). The temperature in the 6 cm^3 leaf chamber was set at 25°C and the leaf temperature measured by a thermocouple, ranged from 24.5 to 25.5°C. The VPD at the leaf surface was between 1.9 and 2.1 kPa. Photosynthetic gas exchange measurements were made between 10:00 and 15:00 h. Each leaf blade was allowed 10 to 15 min to reach steady-state photosynthesis before commencement of P_N/C_i response curves. Initial linear slopes of P_N *vs*. intercellular CO_2 concentration (C_i) were used to estimate maximum carboxylation efficiency of Rubisco (V_{cmax}) according to Farquhar *et al.* (1980). Maximum electron transfer capacity (J_{max}) at 1,700 μmol(quantum) m^{-2} s^{-1} was calculated (von Caemmerer and Farquhar 1981).

Immediately after gas exchange measurements, leaf blades (LFELB and ELB) were sampled for biochemical analysis; Rubisco and leaf N contents were analysed as described by Seneweera *et al.* (2002). ELB was separately sampled for spatial leaf N and Rubisco measurements. This sampling was conducted when the ELB was less than two third of the final length of the previous mature leaf blade (LFELB) (Seneweera and Conroy 2005). After careful dissection of the ELB from the main plant, 17 sequential segments were cut from the ligule to the tip of the leaf blade starting from the leaf base followed by 8 sequential segments of 1 cm (0–8 cm), 5 segments of 2 cm (8–18 cm) and 4 segments of 3 cm (18–30 cm). Immediately after the sampling, tissues were transferred to liquid N_2 and then placed in a −80°C freezer.

The subsamples were freeze-dried and ground with a mortar and pestle for N analysis using a Carbon and Nitrogen Analyser (*NA 2500, Fisons Instrument*, Milano, Italy). For Rubisco determination, about 20 segments from each leaf blade were ground in liquid N_2 and extracted in a buffer containing NaH_2PO_4 (50 mM, pH 7.2), 5% glycerol, mono iodoacetic-sodium salt (2 mM), and 2-mercaptoethanol (0.8% v/v). A portion of the extract was used for measurement of Rubisco content. After centrifugation, an aliquot containing 2 μg of soluble protein was loaded onto a polyacrylamide (12.5%, w/v) gel containing SDS (0.1%). After separating the protein by electrophoresis, the gel was stained with Coommassie Brilliant Blue R250, and large and small subunits were identified from SDS-PAGE. The protein was extracted with formamide, and the concentration of Rubisco in the supernatant was measured spectrophotometrically (Makino *et al*. 1986).

The experiment was a completely randomized design. Analysis of Variance (*ANOVA*) was carried out for P_N, Rubisco, V_{cmax}, and J_{max}. The least significant differences (LSD) and standard errors (SE) were calculated where appropriate to compare the statistical differences between the treatments using *SAS 9.1* (*SAS User Guide, Institute Inc*., Cary, NC, USA). Spatial N and Rubisco protein concentrations were plotted at single replicate level because large numbers of leaf segments (more than 20 leaf segments) were pooled for each analysis. Rubisco and leaf N concentrations were plotted against each other.

When P_N was measured at respective growth CO_2 concentration, P_N was significantly higher at C_e in both LFELB and ELB. However, when P_N was measured at the same CO_2 concentration (either C_a or C_e concentrations), P_N significantly decreased in plants grown at C_e (Table 1). Suppression of V_{cmax} and J_{max} were also observed at C_e but suppression of J_{max} was relatively smaller (Table 1). Consequently, J_{max}/V_{cmax} ratio was higher at C_e suggesting that electron transport was not

Table 1. Influence of elevated CO_2 concentration (C_e) on Rubisco, total leaf N, P_N, V_{cmax} and J_{max} (on whole leaf basis) of rice. The parameters V_{cmax} and J_{max} were estimated from individual CO_2 response curves according to model of Farquhar *et al*. (1980). Values are the mean of four replicates. Significant differences from two-way *ANOVA* for CO_2 and leaf position are shown and they are: ***$P \leq 0.001$; **$P \leq 0.01$; *$P \leq 0.05$, and ns – not significant. Plants were grown at either ambient CO_2 concentration (C_a) or C_e for 70 days. Expanding and fully expanded (ELB – 13^{th} – leaf and LFELB – 12^{th} – leaf, respectively) were sampled for biochemical analysis. Values are the means of four replicates with LSD.

Leaf blade	CO_2 concentration [μmol(CO_2) mol^{-1}]	Rubisco [g m^{-2}]	Leaf N [mmol m^{-2}]	P_N [μmol m^{-2} s^{-1}]	V_{cmax} [μmol(CO_2) m^{-2} s^{-1}]	J_{max} [μmol(electron) m^{-2} s^{-1}]
ELB	390	2.77 ± 0.16	147 ± 11	32.6 ± 1.1	104 ± 5	161 ± 20
	1,000	2.25 ± 0.27	139 ± 6	27.8 ± 1.7	68 ± 5	125 ± 18
LFELB						
	390	2.67 ± 0.13	138 ± 10	35.2 ± 1.2	107 ± 4	153 ± 09
	1,000	2.04 ± 0.3	119 ± 03	30.6 ± 2.34	73 ± 6	138 ± 11
Main effects		Significance				
CO_2		***	***	***	***	***
Leaf position		ns	ns	ns	ns	ns

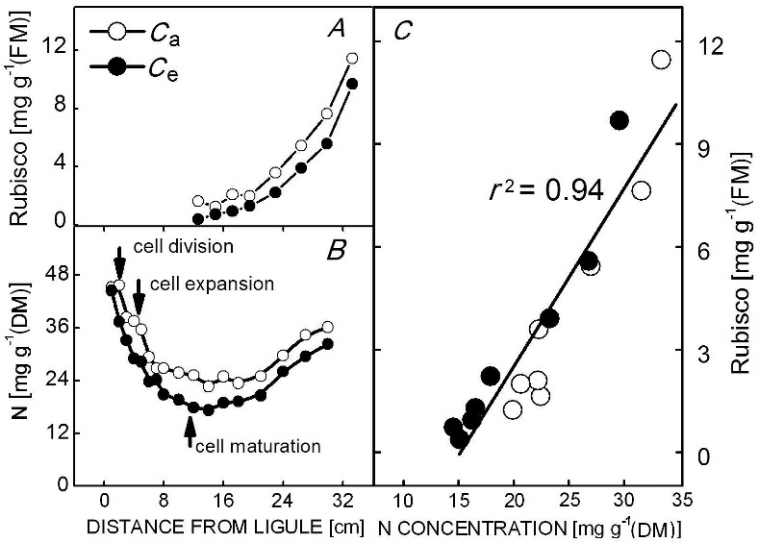

Fig. 1. The effect of elevated CO_2 concentration (C_e) on leaf blade of rice. *A*: spatial Rubisco protein content, *B*: spatial leaf N concentrations on segmental length basis, along the expanding leaf blade from the ligule to the tip. Plants were continuously grown at either ambient CO_2 concentration (C_a) or C_e. *C*: Right panel of the figure shows the relationship between spatial leaf N and Rubisco concentration along the expanding leaf blade. Each data point represents more than twenty samples and samples were pooled for analysis.

limited for P_N at C_e. Similar reductions in Rubisco and leaf N content at C_e were observed in this experiment (Table 1). It has been previously reported that a non-specific reduction in leaf N and Rubisco content at C_e is closely linked to photosynthetic acclimation (Seneweera *et al.* 2002, Nakano *et al* 1997). On the other hand, accumulation of excess soluble carbohydrates at C_e leading to the repression of *rbc*S and *rbc*L genes and thereby suppression of Rubisco synthesis have also been reported (Moore *et al.* 1999). However, the mechanism of photosynthetic acclimation to C_e is still not resolved, as a negative relationship between P_N and soluble sugars content has not always been observed (McCormick *et al.* 2006, Nakano *et al.* 2000).

This is the first report that shows N deposition and Rubisco synthesis on a spatial scale in the expanding leaf blade at C_e (Fig. 1*A*). ELB was chosen because the zones of cell division, cell expansion, and cell maturation can be easily distinguished by variation in spatial N concentration (Gastal and Nelson 1994, Seneweera and Conroy 2005). Cell division and cell expansion zones are marked by the base of the leaf blade to the ligule of the previous leaf blade (high N to the lowest N concentration). The N concentration was highest close to the base of the leaf blade, where cell division is very active [45 mg N g^{-1}(DM), dry mass], and decreased to 15 mg N g^{-1}(DM) at the distal limit where cell maturation has already commenced (Fig. 1*B*). In the cell division zone, N requirement is very high because rapid protein synthesis is required for the production of new cells (Gastal and Nelson 1994). However, no difference in the spatial N content between C_a and C_e treatments was detected during cell division (Fig. 1*B*). For cell expansion and maturation, N is largely needed for the synthesis of photosynthetic proteins, a large proportion of Rubisco and thylakoid proteins (Gastal and Nelson 1994, Skinner and Nelson 1994). Spatial N concentration in cell expansion and maturation zones of the leaf blade was significantly decreased at C_e (Fig 1*B*). The reduction of leaf N concentration during the cell maturation process is not due to an excess in carbohydrate accumulation because these expanding leaf blades are still dependent on source supply as the photosynthetic machinery is not yet fully developed (Gastal and Nelson 1994, Paul and Foyer 2001, Seneweera *et al.* 1995, Seneweera and Conroy 2005). Spatial synthesis of Rubisco in ELB was quantitatively analyzed from ligule to tip of the leaf blade (Fig. 1). Minimal amounts of Rubisco were detected near the base of the leaf blade where cell division and cell expansion are active. Therefore, we measured Rubisco content only in the cell maturation zone of the leaf blade (Fig. 1*A*). Rapid Rubisco synthesis began after cells stopped expansion, and was mostly completed when the leaf blade emerged above the whorl (Gastal and Nelson 1994). Regardless of growth CO_2 concentration, spatial leaf N and Rubisco content in ELB were well correlated (Fig. 1*C*). The magnitude of the reduction in leaf N, Rubisco and P_N at C_e was similar for LEB and LFELB. Further, a strong correlation between spatial Rubisco and leaf N content in ELB suggests that N deposition during leaf expansion plays a key role in Rubisco synthesis and possibly photosynthetic acclimation to C_e.

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