Photosynthetic characteristics of C_4 trait in *chlorina* mutant of rice (*Oryza sativa* L.)

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Abstract

Biao 810S is a *chlorina* mutant of the thermosensitive genic male sterile (TGMS) rice. We compared photosynthetic characteristics of these two lines. The contents of chlorophylls and carotenoids in Biao 810S were approximately half of those in 810S. However, the net photosynthetic rate (P_N) of Biao 810S was higher than that of 810S under high irradiance or low concentration of carbon dioxide, and the photon quantum efficiency was higher than that of 810S. The activity of ribulose-1,5-bisphosphate carboxylase/oxygenase in Biao 810S was only 69.80 % of that in 810S, but the activities of phospho*enol*pyruvate carboxylase and NADP-malic enzyme were 79.50 and 69.06 % higher than those of 810S, respectively, suggesting that the efficiency of photon energy utilization in Biao 810S was enhanced by reduction of thermal dissipation and increase of electron transfer rate to generate sufficient assimilation power for the dark reactions. Consequently, the increased activities of C_4 photosynthetic enzymes lead to more effective fixation of CO_2 and the synergistic effect of light and dark reactions contributed to the higher P_N of Biao 810S.

Additional key words: C_3 and C_4 plants; NADP-malic enzyme; net photosynthetic rate; phospho*enol*pyruvate carboxylase; quantum efficiency; ribulose-1,5-bisphosphate carboxylase/oxygenase.

Introduction

In plants, leaf mutations are frequently mutants of characters (Green *et al*. 1998, Jarvis and Chen 1998) and they are ideal materials for studying photosynthesis, photomorphogenesis, physiological hormones, and disease-resistant mechanisms (Agrawal *et al*. 2001, Fambrini *et al*. 2004). The *chlorina* mutants mostly have low contents of photosynthetic pigments and exhibit low photosynthetic efficiency and competition ability, particularly in etiolation and albino mutants, which cannot grow normally (Chen *et al*. 1981, Dai *et al*. 2000). Therefore, these mutants are rarely utilized for plant production.

C_4 plants release CO_2 at higher rate in the vicinity of ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBPCO) and thereby increase the ratio of RuBP carboxylation/oxygenation substantially (Leegood 1997, 2002). This strategy prevents major losses of CO_2 by photorespiration and is accompanied by an increase in the water and nitrogen use efficiency compared to C_3 plants (Sage and Pearcy 1987). Phospho*enol*pyruvate carboxylase (PEPC) is the key enzyme of C_4 pathway and plays an important fixation role in C_4 and CAM plants in the photosynthetic carbon assimilation (O'Leary 1982, Chollet *et al*. 1996). In C_3 plants, PEPC is active in several anaplerotic reactions to replenish the intermediates of the Krebs cycle, supply carbon skeletons for amino acid biosynthesis following nitrate assimilation, and for the regulation of cytoplasmic pH (Latzko and Kelly 1983, Andrews 1986, Melzer and O'Leary 1987). Moreover, PEPC also plays an important role in stomatal movement (Rainer *et al*. 2002).

Biao 810S is a natural *chlorina* mutant selected from the thermosensitive genic male sterile (TGMS) rice line 810S and its leaves are perennially yellow-green. Previous studies showed the *chlorina* mutant was governed by one recessive gene. Therefore this mutant is an elite genetic marker for testing seed purity caused by self-pollination of TGMS rice line in hybrid seed production. TGMS rice line can fertilize at low temperature and sterilize at high temperature. Biao 810S grows normally, its plant type is almost the same as that of 810S, and it exhibits high photosynthetic rate under higher tempera-

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ture and irradiance, and low CO_2 concentration. We studied photosynthetic characteristics of Biao 810S to elucidate the physiological mechanism of higher net photosynthetic rate (P_N) and promote the utilization of this marker in breeding.

Materials and methods

Plants: Biao 810S, a *chlorina* mutant of TGMS rice, was selected from TGMS rice 810S by the Huaihua Vocational and Technical College of Hunan. Wild type TGMS rice 810S was taken as control. Each material was planted in 3 plots, at 500 plants per plot, and the plants were grown using the normal amounts of fertilizer and water.

For pigment analysis, leaf samples were extracted in 80 % acetone and measured by spectrophotometric method (*Ruili UV-2100*, Beijing, China) at the wavelengths 663, 645, and 470 nm. Contents of chlorophyll (Chl) *a* and *b*, carotenoids and carotene (Car) were calculated using the equations of Arnon (1949).

Gas exchange (Yong *et al*. 2007) was determined in plants with 10−12 fully expanded flag leaves in each ring during 09:00−11:00 (Beijing time) in mid-August (heading stage). For determining the response to irradiance, P_N was measured at photosynthetic photon flux density (PPFD) of 2 000, 1 800, 1 600, 1 400, 1 200, 1 000, 800, 600, 400, 200, 100, 50, 25, and 0 μmol m^{-2} s^{-1} in turn from a *Li-Cor* LED irradiation source. CO_2 concentration was kept at 400 μmol mol^{-1} with *Li-Cor* CO_2 injection system. Leaf temperature was 30 ºC. To determine the dependence of photon-saturated P_N to intercellular CO_2 concentrations (C_i) of 400, 300, 200, 100, 50, 400, 400, 500, 600, 700, and 800 μmol mol^{-1}, PPFD was kept at 1 500 μmol m^{-2} s^{-1} from a *Li-Cor* LED irradiation source and the leaf temperature was 30 ºC.

Carboxylation efficiency was calculated according to the method of Watling *et al*. (2000). After these measurements, the leaf tissue was frozen rapidly between pieces of metal cooled to liquid N_2 temperature. The frozen leaf tissue was stored at 80 °C for determination of enzyme activation state.

Chl fluorescence kinetics (Li 2007): The minimal initial fluorescence (F_0) and maximal fluorescence (F_m) were determined after dark adaptation for 20 min. Then minimal fluorescence (F_0') and maximal fluorescence under light (F_m') were measured after a 1-h irradiation. Other fluorescence parameters were calculated according to Genty *et al*. (1989): $q_P = (F_m' - F_s)/(F_m' - F_0')$; $q_N = (F_m - F_m')/(F_m - F_0)$; $\Phi_{PS2} = (F_m' - F_s)/F_m'$. All these parameters were measured using *Li 6400*.

Enzyme assays: About 0.5 g of leaf tissue was conserved after measuring gas exchange and quickly ground in 3 cm^3 of extraction buffer containing 50 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, 5 mM dithiothreitol (DTT), 2 % (m/v) insoluble polyvinylpolypyrrolidone, 1 mM EDTA-Na_2, and 10 % glycerol. After total maceration, the crude extract was centrifuged at 13 000×*g* for 15 min at 4 ºC, and the supernatant was used immediately for enzyme assay.

RuBPCO activity was assayed in a buffer containing 100 mM Tris-HCl, pH 7.8, 10 mM $NaHCO_3$, 20 mM $MgCl_2$, 10 mM DTT, 0.75 mM reduced nicotinamide adenine dinucleotide (NADH), 5 mM ATP, 10 mM phosphocreatine, 60 units per cm^3 of 3-phosphoglycerate kinase, 300 units per cm^3 of triose-phosphate isomerase, 30 units per cm^3 of creatine phosphokinase, and 30 units per cm^3 of glyceraldehyde-3-phosphate dehydrogenase (Larson *et al*. 1997). Assays were initiated by adding 50 mm^3 of 40 mM RuBP. Absorbance of NADH at the wavelength of 340 nm was measured immediately.

PEPC activity was assayed in a buffer containing 50 mM Hepes-NaOH (pH 8.0), 10 mM $NaHCO_3$, 5 mM $MgCl_2$, 1.5 units of malate dehydrogenase (MDH), 0.2 mM NADH, and 20−50 mm^3 of enzyme extract (Gonzalez *et al*. 1984, Patrizia and Graziano 2000). Assays were initiated by the addition of 2 mM phospho-*enol*pyruvate (PEP). The change in NADH was monitored by a spectrophotometer at 340 nm.

NADP-malic enzyme (NADP-ME) activity was assayed in a buffer containing 150 mM Tris-HCl (pH 8.0), 12 mM $MgCl_2$, 1.5 mM EDTA, 150 mM DTT, 12 mM $NADP^+$, and 20−50 mm^3 of enzyme extract (Johnson and Hatch 1970). Assays were initiated by adding 150 mM L-malic acid to a final concentration. The change in $NADP^+$ was monitored by a spectrophotometer at 340 nm.

Chloroplast structure was observed using the method of Hong *et al*. (2005). Leaves were sampled from individual plants of accordant growth and immediately prefixed in 2.5 % (v/v) glutaraldehyde buffered with 0.1 M phosphate buffer (pH 7.2) and post-fixed in 1 % (m/v) osmium tetroxide buffered with 0.1 M phosphate buffer (pH 7.2). All specimens were dehydrated through a graded series of alcohol and embedded in Spurr's resin. Ultrathin sections were cut using a *Leica* ultramicrotome, double stained with uranyl acetate and lead citrate, and subsequently observed using a *JEM-1230* transmission microscope and photographed.

Statistical analysis: *Excel* and *Sigma Plot for Windows 9.0* were used for correlation analyses.

Results

Photosynthetic pigments: The contents of Chl *a* and *b* and Car in Biao 810S were lower than those of 810S, the total photosynthetic pigment content being approximately 50 % of that of 810S. No significant difference was recorded for the ratios Chl/Car (Table 1).

Table 1. Comparison of photosynthetic pigment contents, chlorophyll (Chl) *a* and *b* and carotenoids (Car) [g kg^{-1}(DM)] and fluorescence characteristics in Biao 810S.

Plants	Chl *a*	Chl *b*	Car	Car/all	F_v'/F_m'	q_P	q_N	Φ_{PS2}	ETR
Biao810S	1.27±0.09B	0.53±0.05B	0.43±0.08B	0.194A	0.627±0.010A	0.743±0.010A	0.462±0.010A	0.466±0.006A	293.58±3.78A
810S	2.46±0.17A	1.01±0.07A	0.83±0.11A	0.186A	0.491±0.010B	0.680±0.010B	0.712±0.007B	0.343±0.007B	216.09±4.41B
±%					+27.70	+9.26	−35.11	+35.86	+35.86

Irradiance response curve: The saturation irradiance of P_N of 810S was 1 570 μmol m^{-2} s^{-1} when P_N reached 22.13 μmol(CO_2) m^{-2} s^{-1} (Fig. 1*A*). However, no saturation irradiance of Biao 810S was observed at 2 000 μmol m^{-2} s^{-1}, when P_N was 27.00 μmol(CO_2) m^{-2} s^{-1}, *i.e.* significantly higher than that of 810S. Thus Biao 810S exhibited stronger response to irradiance and higher photon energy utilization ability.

CO_2 response curve: P_N increased as the CO_2 concentration increased from 0 to 400 μmol mol^{-1} under 30 °C and irradiance of 1 500 μmol m^{-2} s^{-1}(Fig. 1*B*). Under low CO_2 concentration of 200 μmol mol^{-1}, P_N of Biao 810S was 19.37 μmol(CO_2) m^{-2} s^{-1}, *i.e.* 74.03 % higher than that of 810S [11.13 μmol(CO_2) m^{-2} s^{-1}]. P_N of the Biao 810S under 200 μmol(CO_2) mol^{-1} was 92.81 % of the 810S at the 400 μmol(CO_2) mol^{-1} (Fig. 2*A*).

The carboxylation efficiency (CE) of Biao 810S was 0.204, notably higher than that of 810S (0.124). Hence Biao 810S possesses stronger CO_2 assimilation ability at low CO_2 concentration.

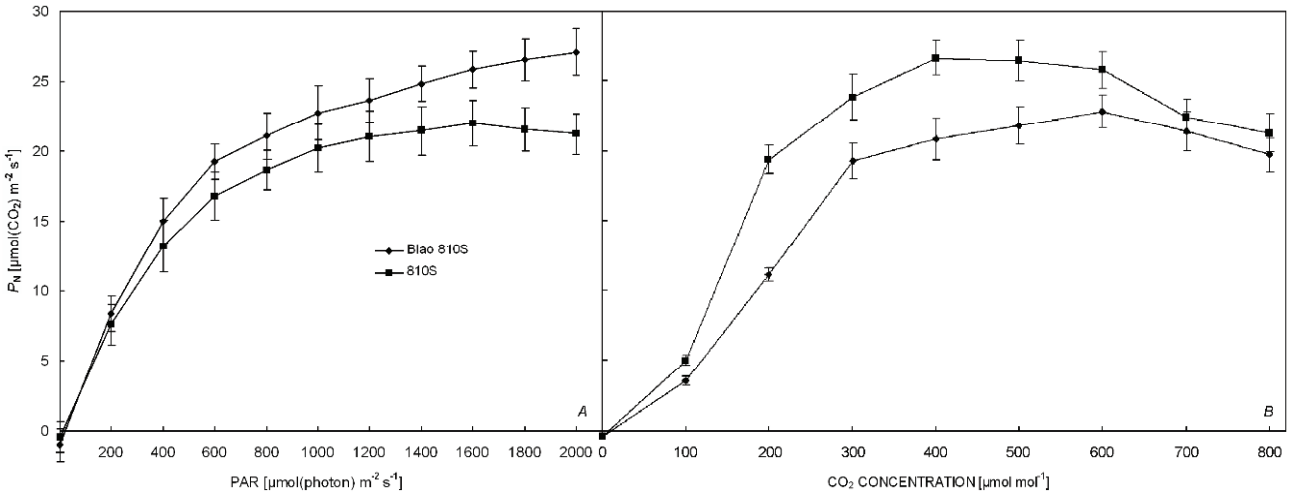

Fig.1. Dependence of net photosynthetic rate (P_N) of 810S and Biao 810S on (*A*) irradiance or (*B*) CO_2 concentration.

Chl fluorescence: The F_v'/F_m', Φ_{PS2}, q_P, and electron transport rate (ETR) of Biao 810S were all notably higher than those of 810S (Table 1), indicating that the mutant possessed higher efficiency of capturing primary photon energy and energy transformation of PS2 reaction centre, exhibited quicker non-cyclic electron flow and lower non-photochemical quenching (q_N). Less photon energy captured by PS2 antenna pigment was dissipated with the thermal form. Thus the photon utilization efficiency of the Biao 810S was significantly higher than that of the wild type, resulting in consistently higher P_N of the *chlorina* mutant Biao 810S.

Activities of key photosynthetic enzymes: The activity of RuBPCO was notably lower than that of 810S, while the activities of PEPC and NADP-ME in Biao 810S were 79.50 and 69.06 % higher than those of 810S, respectively. The increase of the activities of PEPC and NADP-ME in Biao 810S could compensate for the decrease of activity of RuBPC, which resulted in the increase of P_N.

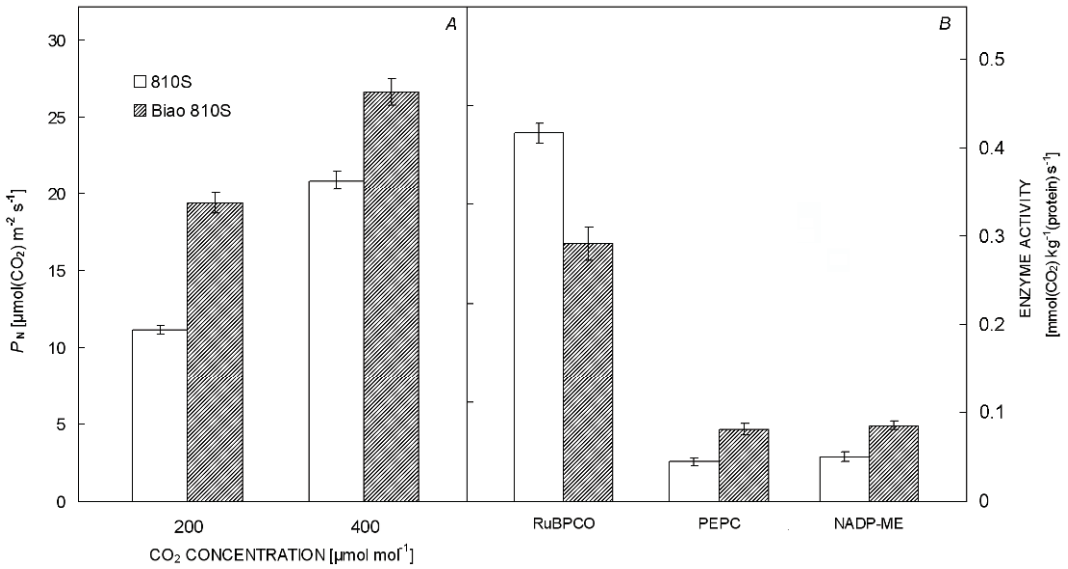

Fig. 2. Differences between 810S and Biao 810S in (*A*) dependence of net photosynthetic rate (P_N) on CO_2 concentration and (*B*) enzyme activities (RuBPCO − ribulose-1,5-bisphosphate carboxylase, PEPC – phospho*enol*pyruvate carboxylase, NADP-ME – NADP-malic enzyme).

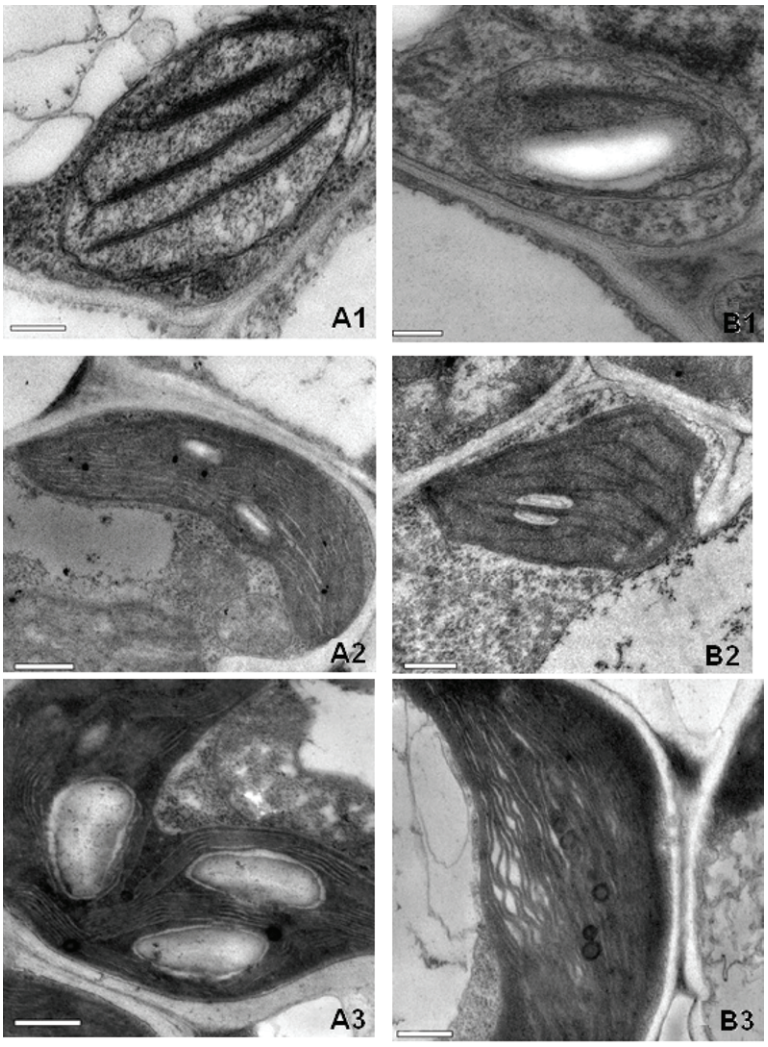

Fig. 3. Chloroplast ultrastructure of (*A*) 810S and (*B*) Biao 810S at different stages (1 − cotyledon, 2 – half-leaf development, 3 – full leaf development). *Scale bars*: A1, B1 − 0.2 μm; A2, A3, B2, B3 − 0.5 μm.

Chloroplast ultrastructure: The chloroplast development was delayed and the number of grana lamellae was lower in Biao 810S. Their structure was relatively looser compared with 810S. The number and volume of starch granules in the chloroplasts were also diminished (Fig. 3).

Discussion

Chl fluorescence analysis is a rapid probe measuring photosynthetic function of leaves (Genty *et al*. 1989, Schreiber *et al*. 1994), used for prediction of yield, breeding for stress tolerance, *etc*. (Baker and Rosenqvist 2004). F_v'/F_m' and Φ_{PS2} of the *chlorina* mutant Biao 810S were significantly higher than those of 810S indicating that the PS2 reaction centre of Biao 810S can more effectively capture photon energy. The q_P of Biao 810S was slightly higher than that of 810S, while the q_N was significantly lower, which resulted in higher P_N of Biao 810S.

The *chlorina* mutant could reduce thermal dissipation and increase ETR to partially reduce deficiency of photon energy (Xu *et al*. 2004, Zhou *et al*. 2006). High ETR under natural irradiance was propitious to RuBP regeneration, and might enhance the activities of photosynthetic enzymes (Harbinson *et al*. 1989). A large reduction in Chl content might not result in a corresponding reduction in photosynthesis and carbon assimilation in a couple of yellow-green maize mutants (Jenkins *et al*. 1989). In rice leaf mutants no relation may exist between Chl content and P_N (Lin *et al*. 2003, Zhou *et al*. 2006), which is consistent with our results. The *chlorina* mutant was limited in capturing photon energy by low Chl content (Table 1) and lesser chloroplast grana lamellae (Fig. 3).

RuBPCO activity is in positive correlation with CE (Farquhar and Sharkey 1982, Collatz 1997). We found that the *chlorina* mutant had high CE, low RuBPCO activity (about 72 % of 810S), but 1.8 times higher activity of PEPC than 810S (Fig. 2*B*) implying that the C_4 photosynthetic enzyme might have play an important role in its photosynthesis.

At low CO_2 concentration, CO_2 was a limiting factor of photosynthesis (Li 2002). The *chlorina* mutant had a similar mechanism of concentrating CO_2 as C_4 plants. P_N of this mutant increased with the increase in irradiance (Fig. 1*A*). C_4-like cycle in C_3 plants and two sets of photosynthetic gene (Matsuoka *et al*. 2001) are responsible for high P_N (74 % higher than that of 810S). Hence the *chlorina* mutant effectively utilizes CO_2.

We verified a C_4-like cycle in the mutant which might compensate the effects of mutation. The amount of large subunit of RuBPCO was reduced and its function was replaced by PEPC.

In summary, Biao 810S with Chl deficiency showed higher P_N. This is due to multivariate factors, enhanced efficiency of radiation utilization by reducing thermal dissipation and increasing ETR to offer sufficient assimilation power to dark reaction. Higher C_4 photosynthetic enzyme activity could more effectively fix CO_2, the synergistic effect of light and dark reactions contributed to higher P_N of Biao 810S. Biao 810S, a C_3 plant, exhibited high activity of PEPC. The mutant promoted development of high photosynthetic efficiency breeding to increase grain yield.

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