

Over- and anti-sense expressions of the large isoform of ribulose-1,5-bisphosphate carboxylase/oxygenase activase gene in *Oryza sativa* affect the photosynthetic capacity

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Abstract

We investigated the effect of large isoform of ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBPCO) activase (RuBPCO-A) on photosynthesis and constructed two plant expression vectors and introduced them into rice cultivars (*Oryza sativa* f. *japonica* cv. Nipponbare) through *Agrobacterium tumefaciens*-mediated transformation. Plasmid pCBrcSRca contained the cDNA of RuBPCO-A large isoform (*rca*) controlled by RuBPCO small subunit gene promoter (*rbcS*), and plasmid pCBUbi-antirca contained a reversed *rca* sequence driven by maize ubiquitin promoter. Transformants were screened by polymerase chain reaction (PCR), Southern and Western blot analysis. Compared to the control rice plants, RuBPCO activity was improved in the pCBrcSRca rice plants, which is opposite to RuBPCO activity in the pCBUbi-antirca rice plants. Net photosynthetic rate, quantum yield of electron transport in photosystem 2, and steady state photochemical fluorescence quenching increased in the pCBrcSRca plants, but decreased in the pCBUbi-antirca plants as compared to the controls. The pCBrcSRca plants had heavier grains and accelerated development, while the pCBUbi-antirca plants showed reverse changes. Thus RuBPCO-A large isoform exerts considerable effect on photosynthesis and is a promising target for plant breeding to improve rice crop yield.

Additional key words: chlorophyll fluorescence; photosynthesis; RuBPCO-A; transgenic rice.

Introduction

In the long history of rice culture, two great breakthroughs for rice yield have been observed: one is a high-yielding semi-dwarf cultivar, IR8 announcing the first green revolution at the end of 1950s; the other is the first hybrid cultivar, which was the signal of the second green revolution at the end of 1970 (Yuan *et al.* 1994, Devanand *et al.* 2000, Peng *et al.* 2000). Since then, yield of rice has been improved slowly. Improving photosynthesis is crucial for the third breakthrough (Ishi 1998, Horton 2000, Xu and Shen 2001).

In higher plants, CO₂ assimilation catalyzed by ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBPCO, EC 4.1.1.39) is the rate-limiting step in photosynthesis (Hartman and Harpel 1994). RuBPCO has eight large and eight small subunits. The expression of small subunit gene is organ-specific, developmentally regulated, light-inducible, and controls the constitutive expression of large subunit at the level of translation (Khrebtukova and Spreitzer 1996, Rodermeil *et al.* 1996). Experiments show that photosynthesis improvement is difficult to accomp-

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Abbreviations: ADP – adenosine 5'-diphosphate; ATP – adenosine 5'-triphosphate; Car – carotene; Chl – chlorophyll; F_v/F_m – maximal photochemical efficiency of PS2; F'_v/F'_m – efficiency of excitation energy captured by open PS2 centres; NADH – reduced nicotinamide adenine dinucleotide; PCR – polymerase chain reaction; PEPC – phosphoenolpyruvate carboxylase; P_N – steady state net photosynthetic rate; PS – photosystem; q_N – proportion of excitation energy dissipated as thermal energy; q_P – proportion of excitation energy captured by PS2 and used in photochemical electron transport; *rbcS* – gene for ribulose-1,5-bisphosphate carboxylase/oxygenase small subunit; RC – reaction centre; *rca* – cDNA of ribulose-1,5-bisphosphate carboxylase/oxygenase activase; RuBP – ribulose-1,5-bisphosphate; RuBPCO – ribulose-1,5-bisphosphate carboxylase/oxygenase; RuBPCO-A – ribulose-1,5-bisphosphate carboxylase/oxygenase activase; Xan – xanthophyll; Γ – CO₂ compensation concentration; Φ_{PS2} – quantum yield of electron transport in PS2.

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lish by a direct modulation of RuBPCO (Kostov and McFadden 1995, Spreitzer *et al.* 1995, Spreitzer 1999, Satagopan and Spreitzer 2004).

RuBPCO activase (RuBPCO-A), a chloroplast protein, keeps RuBPCO in an activation state by dissociation of any inhibitory sugar phosphates from RuBPCO active site and promotion of RuBPCO catalysis at the cost of adenosine 5'-triphosphate (ATP) hydrolyzation during the process of RuBPCO activation (Somerville *et al.* 1982, Liu *et al.* 1996, Portis 2003). The expression of RuBPCO-A genes, similar to the expression of RuBPCO small subunit gene, is organ-specific, developmentally regulated, and light-inducible (Zhang and Komatsu 2000). Most species, including rice, contain two forms of RuBPCO-A produced by alternative splicing of its mRNA (Werneke *et al.* 1989). The large isoform regulates the activity of RuBPCO in response to irradiance

via redox changes in chloroplast stroma (Zhang and Potis 1999). In contrast, the small isoform itself is not regulated by irradiance. Interestingly, when both isoforms are present together, the changes in the activity of the large isoform induced by irradiance are sufficient to regulate the activity of the small one (Zhang *et al.* 2002).

As the large isoform of RuBPCO-A regulates the activity of RuBPCO in response to irradiance (Zhang *et al.* 2002), the effect of the increasing/decreasing amount of large isoform of RuBPCO-A on photosynthesis was investigated in our experiments. For more levels of RuBPCO-A large isoform, promoter of RuBPCO small subunit gene (*rbcS*) was chosen to control the cDNA of rice RuBPCO-A large isoform (*rca*) in order to confer the regulation specificity of the *rca*. To decrease the enzyme contents, reversed *rca* sequence under control of maize ubiquitin promoter was introduced into rice plants.

Materials and methods

Construction of vectors: A 1.6-kb cDNA fragment of *rca* (Genbank accession No. OSU74321) was isolated from rice by PCR amplification. The binary plasmid pCBrbcSRca was constructed by inserting a *Hind*III-*Eco*RI fragment containing *rca* sequence under the control of *rbcS* promoter into the plasmid pCAMBIA 1301 between the sites of *Hind*III and *Eco*RI (Fig. 1). The plasmid pGRN73 including *rbcS* promoter was provided by Prof. Wu Ray, Cornell University, USA. A reversed *rca* fragment controlled by maize ubiquitin promoter was introduced into plasmid pCAMBIA 1301 between the sites of *Hind*III and *Eco*RI to generate plasmid pCBUbi-antirca (Fig. 1). Both constructed plasmids (pCBrbcSRca and pCBUbi-antirca) contained hygromycin phosphotransferase gene, and were transferred into *Agrobacterium tumefaciens* strain LBA4404 via tri-parental mating

method (Ditta *et al.* 1980).

Transformation and growth processes: Seeds of rice cultivar *Oryza sativa f. japonica* cv. Nipponbare, which were obtained from the Institute of Genetics and Developmental Biology, Chinese Academy of Sciences, were germinated on NB medium (Rance *et al.* 1994) for calli induction at 25 °C under 16/8 h (light/dark) photoperiod. After that, global calli were infected with the *A. tumefaciens* strain LBA4404 for 3 d at 25 °C under dark on NB medium plus 0.1 mM acetosyringone, and were transferred to selection medium which was composed of NB medium and 50 g m⁻³ hygromycin at 25 °C under dark. Four weeks later, vigorously compacted calli were selected for rice regeneration on NB medium plus 2 g m⁻³ kinetin, 0.2 g m⁻³ α -naphthaleneacetic acid, 30 kg m⁻³

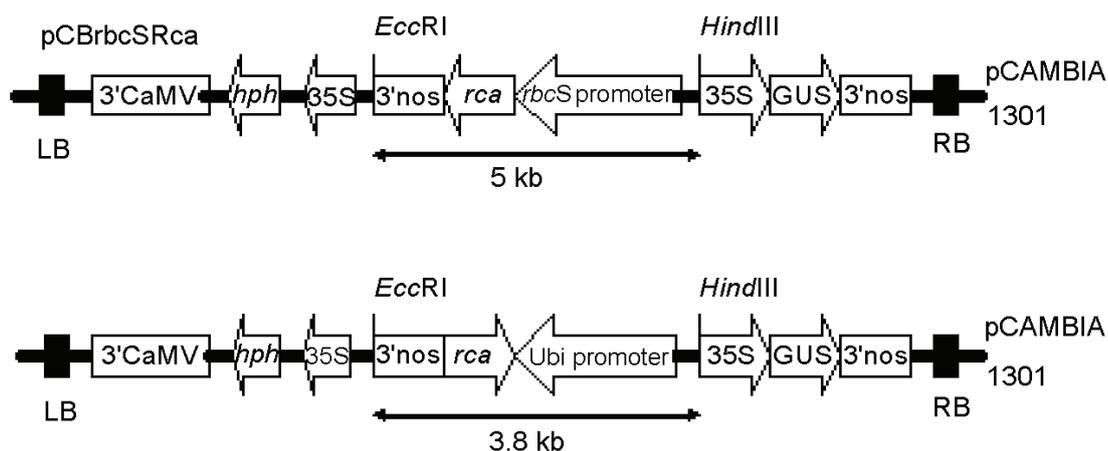


Fig. 1. Construction of pCBrbcSRca and pCBUbi-antirca plant expression vectors. The cDNA of RuBPCO-A large isoform of *Oryza sativa* (*rca*) was inserted into pCAMBIA 1301 under the control of RuBPCO small subunit gene (*rbcS*) promoter and termination of 35S CaMV polyadenylation sequence (3'nos) in plasmid pCBrbcSRca. The expression cassette including maize ubiquitin promoter, *rca* sequence with reversed direction, 3'nos was inserted into pCAMBIA 1301 in plasmid pCBUbi-antirca. The *Eco*RI and *Hind*III sites were used during the construction of both plasmids.

sorbital, and 30 g m⁻³ hygromycin at 28 °C under a 16/8 h (light/dark) photoperiod. Lastly, the recovered young plantlets were transferred to soil in pots in a greenhouse at 30/25 °C under a 16/8 h (light/dark) photoperiod. Seeds were harvested from these plants.

T₁ transgenic rice seeds were germinated in the presence of hygromycin. Ten young plantlets of the pCBrcSRca (or pCBUbi-antirca) transformed rice plants were transplanted to soil in a greenhouse at 30/25 °C under a 16/8 h (light/dark) photoperiod. Plant height as well as leaf number of each plant were recorded at the same time from the transplanting. The mass of one thousand grains was determined.

Molecular analysis of the transformants: Genomic DNA for polymerase chain reaction (PCR) and Southern blot analysis was isolated according to the SDS method (Pich and Schubert 1993). The pair of primers used to amplify the 1.6-kb *rca* cDNA were 5'-ATC ATC GAC TTT CAG CAA ATT AAG A-3' and 5'-CTT GTC ATG CCC AGC TAT GG-3'. Twenty mm³ PCR reaction was performed in a *touchgene* thermocycler (UK) as follows: initiation at 94 °C for 3 min, followed by 35 cycles at 94 °C for 0.5 min, 58 °C for 1 min, 72 °C for 1.5 min, and extension at 72 °C for 10 min for terminal. The primers used to generate a 687-bp *rca* cDNA fragment as probe for Southern blot analysis were 5'-ACC GTG AGG CGG CAG ACA T-3' and 5'-CTT GCC CGT AGA AGG AAC CA-3'. About 15 µg genomic DNA was digested by *Hind*III, separated on 0.8 % agarose gel in 1×TAE buffer at 4 v/cm³, and transferred to a nylon⁺ membrane. The membrane was washed with 6×SSC buffer, pre-hybridized and hybridized with *rca* probe labelled by ³²P-dCTP (Sambrook *et al.* 1989).

For Western blotting, leaf tissue was ground in liquid nitrogen followed by protein extraction buffer (0.1 mM Tris-HCl, 10 mM MgCl₂, 1.0 mM Na₂EDTA, 20 mM mercaptoethanol, 20 kg m⁻³ polyvinylpyrrolidone, pH 7.8). According to Sambrook *et al.* (1989), 15 µg total soluble protein was separated by 15 % SDS polyacrylamide gel electrophoresis, transferred onto a nitrocellulose membrane, and hybridized with anti RuBPCO-A antiserum (provided by Prof. Daquan Xu and Liren Li, Institute of Plant Physiology and Ecology, Chinese Academy of Science).

Net photosynthetic rate (P_N): An infrared gas analyzer (*Ciras-1*, PP Systems, UK) was used to determine P_N of the attached 2nd upright leaves of T₁ rice when flag leaves had already emerged but were not yet fully expanded. Five replications were made. The linear slope of the P_N to photon flux density from irradiance-response curve was calculated as apparent quantum yield at 25 °C, 360 µmol(CO₂) mol⁻¹ concentration, irradiance below 200 µmol m⁻² s⁻¹. The linear slope of P_N to intercellular CO₂ concentration from CO₂-response curve was calculated as carboxylation efficiency in low C_i at saturation

irradiance of 1 000 µmol m⁻² s⁻¹, 25 °C, 80 % relative humidity, and a series of CO₂ concentrations (Tenhunen *et al.* 1984).

Chlorophyll (Chl) fluorescence: According to Genty *et al.* (1989), Chl fluorescence was measured on the detached 2nd upright leaves of T₁ rice at 25 °C with five replications using a portable fluorometer (*PAM-2000*, H. Walz, Effeltrich, Germany). Leaves were dark adapted for 30 min before measurement. Fluorescence parameters were calculated: (a) maximum photochemical efficiency of photosystem 2 (PS2): F_v/F_m = (F_m - F₀)/F_m, (b) quantum yield of electron transport of PS2: Φ_{PS2} = (F'_m - F_s)/F'_m, (c) efficiency of excitation energy capture by open PS2 reaction centres (RCs): F'_v/F'_m = (F'_m - F'₀)/F'_m, (d) photochemical quenching coefficient: q_p = (F'_m - F_s)/(F'_m - F'₀), (e) the non-photochemical quenching coefficient: q_N = 1 - (F'_m - F'₀)/(F_m - F₀). Fluorescence nomenclature was used according to van Kooten and Snel (1990).

Pigment contents, activities of RuBPCO and PEPC: For pigment analysis, leaf samples were extracted in 80 % acetone. Measurement was carried out by spectrophotometric method (*UV8500*, Shanghai, China) at the wavelengths 663, 645, and 470 nm. Contents of Chl *a*, Chl *b*, xanthophyll (Xan), and carotene (Car) were calculated using the equations of Arnon (1949).

About 1 g leaf tissue was ground in 10 cm³ extraction buffers (0.1 mM Tris-HCl, 10 mM MgCl₂, 1.0 mM Na₂EDTA, 20 mM mercaptoethanol, 20 kg m⁻³ polyvinylpyrrolidone, pH 7.8) at 0 °C with the help of quartz granules. The mixture was then centrifuged at 11 000×g for 10 min. For RuBPCO initial activity analysis, 20 mm³ supernatant were added into 930 mm³ of reaction buffer [100 mM Tris-HCl (pH 7.8), 10 mM NaHCO₃, 20 mM MgCl₂, 10 mM dithiothreitol, 0.75 mM reduced nicotinamide adenine dinucleotide (NADH), 5 mM ATP, 10 mM phosphocreatine, 60 units per cm³ of 3-phosphoglycerate kinase, 300 units per cm³ of triose-phosphate isomerase, 30 units per cm³ of creatine phosphokinase, glyceraldehyde-3-phosphate dehydrogenase, and glycerol-3-phosphate dehydrogenase] at 25 °C. After that, 50 mm³ of 40 mM RuBP was added into the mixture. Absorbance of NADH at the wavelength of 340 nm was measured immediately (Larson *et al.* 1997). For RuBPCO total activity analysis, 20 mm³ supernatant were also mixed with 930 mm³ of reaction buffer. However, the mixture was placed at 25 °C for 20 min. After that, 50 mm³ of 40 mM RuBP was added into the mixture and the absorbance of NADH was measured immediately.

Phosphoenolpyruvate carboxylase (PEPC) activity was measured according to the enzyme's activity of malate dehydrogenase-catalysed NADH oxidation (De Nisi and Zocchi 2000). About 0.2 g leaf tissue was ground in 1 cm³ extraction buffer, which contained 50 mM Tris-HCl (pH 7.5), 10 mM MgCl₂, 1.0 mM

Na₂EDTA, 100 kg m⁻³ glycerol, 14 mM mercaptoethanol, 1.0 mM phenylmethylsulphonyl fluoride, and 10 kg m⁻³ leupeptin. The mixture was centrifuged at 13 000×g for 15 min and 100 000×g for 30 min. At room temperature, 100 mm³ of soluble mixture was put into 900 mm³ of buffer [100 mM Tris-HCl (pH 8.0), 5 mM MgCl₂, 2.5 mM PEP, 0.2 mM NADH, 10 mM NaHCO₃, and

15 kg m⁻³ malate dehydrogenase]. NADH oxidation was determined at the wavelength of 340 nm.

Statistical analysis: Statistics Package for Social Science (SPSS) 9.0 for Windows was used for correlation analyses.

Results

PCR, Southern and Western blot analysis of the transformed rice plants: In our experiments, more than 30 T₀ plants transformed, respectively, by pCBrbcSRca and pCBUbi-antirca with different copies and inserted sizes of foreign DNA were obtained (data not shown). Among them, 6 ones were selected for T₁ generation analyses. Each T₁ generation line had 10 replicates, which were used to do all the analyses.

PCR reaction showed that 52 out of 71 pCBrbcSRca and 45 out of 57 pCBUbi-antirca transgenic rice plants had the 1.6-kb *rca* fragment (Fig. 2A). From Southern blot analysis, one *rca* band in the genomic DNA of the control plants, two *rca* bands in the genomic DNA of pCBUbi-antirca plants, and three *rca* bands in the genomic DNA of pCBrbcSRca plants were detected (Fig. 2B). Western blot analysis showed that the levels of 47 kDa large isoform of RuBPCO-A were almost equal to that of 43 kDa small one in the control rice plants, the levels of 47 kDa isoform were obviously reduced in pCBUbi-antirca plants, and the levels of 47 kDa isoform significantly increased in pCBrbcSRca plants (Fig. 2C). These results implied that the expression of inserted *rca* in the transgenic rice plants alters the RuBPCO-A contents.

Activities of RuBPCO and PEPC: RuBPCO initial activity was decreased down to near one-quarter of the control in the pCBUbi-antirca plants, and increased up to 2-fold of the control in the pCBrbcSRca plants (Table 1). However, RuBPCO total activity and PEPC activity of the both transgenic rice plants did not differ from those of the control (Table 1).

P_N response to irradiance and CO₂: Irradiance- and CO₂-response curves showed that P_N of pCBUbi-antirca plants was lower than in the control while P_N of pCBrbcSRca plants was higher than in the control. For an example, under saturation irradiance (1 000 μmol m⁻² s⁻¹) and atmospheric CO₂ concentration, P_N was 9.5, 5.2, and 13.8 μmol (CO₂) m⁻² s⁻¹ in the control, pCBUbi-antirca, and pCBrbcSRca rice plants, respectively.

Apparent quantum efficiency, the ability of leaves to use photons under low irradiance, was 0.025 mol(CO₂) mol⁻¹(photon) in the pCBrbcSRca rice plant, similar to 0.020 mol(CO₂) mol⁻¹(photon) of the control, but significantly higher than 0.013 mol(CO₂) mol⁻¹(photon) of the pCBUbi-antirca plants (Fig. 3A). Carboxylation

efficiency representing the activity of RuBPCO was calculated at low CO₂ concentrations (less than 200 μmol mol⁻¹). As shown in Fig. 3B, the carboxylation efficiency was 0.018 μmol(CO₂) m⁻² s⁻¹ Pa⁻¹ for the control, 0.016 μmol(CO₂) m⁻² s⁻¹ Pa⁻¹ for the pCBUbi-antirca, and 0.036 μmol(CO₂) m⁻² s⁻¹ Pa⁻¹ for the pCBrbcSRca

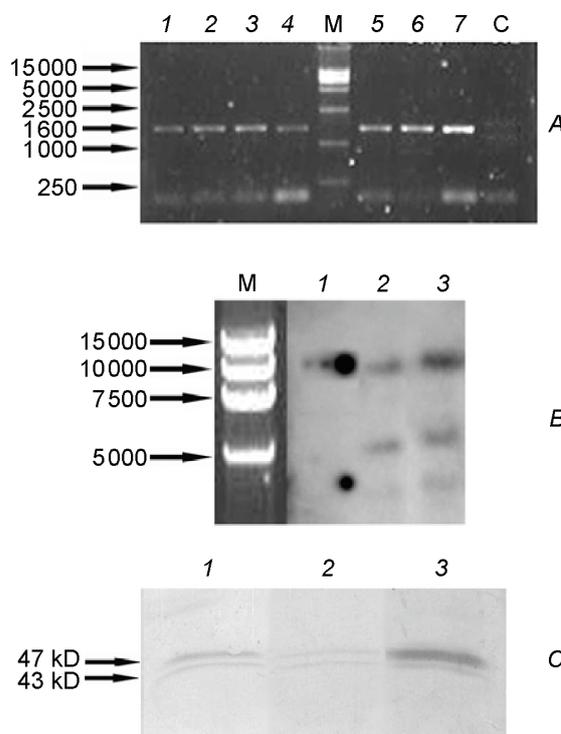


Fig. 2. Molecular analyses of the pCBrbcSRca and pCBUbi-antirca transgenic rice plants. (A) Polymerase chain reaction (PCR) for amplification of 1.6 kb *rca* fragment. M – DL-15 000 DNA size marker (Takara); C, the control rice; lanes 1–4, PCR products from different pCBrbcSRca transgenic rice plants; lanes 5–7, PCR products from different pCBUbi-antirca transgenic rice plants. (B) Southern blot analysis of the transgenic plants. Fifteen μg of genomic DNA was digested by *Hind*III and hybridized with 687 bp *rca* probe. M – DL-15 000 DNA size marker (Takara); lane 1, the control rice; lane 2, the pCBUbi-antirca transgenic rice plant; lane 3, the pCBrbcSRca transgenic rice plant. (C) Western blot analysis of the transgenic plants. Ten μg of total soluble protein was separated by 15 % SDS-PAGE in each lane. Lane 1, the control rice; lane 2, the pCBUbi-antirca transgenic rice plant; lane 3, the pCBrbcSRca transgenic rice plant.

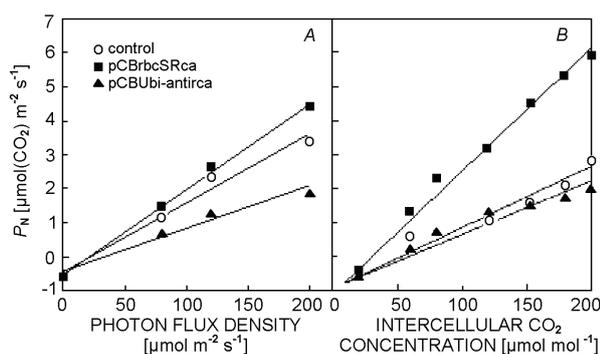


Fig. 3. Apparent quantum yield (A) and carboxylation efficiency (B) of the pCBrbcSRca, pCBUbi-antirca, and control rice plants.

rice plants. Γ is CO_2 compensation concentration (Γ) is CO_2 concentration when photosynthetic rate equals to photorespiration plus respiration. Γ was 54.5 ± 1.8 , 58.9 ± 1.5 , and $49.8 \pm 1.2 \mu\text{mol}(\text{CO}_2) \text{mol}^{-1}$ in the control, pCBUbi-antirca, and pCBrbcSRca rice plants, respectively.

Table 1. RuBPCO and PEPC activities [$\text{mmol}(\text{CO}_2) \text{kg}^{-1}(\text{protein}) \text{s}^{-1}$] of transgenic and control rice plants. Means \pm SE of 5–7 leaves.

Enzyme activities	Control	pCBUbi-antirca	pCBrbcSRca
RuBPCO initial activity	14.17 \pm 0.24	3.83 \pm 0.19	27.33 \pm 0.47
RuBPCO total activity	33.50 \pm 0.52	32.47 \pm 0.44	34.66 \pm 0.69
PEPC activity	48.68 \pm 1.33	50.51 \pm 1.83	40.84 \pm 0.17

Table 2. The parameters of chlorophyll fluorescence in detached 2nd upright leaves of the transgenic and control rice plants. Means \pm SE of 5 leaves. The significant levels of difference between the transgenic and control are indicated by * for $p < 0.05$, ** for $p < 0.01$.

Plant	F_v/F_m	Φ_{PS2}	F'_v/F'_m	q_p	q_N
Control	0.843 \pm 0.005	0.402 \pm 0.020	0.580 \pm 0.019	0.695 \pm 0.023	0.746 \pm 0.013
pCBrbcSRca	0.846 \pm 0.004	0.440 \pm 0.008**	0.593 \pm 0.015	0.741 \pm 0.007*	0.734 \pm 0.023
pCBUbi-antirca	0.837 \pm 0.002	0.235 \pm 0.015**	0.440 \pm 0.015**	0.535 \pm 0.017**	0.870 \pm 0.003**

Table 3. Pigment contents [mg kg^{-1}] and chlorophyll (Chl) a/b ratios in the transgenic and control rice plants.

Plant	Chl a	Chl b	Xan+Car	Chl $a+b$	Chl a/b
Control	2 579.95 \pm 91.24	655.78 \pm 52.77	552.55 \pm 66.54	3 235.73 \pm 144.02	3.93 \pm 0.11
pCBrbcSRca	2 808.49 \pm 115.92	710.63 \pm 68.60	616.15 \pm 34.83	3 519.12 \pm 184.52	3.95 \pm 0.21
pCBUbi-antirca	2 414.92 \pm 89.34	695.74 \pm 65.40	548.86 \pm 61.68	3 110.67 \pm 154.74	3.47 \pm 0.16

Pigment composition: As Table 3 shows, contents of Chl a , Chl b , Xan+Car, Chl $a+b$, and Chl a/b ratio in both transgenic rice plants were not significantly different

Modification of growth process of the transgenic rice plants: During the greater part of the growth process, both the two types of transgenic plants showed no difference in appearance including the number of leaves, the number of tillers, leaf area, and plant height (data not

Different changes of Chl fluorescence in the two transgenic rice plants (Table 2): Both transgenic and control rice plants showed no difference in F_v/F_m . Φ_{PS2} , which represents the efficiency of transfer of absorbed photons to the RC of PS2, was 9.45 % higher in the pCBrbcSRca plants, but 41.54 % lower in the pCBUbi-antirca plants as compared with the controls. F'_v/F'_m of the pCBrbcSRca plants was almost equal to that of the control, but in the pCBUbi-antirca plants it was reduced to 75.86 % of that of the control. Compared to the control, q_p was increased by 6.62 % in the pCBrbcSRca plants, but decreased by 23.02 % in the pCBUbi-antirca plants. q_N of the pCBrbcSRca plants was similar to that of the control while q_N of the pCBUbi-antirca plants was 16.62 % higher than the control. These results imply that more energy participates in CO_2 fixation in the pCBrbcSRca rice plants. However, in term of the pCBUbi-antirca rice plants, less energy is captured by RCs of PS2 and utilized for CO_2 fixation, and more energy dissipates as thermal energy.

from those of the control ones. Thus the contents of RuBPCO-A did not affect pigment composition.

shown). However, after the 3rd upright leaves appeared, the pCBrbcSRca plants became taller and the pCBUbi-antirca plants became shorter than the control. When flag leaves fully expanded, the average heights of the pCBrbcSRca, pCBUbi-antirca transgenic, and control rice

plants were 0.99, 0.87, and 0.94 m, respectively (Table 4). The heading time of pCBrbcSRca plants was 112 d after transplanting into the soil, 7 d ahead of the control, and 15 d ahead of the pCBUbi-antirca plants (Table 4). Time for flowering and seed-setting of each transgenic rice plant were ahead or postponed

Discussion

We investigated the effect of the large isoform of RuBPCO-A on photosynthesis with a strategy of increasing or decreasing its content in rice. Previous research of the *rca* mutants with reduced contents of RuBPCO-A of *Arabidopsis* and tobacco showed reduced RuBPCO activity, impaired CO₂ assimilation rate, and delayed plant development (Robinson and Portis 1988, Mate *et al.* 1993, Jiang *et al.* 1994, Eckardt *et al.* 1997, He *et al.* 1997). Recent reports showed that the activity of

accordingly. One of the pCBUbi-antirca transgenic plants started to tiller at 109 d, flower at 145 d, and set seeds at 185 d. One thousand grain masses of the pCBrbcSRca, pCBUbi-antirca transgenic, and control rice plants were 22.97, 17.50, and 19.40 g, respectively.

RuBPCO-A large isoforms determines the activities of both isoforms in response to irradiance. In our experiments, the pCBrbcSRca transgenic rice plants with increased contents of RuBPCO-A large isoform and pCBUbi-antirca transgenic rice plants with decreased contents of RuBPCO-A large isoform were generated. Hence the promotion of the RuBPCO-A large isoform synthesis stimulated photosynthesis and plant growth, and the detention of this synthesis had reverse effects.

Table 4. Growth characteristics of the pCBrbcSRca and pCBUbi-antirca rice plants.

Traits	Control	pCBrbcSRca	pCBUbi-antirca
Plant height [m]	0.94±0.01	0.99±0.02	0.87±0.01
Heading time [d]	119±1	112±1	127±2
Mass of 1 000 grains [g]	19.40±0.09	22.97±0.72	17.50±0.37

RuBPCO activity was improved in the pCBrbcSRca plants, but decreased in the pCBUbi-antirca plants. Carboxylation efficiency and RuBPCO initial activity representing the activity of RuBPCO were increased to a similar extent in pCBrbcSRca plants. However, in pCBUbi-antirca plants both the parameters were decreased with the RuBPCO initial activity depression to an even greater extent. Considering Γ in all examined plants, the carboxylation/oxygenation specificities of RuBPCO were not altered in both transgenic rice plants with either more or less amount of RuBPCO-A. RuBPCO total activity and PEPC activity in both transgenic plants were close to those of the control. Thus the contents of RuBPCO-A did not affect the amount or specificity of RuBPCO, but affected its activity directly under saturation irradiance. This is consistent with the studies of *rca* mutants that RuBPCO-A inhibition had no impact on the accumulation of RuBPCO (Jiang *et al.* 1994) but affected its activity (Eckardt *et al.* 1997, Hammond *et al.* 1998).

In the pCBrbcSRca rice plants P_N was enhanced, which was opposite to the situation in the pCBUbi-antirca rice plants. Φ_{PS2} and q_p , parameters of Chl fluorescence, reflect to some extent the irradiance-dependent reactions of photosynthesis. Both parameters were elevated in the pCBrbcSRca plants as compared to the control plants, implying a greater percentage of open RCs of PS2 and more excitation energy captured by PS2 RCs. Therefore, more energy is used for photochemical reaction, the electron transport is propelled to form more ATP and NADPH (Khoo *et al.* 1997), and more CO₂ can be

assimilated. However, in the pCBUbi-antirca rice plants, Φ_{PS2} and q_p were decreased. Analyses of F'_v/F'_m , q_N , and apparent quantum efficiency showed that the pCBUbi-antirca plants were characterized by the decreased efficiency of excitation energy capture by open PS2 RCs, the reduced ability of leaves to make use of photon energy under low irradiance, and the increased capacity of PS2 to dissipate excessive energy. No such changes were observed in the pCBrbcSRca plants. Pigment compositions of antenna in both transgenic plants were not altered (Table 3). Consistent with the changes of RuBPCO activity, P_N in the pCBrbcSRca plants was increased up to 45.26 % of control plants, but decreased to a similar extent in the pCBUbi-antirca plants at saturating irradiance. These results support the hypothesis that dark reactions (such as RuBPCO catalyzing CO₂ assimilation) and light reactions (such as excitation energy captured by PS2 RCs) cooperate together and provide the evidence that RuBPCO-A exerts considerable control on photosynthesis under a series of conditions (Quick *et al.* 1991, Stitt *et al.* 1991).

The promotion of photosynthetic capacity stimulates plant growth, and *vice versa*. Saccharides produced by photosynthesis provide material for plant growth. Thus, it was reported for antisense *rbcS* tobacco that the relation between plant growth rate and RuBPCO activity is positive (Quick *et al.* 1991, Krapp *et al.* 1994). In our experiments, the improved photosynthetic capacity in the pCBrbcSRca rice plants was associated with higher plant height, heavier grains, and accelerated development. In

contrast, the reduced P_N in the pCBUbi-antirca rice plants led to lower plant height, lighter grains, and retarded development, which is close to the phenomena observed in antisense *rca* mutants of *Arabidopsis* or tobacco. Mechanism for those phenomena remains elusive, but decreased growth may be attributed to reduced relative growth rates (Gifford and Jenkins 1982). Under long-term CO₂ enrichment, the activity of RuBPCO and photosynthetic rate decrease, but an increased accumulation of the saccharide complex occurs so as to form larger leaf numbers, leaf area index, and number of effective tillers (Koch *et al.* 1986, Baker *et al.* 1990, Stitt 1991, Long and Drake 1992, Bowes 1993, Cheng *et al.* 1998). Further-

more, the days of vegetative growth are reduced (Baker *et al.* 1990, Wittwer 1995). So, we speculate that the higher saccharide accumulation from the promoted P_N in the pCBrcSRca rice plants accounts for the promotion of plant growth. However, more experiments are necessary to test it.

In conclusion, we observed that improving the contents of RuBPCO-A large isoform in rice plants significantly enhances their photosynthesis capacity and promotes their growth, but decreasing the accumulation of RuBPCO-A leads to the opposite results. This shows a realistic opportunity to improve the yield of rice *via* the increased contents of RuBPCO-A enzyme.

References

- Arnon, D.I.: Copper enzymes in isolated chloroplasts. Polyphenoloxidase in *Beta vulgaris*. – *Plant Physiol.* **24**: 1-15, 1949.
- Baker, J.T., Allen, L.H., Jr., Boote, K.J., Jones, P., Jones, J.W.: Developmental responses of rice to photoperiod and carbon dioxide concentration. – *Agr. Forest Meteorol.* **50**: 201-210, 1990.
- Bowes, G.: Facing the inevitable: Plants and increasing atmospheric CO₂. – *Annu. Rev. Plant Physiol. Plant mol. Biol.* **44**: 309-332, 1993.
- Cheng, S.H., Moore, B.D., Seemann, J.R.: Effects of short and long term elevated CO₂ on the expression of ribulose-1,5-bisphosphate carboxylase/oxygenase genes and carbohydrate accumulation in leaves of *Arabidopsis thaliana* (L.) Heynh. – *Plant Physiol.* **116**: 715-723, 1998.
- De Nisi, P., Zocchi, G.: Phosphoenolpyruvate carboxylase in cucumber (*Cucumis sativus* L.) roots under iron deficiency: activity and kinetic characterization. – *J. exp. Bot.* **51**: 1903-1909, 2000.
- Devanand, P.S., Rangaswamy, M., Ikehashi, H.: Identification of hybrid sterility gene loci in two cytoplasmic male sterile lines in rice. – *Crop Sci.* **40**: 640-646, 2000.
- Ditta, G., Stanfield, S., Corbin, D., Helinski, D.R.: Broad host range cloning system for gram negative bacteria: construction of gene bank of *Rhizobium meliloti*. – *Proc. nat. Acad. Sci. USA* **77**: 7374-7351, 1980.
- Eckardt, N.A., Snyder, G.W., Portis, A.R., Jr., Ogren, W.L.: Growth and photosynthesis under high and low irradiance of *Arabidopsis thaliana* antisense mutants with reduced ribulose-1,5-bisphosphate carboxylase/oxygenase activase content. – *Plant Physiol.* **113**: 575-586, 1997.
- Genty, B., Briantais, J.-M., Baker, N.R.: The relationship between the quantum yield of photosynthetic electron transport and quenching of chlorophyll fluorescence. – *Biochim. biophys. Acta* **990**: 87-92, 1989.
- Gifford, R.M., Jenkins, C.L.D.: Prospects of applying knowledge of photosynthesis toward improving crop production. – In: Govindjee (ed.): *Photosynthesis*. Vol. II. Pp. 419-457. Academic Press, New York – London – Paris – San Diego – San Francisco – São Paulo – Sydney – Tokyo – Toronto 1982.
- Hammond, E.T., Andrews, T.J., Mott, K.A., Woodrow, I.E.: Regulation of Rubisco activation in antisense plants of tobacco containing reduced levels of Rubisco activase. – *Plant J.* **14**: 101-110, 1998.
- Hartman, F.C., Harpel, M.R.: Structure, function, regulation, and assembly of D-ribulose-1,5-bisphosphate carboxylase/oxygenase. – *Annu. Rev. Biochem.* **63**: 197-234, 1994.
- He, Z., Caemmerer, S. von, Hudson, G.S., Price, G.D., Badger, M.R., Andrews, T.J.: Ribulose-1,5-bisphosphate carboxylase/oxygenase activase deficiency delays senescence of ribulose-1,5-bisphosphate carboxylase/oxygenase but progressively impairs its catalysis during tobacco leaf development. – *Plant Physiol.* **115**: 1569-1580, 1997.
- Horton, P.: Prospects for crop improvement through the genetic manipulation of photosynthesis: morphological and biochemical aspects of light capture. – *J. exp. Bot.* **51**: 475-485, 2000.
- Ishi, R.: Leaf/canopy photosynthesis and crop productivity. – In: Raghavendra, A.S. (ed.): *Photosynthesis: A Comprehensive Treatise*. Pp. 215-225. Cambridge University Press, Cambridge 1998.
- Jiang, C.Z., Quick, W.P., Alred, R., Kliebenstein, D., Rodermeier, S.R.: Antisense RNA inhibition of Rubisco activase expression. – *Plant J.* **5**: 787-798, 1994.
- Khoo, G.H., He, J., Hew, C.S.: Photosynthetic utilization of radiant energy by CAM *Dendrobium* flowers. – *Photosynthetica* **34**: 367-376, 1997.
- Khrebukova, I., Spreitzer, R.J.: Elimination of the *Chlamydomonas* gene family that encodes the small subunit of ribulose-1,5-bisphosphate carboxylase/oxygenase. – *Proc. nat. Acad. Sci. USA* **93**: 13689-13693, 1996.
- Koch, K.E., Jones, P.H., Avigne, W.T., Allen, L.H., Jr.: Growth, dry matter partitioning, and diurnal activities of RuBP carboxylase in citrus seedlings maintained at two levels of CO₂. – *Physiol. Plant.* **67**: 477-484, 1986.
- Kostov, R.V., McFadden, B.A.: A sensitive, simultaneous analysis of ribulose 1,5-bisphosphate carboxylase/oxygenase efficiencies: Graphical determination of the CO₂/O₂ specificity factor. – *Photosynth. Res.* **43**: 57-66, 1995.
- Krapp, A., Chaves, M.M., David, M.M., Rodriguez, M.L., Pereira, J.S., Stitt, M.: Decreased ribulose-1,5-bisphosphate carboxylase/oxygenase in transgenic tobacco transformed with “antisense” *rbcS*. VIII. Impact on photosynthesis and growth in tobacco growing under extreme high irradiance and high temperature. – *Plant Cell Environ.* **17**: 945-953, 1994.
- Larson, E.M., O'Brien, C.M., Zhu, G., Spreitzer, R.J., Portis, A.R., Jr.: Specificity for activase is changed by a Pro-89 to Arg substitution in the large subunit of ribulose-1,5-bisphosphate carboxylase/oxygenase. – *J. biol. Chem.* **272**: 17033-17037, 1997.

- Liu, Z., Taub, C.C., McClung, C.R.: Identification of an *Arabidopsis thaliana* ribulose-1,5-bisphosphate carboxylase/oxygenase activase (RCA) minimal promoter regulated by light and the circadian clock. – *Plant Physiol.* **112**: 43-51, 1996.
- Long, S.P., Drake, B.G.: Photosynthetic CO₂ assimilation and rising atmospheric CO₂ concentrations. – In: Baker, N.R., Thomas, H. (ed.): *Crop Photosynthesis: Spatial and Temporal Determinants*. Pp. 69-103. Elsevier Science Publ., Amsterdam 1992.
- Mate, C.J., Hudson, G.S., Caemmerer, S. von, Evans, J.R., Andrews, T.J.: Reduction of ribulose bisphosphate carboxylase activase levels in tobacco (*Nicotiana tabacum*) by antisense RNA reduces ribulose bisphosphate carboxylase carboxylation and impairs photosynthesis. – *Plant Physiol.* **102**: 1119-1128, 1993.
- Peng, S., Laza, R.C., Visperas, R.M., Sanico, A.L., Cassman, K.G., Khush, G.S.: Crop breeding, genetics and cytology. Grain yield of rice cultivars and lines developed in the Philippines since 1966. – *Crop Sci.* **40**: 307-314, 2000.
- Pich, U., Schubert, I.: Midiprep method for isolation of DNA from plants with a high content of polyphenolics. – *Nucleic Acids Res.* **21**: 3328, 1993.
- Portis, A.R., Jr.: Rubisco activase – Rubisco's catalytic chaperone. – *Photosynth. Res.* **75**: 11-27, 2003.
- Quick, W.P., Schurr, U., Fichtner, K., Schulze, E.-D., Rodermeil, S.R., Bogorad, L., Stitt, M.: The impact of decreased Rubisco on photosynthesis, growth, allocation and storage in tobacco plants which have been transformed with antisense *rbcS*. – *Plant J.* **1**: 51-58, 1991.
- Rance, I.M., Tian, W.Z., Mathews, H., Kochko, A.D., Beachy, R.N., Fauquet, C.: Partial desiccation of mature embryo-derived calluses, a simple treatment that dramatically enhances the regeneration ability of India rice. – *Plant Cell Rep.* **13**: 647-651, 1994.
- Robinson, S.P., Portis, A.R., Jr.: Release of the nocturnal inhibitor, carboxyarabinitol-1-phosphate, from ribulose bisphosphate carboxylase/oxygenase by rubisco activase. – *FEBS Lett.* **233**: 413-416, 1988.
- Rodermeil, S., Haley, J., Jiang, C., Tsai, C., Bogora, L.: A mechanism for intergenomic integration: Abundance of ribulose bisphosphate carboxylase small-subunit protein influences the translation of the large-subunit mRNA. – *Proc. nat. Acad. Sci. USA* **93**: 3881-3885, 1996.
- Sambrook, J., Fritsch, E.F., Maniatis, T.: *Molecular Cloning – a Laboratory Manual*. 2nd Ed. – Pp. 469-490. Cold Spring Harbor Laboratory Publ., Cold Spring Harbor 1989.
- Satagopan, S., Spreitzer, R.J.: Substitutions at the Asp-473 lath residue of *Chlamydomonas* ribulosebisphosphate carboxylase/oxygenase cause decreases in carboxylation efficiency and CO₂/O₂ specificity. – *J. Biol. Chem.* **279**: 14240-14244, 2004.
- Somerville, C.R., Portis, A.R., Jr., Ogren, W.L.: A mutant of *Arabidopsis thaliana* which lacks activation of RuBP carboxylase *in vivo*. – *Plant Physiol.* **70**: 381-387, 1982.
- Spreitzer, R.J.: Questions about the complexity of chloroplast ribulose-1,5-bisphosphate carboxylase/oxygenase. – *Photosynth. Res.* **60**: 29-42, 1999.
- Spreitzer, R.J., Thow, G., Zhu, G.H.: Pseudoreversion substitution at large-subunit residue 54 influences the CO₂/O₂ specificity of chloroplast ribulose-bisphosphate carboxylase/oxygenase. – *Plant Physiol.* **109**: 681-685, 1995.
- Stitt, M.: Rising CO₂ levels and their potential significance for carbon flow in photosynthetic cells. – *Plant Cell Environ.* **14**: 741-762, 1991.
- Stitt, M., Quick, W.P., Schurr, U., Schulze, E.-D., Rodermeil, S.R., Bogorad, L.: Decreased ribulose-1,5-bisphosphate carboxylase-oxygenase in transgenic tobacco transformed with 'antisense' *rbcS*. II. Flux-control coefficients for photosynthesis in varying light, CO₂, and air humidity. – *Planta* **183**: 555-566, 1991.
- Tenhunen, J.D., Lange, O.L., Gebel, J., Beyschlag, W., Weber, J.A.: Changes in photosynthetic capacity, carboxylation efficiency and CO₂ compensation point associated with midday stomatal closure and midday depression of net CO₂ exchange in leaves of *Quercus suber*. – *Planta* **193**: 193-203, 1984.
- van Kooten, O., Snel, J.F.H.: The use of chlorophyll fluorescence nomenclature in plant stress physiology. – *Photosynth. Res.* **25**: 147-150, 1990.
- Xu, D.Q., Shen, Y.K.: Photosynthetic efficiency and crop yield. – In: Pessarakli, M. (ed): *The Handbook of Plant and Crop Physiology*. 2nd Ed. Pp. 821-834. M. Dekker Publ., New York 2001.
- Yuan, L.P., Yang, Z.Y., Yang, J.B.: Hybrid rice technology: New developments and future prospects. – In: Virmani, S.S. (ed.): *Hybrid Rice in China*. Pp. 143-147. IRRI Publ., Manila 1994.
- Werneke, J.W., Chatfield, J.M., Ogren, W.L.: Alternative mRNA splicing generates the two ribulosebisphosphate carboxylase/oxygenase activase polypeptides in spinach and *Arabidopsis*. – *Plant Cell* **1**: 815-825, 1989.
- Wittwer, S.H.: *Food, Climate and Carbon Dioxide: The Global Environment and World Food Production*. – Lewis Publ., New York 1995.
- Zhang, N., Kallis, R.P., Ewy, R.G., Potis, A.R., Jr.: Light modulation of Rubisco in *Arabidopsis* requires a capacity for redox regulation of the larger Rubisco activase isoform. – *Proc. nat. Acad. Sci. USA* **99**: 3330-3334, 2002.
- Zhang, N., Potis, A.R., Jr.: Mechanism of light regulation of Rubisco: A specific role for the larger Rubisco activase isoform involving reductive activation by thioredoxin-f. – *Proc. nat. Acad. Sci. USA* **96**: 9438-9443, 1999.
- Zhang, Z.L., Komatsu, S.: Molecular cloning and characterization of cDNAs encoding two isoforms of ribulose-1,5-bisphosphate carboxylase/oxygenase activase in rice (*Oryza sativa* L.). – *J. Biochem.* **128**: 383-389, 2000.