Expression of the large isoform of ribulose-1,5-bisphosphate carboxylase/oxygenase activase gene driven by *rbcS* promoter in *Oryza sativa* enhances the photosynthetic capacity

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Abstract

In order to investigate the effect of large isoform of ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBPCO) activase (RuBPCO-A) on photosynthesis, cDNA of the enzyme (*rca*) was transferred to rice cultivars (*Oryza sativa* f. *japonica* cv. Nipponbare) under the control of RuBPCO small subunit gene promoter (*rbcS*) *via Agrobacterium tumefaciens*-mediated transformation. Transgenic rice plants were identified by polymerase chain reaction (PCR) and Southern and Western blot analyses. Net photosynthetic rate (P_N) values of the T_1 transgenic lines 34 (T34) and 40 (T40) were 45.26 and 46.32 % higher than that of the control plants, respectively. At the same time, their carboxylation efficiency and RuBPCO initial activity, quantum yield of electron transport in photosystem 2 (Φ_{PS2}), and steady state photochemical fluorescence quenching (q_P) increased. In addition, heading time of the transgenic rice was advanced. Thus increasing the amount of large isoform of RuBPCO-A in the transgenic rice might have a stimulatory effect on both photosynthesis and plant growth.

Additional key words: carboxylation efficiency; chlorophyll fluorescence; net photosynthetic rate; photosystem 2; transgenic rice.

Introduction

In higher plants, photosynthesis is limited at the step of CO_2 assimilation catalyzed by ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBPCO, EC 4.1.1.39). There are many problems that must be overcome in the process of CO_2 fixation, such as carbamylation of RuBPCO before catalysis, slow turnover of carbamylated RuBPCO, inhibition of RuBPCO by various sugar phosphates, *etc*. Given that RuBPCO catalyses the carboxylation or oxygenation of ribulose-1,5-bisphosphate (RuBP) at the same time, many scientists tried to promote its specificity for carboxylation/oxygenation (Kostov and McFadden 1995, Spreitzer *et al.* 1995, Satagopan and Spreitzer 2004), even though most of them failed (Spreitzer 1999). Eight large and small subunits form RuBPCO. Small subunit, encoded by multi-nuclear-gene family, is regulated by visible radiation at the level of its mRNA accumulation (Kyozuka *et al*. 1993).

RuBPCO activase (RuBPCO-A), a nuclear-encoded radiation-inducible chloroplast protein, activates RuBPCO by releasing any inhibitory sugar phosphates from the active site of RuBPCO and accelerating the catalytic activity of carbamylated enzyme (Portis 2003). Adenosine 5'-triphosphate (ATP) is hydrolysed during the process of RuBPCO activation. In most species, there are two (large and small) isoforms of RuBPCO-A (Werneke *et al*. 1989). The large one regulates the activity of

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Abbreviations: ADP – adenosine-5'-diphosphate; ATP – adenosine-5'-triphosphate; Chl – chlorophyll; F_v/F_m – maximal photochemical efficiency of PS2; F'_v/F'_m, – efficiency of excitation energy captured by open PS2 centres; PCR – polymerase chain reaction; 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; *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; Γ – CO_2 compensation concentration; Φ_{PS2} – quantum yield of electron transport in PS2.
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RuBPCO in response to radiation-induced changes in both the adenosine-5'-diphosphate (ADP)/ATP ratio and the redox potential in stroma *via* thioredoxin-*f* (Zhang and Portis 1999, Zhang *et al*. 2002, Portis 2003). Although the ATP hydrolysis and RuBPCO activation of both the isoforms are regulated by irradiation/dark *via* ADP/ATP ratio changes, the large isoform is more susceptible to inhibition by ADP (Shen *et al*. 1991). In contrast, the small isoform alone is not regulated by irradiance (Zhang and Portis 1999). However, mixing both isoforms together *in vitro*, the activity changes of the large isoform induced by irradiance *via* reduction/ oxidation change in stroma are sufficient to regulate the activity of both isoforms (Zhang and Portis 1999).

Considering that the large isoform of RuBPCO-A plays more important role than the small one in regulating RuBPCO activity under various environmental conditions (Portis 2003), we investigated the effect of increasing the amount of large isoform of RuBPCO-A on photosynthesis. The cDNA of rice RuBPCO-A large isoform (*rca*) under the control of light-inducible, organ-specific RuBPCO small subunit gene (*rbc*S) promoter was inserted into its genome *via* the *Agrobacterium*-mediated transformation method.

Materials and methods

Construction of a transformation vector: The cDNA of *rca* was obtained by PCR amplification using a pair of designed primers according to the sequences of rice RuBPCO-A gene (Accession No. OSU74321). A 1.6-kb magnified fragment verified by sequencing was inserted into the plasmid pCAMBIA 1301 under control of the rice *rbc*S promoter from the plasmid pGRN73, which was kindly provided by Prof. Wu Ray, Cornell University, USA. A binary recombinant plasmid pCBrbcSRca (Fig. 1) containing hygromycin phosphotransferase gene was introduced into *Agrobacterium tumefaciens* strain LBA4404 by tri-parental mating method (Ditta *et al*. 1980).

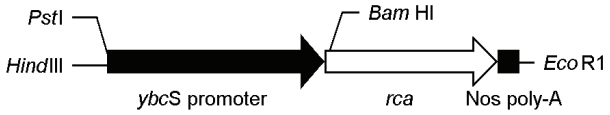

Fig. 1. Construction of plant expression vector pCBrbcSRca. The cDNA of RuBPCO-A of *Oryza sativa* (*rca*) was inserted into pCAMBIA 1301 under the control of RuBPCO small subunit (*rbc*S) promoter and termination of 35S CaMV polyadenylation sequence. The *EcoR*1 and *Hind*III sites were used during the process of pCBrbcSRca construction.

Transformation, selection, and regeneration of rice transformants: *Oryza sativa* f. *japonica* cv. Nipponbare was obtained from the Institute of Genetics and Developmental Biology, Chinese Academy of Sciences. *Agrobacterium tumefaciens* LBA4404 carrying the binary vector pCBrbcSRca was used to infect calli. Screening the calli was carried out in the presence of 50 g m^{-3} hygromycin on NB-medium (Rance *et al.* 1994). Regenerated young plantlets were grown in pots at 30/25 °C under a 16/8 h (light/dark) photoperiod.

Molecular analyses of the rice transformants: Genomic DNA was isolated from leaf tissue by SDS method (Pich and Schubert 1993). The polymerase chain reaction (PCR) primers for amplification of the 1.6-kb *rca* fragment were 5'-ATC ATC GAC TTT CAG CAA ATT AAG A-3' and 5'-CTT GTC ATG CCC AGC TAT GG-3'. A 0.8-kb fragment of hygromycin phosphotransferase gene was also amplified with a pairs of primers 5'-AAA AAG CCT GAA CTC ACC GC-3' and 5'-CGA AAT TGC CGT CAA CCA AG-3'. The reaction was conducted in a *touchgene* thermocycler (UK) initiation at 94 °C for 3 min (denaturation of DNA), then 35 cycles at 94 °C for 0.5 min (denaturation), 58 °C for 1 min (hybridisation of primers), 72 °C for 1.5 min (polymerase reaction), and extension at 72 °C for 10 min (termination). The probe used for Southern blotting was a 687 bp long *rca* fragment. Primers used to produce the probe were 5'-ACC GTG AGG CGG CAG ACA T-3' and 5'-CTT GCC CGT AGA AGG AAC CA-3'. Genomic DNA was digested by *Hind*III, separated on agarose gel, and blotted to *nylon*$^{+}$ membranes. Southern blotting was done according to Sambrook *et al*. (1989).

For protein analyses, transgenic rice leaf tissue was ground into powder in liquid nitrogen and further ground in extraction buffer containing 0.1 mM Tris-HCl, 10 mM $MgCl_2$, 1.0 mM Na_2EDTA, 20 mM mercaptoethanol, 20 kg m^{-3} polyvinylpyrrolidone, pH 7.8. Protein content was determined according to Alexander *et al*. (1985). Total soluble protein was separated by 15 % SDS polyacrylamide gel electrophoresis, and then transferred to a nitrocellulose membrane for Western blotting. The rabbit antiserum against rice RuBPCO-A was provided by Prof. Daquan Xu and Liren Li (Ruhang *et al*. 1997), Institute of Plant Physiology and Ecology, Chinese Academy of Sciences. Western blotting was conducted after Sambrook *et al*. (1989).

Germination test and mass of the transgenic rice seeds: Seeds of T_1 generation were germinated in the presence of hygromycin. When 3–4 leaves emerged, the plantlets were transplanted into soil in a greenhouse with 10 replications of each line. Plant height and the number of leaves had been recorded since then. Mass of 1 000 grains was determined.

Net photosynthetic rate, P_N was determined on the attached 2nd upright leaf of T_1 rice with five replications using an open system (*Ciras-1*, *PP System*, UK) when the

flag leaf emerged but was not fully expanded. P_N was determined as irradiance- and CO_2-response curves. Apparent photon efficiency was calculated as the initial linear slope of the irradiance-response curve at the conditions of 25 °C, 360 μmol(CO_2) mol^{-1} concentration, and a range of irradiances. For the CO_2-response curve, various CO_2 concentrations were realised by mixing pure CO_2 with CO_2-free air at 80 % relative humidity and 25 °C within leaf chamber. Photon flux density was 1 000 μmol m^{-2} s^{-1} (saturation irradiance for the greenhouse-grown rice). Carboxylation efficiency, the initial linear slope of the CO_2 response curve, was calculated according to Tenhunen *et al*. (1984).

Chlorophyll (Chl) fluorescence was measured at room temperature (25 °C) with a portable fluorometer (*PAM-2000*, *Walz*, Effeltrich, Germany) according to Genty *et al*. (1989). After 30-min dark-adaptation, the fluorescence of the 2nd upright detached leaf was measured in at least five replications for each line. Fluorescence nomenclature was adopted from van Kooten and Snel (1990) and fluorescence parameters were calculated: (*a*) maximum photochemical efficiency of photosystem 2 (PS2): $F_v/F_m = (F_m - F_0)/F_m$, (*b*) efficiency of excitation energy captured by open PS2 centres: $F_v'/F_m' = (F_m' - F_0')/F_m'$, (*c*) quantum yield of electron transport in PS2: $\Phi_{PS2} = (F_m' - F_s)/F_m'$, (*d*) proportion of excitation energy dissipated as thermal energy: $q_N = 1 - (F'_m - F'_0)/(F_m - F_0)$, (*e*) proportion of excitation energy captured by PS2 and used in photochemical electron transport: $q_p = (F_m' - F_s)/(F_m' - F_0')$.

Determination of RuBPCO activities: Leaf samples were taken at midday and immediately frozen in liquid nitrogen. About 1 g leaf tissue was ground into powder in a mortar and a pestle in liquid nitrogen and further ground in 10 cm^3 extraction buffers (0.1 mM Tris-HCl, 10 mM $MgCl_2$, 1.0 mM Na_2EDTA, 20 mM mercaptoethanol, 20 kg m^{-3} polyvinylpyrrolidone, pH 7.8) in the presence of quartz granules at 0 °C. The mixture was centrifuged at 11 000×*g* for 10 min and the supernatant was used for measuring the activity of RuBPCO according to the method of Larson *et al.* (1997). Reaction buffer contained 100 mM Tris-HCl (pH 7.8), 10 mM $NaHCO_3$, 20 mM $MgCl_2$, 10 mM dithiothreitol, 0.75 mM 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, glyceraldehyde-3-phosphate dehydrogenase, and glycerol-3-phosphate dehydrogenase. For RuBPCO initial activity analysis, 20 mm^3 of extraction buffers were added into 930 mm^3 of reaction buffers at 25 °C. Then, 50 mm^3 of 40 mM RuBP was added into the mixture quickly. Absorbance of NADH was measured with spectrophotometer at 340 nm. For RuBPCO total activity analysis, the same method as for RuBPCO initial activity analysis was used except for reaction time of 20 min.

Results

Hygromycin-resistant test and molecular identification of putative transgenic rice: Germination experiment showed that 191 out of 257 seeds of T_1 transgenic rice were resistant to hygromycin (Fig. 2*A,B*). Total 71 regenerated transgenic plants were identified by PCR reaction, among them 52 plants contained the 1.6-kb *rca* fragment (Fig. 2*C*). The 0.8-kb fragment of hygromycin phosphotransferase gene was also amplified (Fig. 2*D*). Two or three bands of genomic DNA that hybridized with *rca* were detected in the transgenic rice and only one band was detected in the control rice by Southern blotting (Fig. 2*E*). This suggested that *rca* is integrated into the genome of rice. Western blot analysis showed that in the transgenic rice the contents of 47 kDa large isoform were more than that of 43 kDa small isoform, while in the non-transgenic rice the amount of 47 kDa large isoform was almost equal to that of 43 kDa small one (Fig. 2*F*). This suggests that the inserted *rca* is stably expressed in the transgenic rice.

Growth of the transgenic rice plants: During the initial growth process, both the transgenic rice lines 34 (T34) and 40 (T40), and the controls grew similarly in height. However, after the 3rd upright leaves appeared, the transgenic lines became taller than the control plants. When the flag leaves fully expanded, the average height of T34 and T40 reached 0.99 and 1.01 m, respectively, while that of the control rice was 0.94 m (Table 1). The heading time of T34 and T40 was 112 d after planting into the soil, while that of the control rice was 119 d (Table 1). Consequently, the transgenic rice plants also flowered and set seeds ahead of the control rice. One thousand grains weighed 22.97 (T34) and 20.51 (T40) g (Fig. 3), respectively, which was significantly more than that of the non-transgenic rice (19.40 g).

***P_N* response to irradiance**: Higher P_N was observed in the transgenic lines under a wide range of irradiances. Under saturation irradiance (1 000 μmol m^{-2} s^{-1}), the P_N of T34 and T40 was 13.8 and 13.9 μmol(CO_2) m^{-2} s^{-1}, which was significantly more than 9.5 μmol(CO_2) m^{-2} s^{-1} of the control rice. Apparent quantum efficiency, which indicates the ability of leaves to make use of photon energy at low irradiances, was calculated at the irradiance below 200 μmol m^{-2} s^{-1}. As shown in Fig. 4*A*, the apparent quantum efficiency of both the transgenic lines was 0.025 mol(CO_2) mol^{-1}(photon) and was not significantly different from the control, 0.020 mol(CO_2) mol^{-1}(photon).

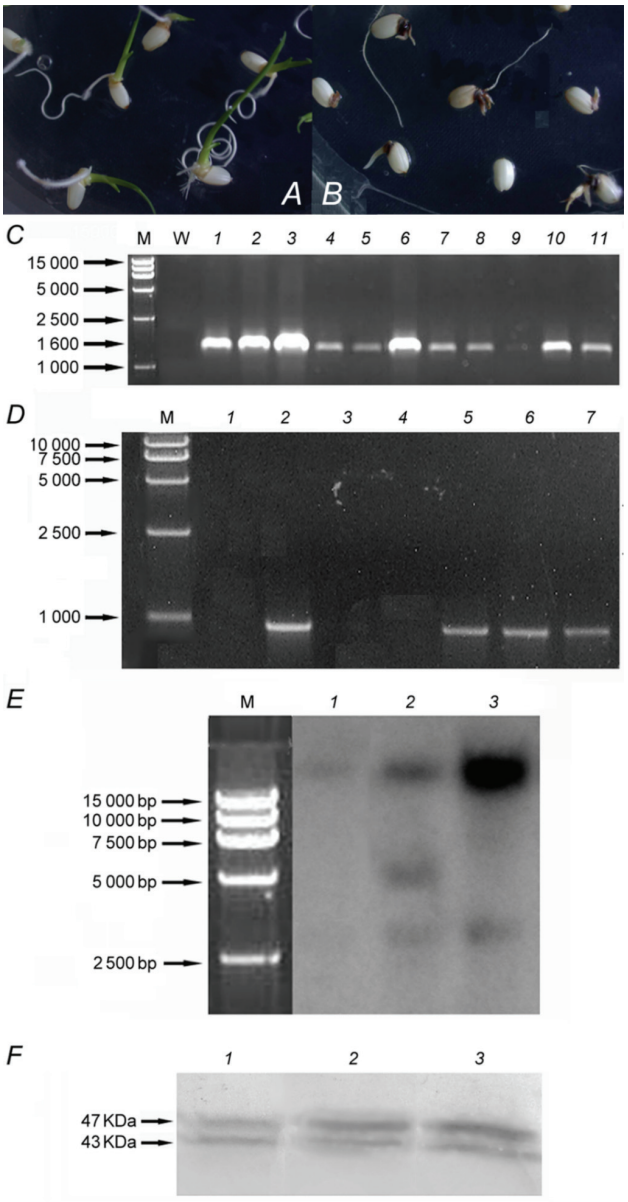

Fig. 2. *A,B*: Seeds of the T_1 transgenic plants (*A*) and control rice (*B*) germinated in the presence of hygromycin. *C*: Polymerase chain reaction (PCR) for amplification of 1.6 kb *rca* fragment. M – DL-15 000 DNA size marker (Takara); W – control rice; lanes *1-11* – PCR products from the different transgenic rice. *D*: PCR amplification of 841 bp fragment of hygromycin phosphotransferase gene. M – DL-15 000 DNA size marker (Takara); lane *1* – PCR products from the control rice; lanes *2–7* – PCR products from the different transgenic rice. *E*: Southern blot analysis of the transgenic lines T34, T40, and control rice. Fifteen μg of genomic DNA were digested by *Hind*III and hybridized with 687 bp *rca* probe. M – DL-15 000 DNA size marker (Takara); lane *1* – the control rice; lanes *2–3* – the transgenic lines T34 and T40, respectively. *F*: Western blot analysis of the transgenic lines T34 and T40, and of the control rice. Ten μg of total soluble protein were separated by 15 % SDS-PAGE in each lane. Lane *1* – the control rice; lanes *2–3* – the transgenic lines T34 and T40.

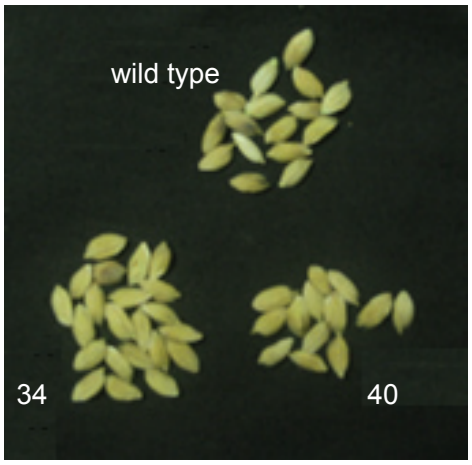

Fig. 3. Grain size of the T_1 transgenic rice lines T34 and T40, and the control (wild type).

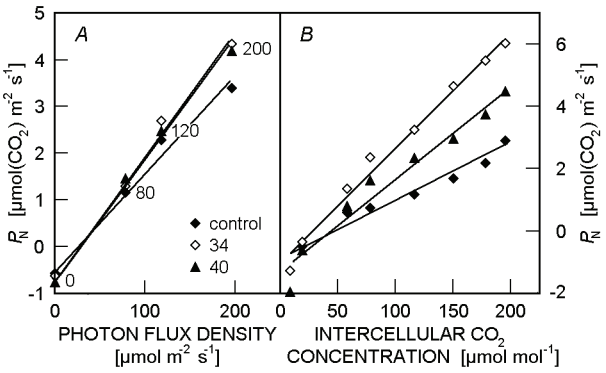

Fig. 4. Apparent quantum efficiency (*A*) and carboxylation efficiency (*B*) of the transgenic rice lines T34 and T40, and the control plants.

***P*$_N$ response to CO$_2$**: Under different CO_2 concentrations and saturation irradiance (1 000 μmol m^{-2} s^{-1}), P_N was significantly higher in transgenic plants than in the controls. The carboxylation efficiency, representing the activity of RuBPCO, was 0.036 and 0.029 μmol(CO_2) m^{-2} s^{-1} Pa^{-1} for the transgenic lines T34 and T40, respectively, and 0.018 μmol(CO_2) m^{-2} s^{-1} Pa^{-1} for the controls at low CO_2 concentration (less than 200 μmol mol^{-1}) (Fig. 4*B*). The CO_2 compensation concentration (Γ) is the concentration where photosynthetic rate equals to photorespiration plus respiration. In the transgenic plants, Γ was 49.8±1.2 (T34) and 51.6±1.7 (T40) μmol(CO_2) mol^{-1}, which was similar to 54.5±1.8 μmol(CO_2) mol^{-1} in the control.

Change of Chl fluorescence in the transgenic rice: In Table 2, maximum photochemical efficiency of PS2 (F_v/F_m) showed no difference in both the transgenic and the control plants. Φ_{PS2}, representing the activity of PS2, was 9.45 % (T34) and 10.95 % (T40) higher than in the controls. These results suggest that the transgenic plants have a higher capacity for photon energy conversion.

Efficiency of excitation energy capture by open PS2 centres (F'_v/F'_m) of the transgenic plants did not differ from the controls. Photochemical quenching (q_P) increased in both T34 and T40 as compared with the controls, implying that the percentage of open reaction centres of PS2 and the portion of energy participating in CO_2 fixation are increased. Non-photochemical quenching (q_N) in the transgenic plants was not different from that of the control plants, suggesting that the former had the ability of dissipating excessive energy similar to the latter.

Table 1. Growth characteristics of transgenic rice lines T34 and T40 in comparison with controls.

Traits	Control	T34	T40
Plant height [m]	0.94±0.01	0.99±0.02	1.01±0.02
Heading time [d]	119	112	112
Mass of 1 000 grains [g]	19.40	22.97	20.51

Table 2. The parameters of fluorescence induction in the 2nd upright leaves of both the transgenic and control rice plants. Means ± SE from 5 leaves. The significant levels of difference between the transgenic and the control plants are indicated by $^{*}p<0.05$ and $^{**}p<0.01$.

Lines	F_v/F_m	Φ_{PS2}	F'_v/F'_m	q_P	q_N
Control	0.843±0.005	0.402±0.020	0.580±0.019	0.695±0.023	0.746±0.013
T34	0.846±0.004	0.440±0.008**	0.593±0.015	0.741±0.007*	0.734±0.023
T40	0.838±0.007	0.446±0.007**	0.601±0.009	0.732±0.001*	0.722±0.012

Activities of RuBPCO: RuBPCO initial activity of the transgenic lines T34 and T40 were 92.94 and 64.71 % higher than in the controls, respectively (Table 3). However, RuBPCO total activity (Table 3) and phospho*enol*pyruvate carboxylase (PEPC) activities (data not shown) of the transgenic lines were not different from the controls.

Table 3. RuBPCO activities [mmol(CO_2) kg^{-1}(protein) s^{-1}] of transgenic and control rice plants.

RuBPCO activity	Control	T34	T40
initial	14.17	27.33	23.33
total	33.50	34.66	32.17

Discussion

Much attention has been focused on the biochemistry and molecular biology of RuBPCO-A since its discovery. Achieved results show that RuBPCO-A may exert considerable control of photosynthesis at saturating irradiance (Jiang *et al*. 1994). So far, the experiments investigating the effect of RuBPCO-A on photosynthesis have been done by utilizing *rca* mutants of *Arabidopsis* and tobacco with reduced contents of RuBPCO-A (Somerville *et al*. 1982, Jiang *et al*. 1994, Eckardt *et al*. 1997, He *et al*. 1997). Their results show decreased activity of RuBPCO, impaired CO_2 assimilation rate, and retarded growth rate in those mutants. We investigated the effect of elevating the amount of large isoform of RuBPCO-A on photosynthesis in rice with a strategy different from those of *rca* mutants. Our results indicated that this strategy is beneficial to improvement of plant photosynthesis and growth.

We found that the RuBPCO initial activity and the carboxylation efficiency in the transgenic rice lines T34 and T40 were higher than in the control plants (Table 3, Fig. 4*B*), indicating that the RuBPCO activity is improved and more CO_2 can be assimilated. However, the amount of RuBPCO in the transgenic plants was not different from that of the controls because of unchanged RuBPCO total activity. Regarding the carboxylation/oxygenation specificity of RuBPCO, it was not altered in the transgenic plants on account of unchanging CO_2 compensation concentration in all plants studied. These results indicate that increasing the content of RuBPCO-A has no effect on the amount of RuBPCO, but has an effect on the activity of RuBPCO.

At saturating irradiance, P_N in the transgenic lines T34 and T40 were 45.26 and 46.32 % higher than that in the controls, respectively, which is consistent with the elevated RuBPCO initial activity in those plants. This result supports the hypothesis that the RuBPCO activity determines photosynthetic rate at saturating irradiance (Quick *et al*. 1991, Stitt *et al*. 1991). Considering the Chl fluorescence, Φ_{PS2} and q_P were also elevated, indicating that there is a larger proportion of active PS2 reaction centres present in the transgenic rice plants and more excitation energy can be captured. Thus, more energy is used for photochemical reaction and propels the electron transport to form more ATP and reduced nicotinamide

adenine dinucleotide phosphate to be used for CO_2 assimilation. Therefore, with the increase of RuBPCO activity, the photosynthetic capacity is improved. However, F_v/F_m, q_N, F'_v/F'_m, and apparent quantum efficiency in the transgenic plants were not altered, revealing that the ability of antennae to capture photon energy, the ability of PS2 to dissipate excessive energy, and the ability of leaves to make use of quantum at low irradiance were not influenced.

The transgenic rice plants we obtained were higher, headed and flowered earlier, and had heavier grains. Therefore, a positive relationship between photosynthesis and plant growth occurs in the transgenic rice with increased contents of RuBPCO-A. Plants that grow with long-term CO_2 enrichment usually have a decreased activity of RuBPCO and P_N, but accumulate more saccharides (Stitt 1991, Long and Drake 1992, Bowes 1993, Griffin and Seemann 1996, Cheng *et al*. 1998). Under this condition, it results in increased leaf numbers, leaf area index, effective tillers, and, finally, accelerated life cycle (Carter and Peterson 1983, Omer and Horvath 1983, Baker *et al*. 1990). Mechanism for those phenomena remains elusive, but they provide an explanation that large saccharide accumulation promotes plant growth. We show that the number of leaves and effective tillers of both the transgenic and the control rice (data not shown) were not different from each other. The promoted plant growth rate in the transgenic rice might also be the reason of increased saccharide complex due to the improved photosynthesis.

In summary, we adopted the research strategy to enhance the contents of large isoform of RuBPCO-A in rice. We found a significant enhancement of the photosynthetic capacity and promotion of rice growth. This would provide an opportunity to improve the yield of rice *via* RuBPCO-A.

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