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BRIEF COMMUNICATION

Photosynthetic characteristics and effect of ATP in transgenic rice with phospho*enol*pyruvate carboxylase and pyruvate orthophosphate dikinase genes

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Abstract

In the untransformed rice (WT) and transgenic rice with the PEPC and PPDK genes (CK) we determined activities of C_4 photosynthetic enzymes, photosynthetic response to irradiance and temperature, the metabolic index of active oxygen, and the yield component factors. The activities of C_4 photosynthetic enzymes in WT were very low, while those of corresponding enzymes in CK were highly observable. Moreover, after adenosine triphosphate (ATP) treatment, and under high irradiance and high temperature, the net photosynthetic rate of CK increased by 17 and 12 %, respectively, as compared to that achieved without ATP treatment. The resistance of CK against photo-oxidation was enhanced under these conditions, and CK yield increased by 15 %. ATP treatment enhanced the photosynthetic productivity of CK, thereby proving that ATP is the key factor in enhancing the photosynthetic capacity of transgenic rice with C_4 gene. Our new technical approach can be used in breeding rice with high photosynthetic efficiency and high grain yield.

Additional key words: active oxygen; ATP; enzymes of C_4 photosynthetic pathway; irradiance; net photosynthetic rate; *Oryza*; temperature.

Several genes encoding C_4 photosynthetic enzymes, such as phospho*enol*pyruvate carboxylase (PEPC), pyruvate orthophosphate dikinase (PPDK), and NADP-malic enzyme (ME), have been successfully introduced into the C_3 rice (Fukayama *et al*. 1999, Ku *et al.* 1999, Tsuchida *et al.* 2001, Huang *et al*. 2002). Previous studies reported high photosynthetic capability of the PEPC transgenic rice (Chi *et al.* 2001, Jiao *et al. 2*001, 2002) and its tolerance to photo-oxidation under high irradiance (Jiao *et al.* 2005). In addition, the photon-saturated photosynthetic rate of transgenic rice with the PEPC and PPDK genes (CK) was not enhanced significantly, as compared to that in PEPC transgenic rice plants (Chi *et al.* 2001). Photosynthetic activity of rice may be affected by ATP content in leaves (Yong *et al*. 2007). Our present study reports on the effects of adenosine triphosphate (ATP) on the characteristics of photosynthesis, photo-oxidation, and yield component factors of CK, in order to demonstrate whether or not ATP is a key factor in enhancing photosynthetic productivity of transgenic rice with the C_4 gene. We also introduce a new approach that can be used in breeding rice with high photosynthetic efficiency and grain yield.

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Abbreviations: ATP – adenosine triphosphate; CK – transgenic rice with the PEPC and PPDK genes; DTT – dithiothreitol; MDA – malondialdehyde; MDH – NADP-malate dehydrogenase; ME – NADP-malic enzyme; NADH – nicotinamide adenine dinucleotide phosphate; $O_2^{\cdot-}$ – superoxide anion radical; PEPC – phospho*enol*pyruvate carboxylase; PCR – polymerase chain reaction; PFD – photon flux density; POD – peroxidase; PPDK – pyruvate orthophosphate dikinase; PVP – polyvinylpolypyrrolidone; RuBPC – ribulose-1,5-biphosphate carboxylase; SOD – superoxide dismutase; WT– wild type.
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Seeds of CK, obtained by conventional hybridization between PEPC and PPDK transgenic rice, and wild type (WT) germplasm, were grown in 4 000 cm^3 pots in a net house in Nanjing. There were five hills per pot and one seedling per hill. Plants were watered and fertilized in soil conventionally. The 4 μM ATP was used to spray the upper surfaces of the attached leaves of the rice for three times at elongation stage and heading stage, respectively. The rice was reaped at maturity and dried for analysis of yield components. According to Wang and Fang (2003), total DNA was extracted using the improved method of SDS dram extraction. Through the software of *GENTYX* on the basis of the analysis of maize C_4-specific PEPC and PPDK genome di-sequence, two pairs of primers were designed as follows: PEPC (PC1: 5'-GAT CTG GAC GAA GAG CAT CAG GGG C-3'; PC2: 5'-TGA GGA GAG AGG TGG ATT TGG GT TG-3'), PPDK (PK1: 5'-TAG TTT TCC CTA CCT CAT CAG CC- 3'; PK2: 5'-TTG GAC ATT TTA CTC TTC CTT TA-3'). The cycling parameters for PCR were (*a*) 94 ºC for 5 min (initial de-naturation), (*b*) 94 ºC for 45 s (de-naturation), (*c*) PEPC primers: 60 ºC for 40 s (annealing); PPDK primers: 56 ºC for 40 s (annealing), (*d*) 72 ºC for 60 s (extension), and (*e*) 72 ºC for 10 min (final extension). The steps *b*−*d* were repeated for 30 cycles. PCR amplification was performed with a *Gene Amp PCR System* 9600 (*Perkin Elmer*, USA). The PCR amplification was carried out in a 35 mm^3 reaction mixture containing 2.0 mm^3 of 1 mM of each primer set (about 50 ng) of DNA as a template, 2.0 mm^3 of 2 mM each of dNTP, 3.5 mm^3 of 10×PCR buffer, 0.2 mm^3 of Taq DNA polymerase (5 U per mm^3) (*TaKaRa,* China*)*, and 16.3 mm^3 of distilled H_2O. PCR products were electrophoresed in a 1.4 % agarose gel.

PEPC activity was assayed according to the method of Gonzales *et al.* (1984) and Ku *et al.* (1999). About 0.25 g leaf tissue was harvested from flag leaves at heading stage in the light and quickly ground in 1.5 cm^3 extraction buffer containing 50 mM Tris-HCl pH 7.5, 10 mM $MgCl_2$, 5 mM dithiothreitol (DTT), 2 % (m/v) insoluble polyvinylpolypyrrolidone (PVP), and 10 % glycerol. After total maceration, the crude extract was centrifuged at 13 000×*g* for 10 min at 4 ºC, and the supernatant was used immediately for assay of various C_4 enzymes. PEPC was assayed spectrophotometrically at room temperature (30 ºC) in a mixture containing 50 mM Hepes-KOH pH 8.0, 10 mM $NaHCO_3$, 5 mM $MgCl_2$, 1.5 units NAD-malate dehydrogenase (MDH), 0.2 mM NADH, and 20–50 mm^3 enzyme extract. The reaction was started by adding PEP to a final concentration of 2 mM. The change in NADH was monitored in a spectrophotometer at wavelength of 340 nm. The PPDK activity was examined using the method of Hatch and Slack (1975). RuBPC activity was extracted and assayed according to Kung *et al.* (1980): fresh leaves (0.5 g) were harvested from irradiated plants, cut into small pieces, and immediately homogenized in a pre-chilled pestle and mortar with acid-washed quartz sand in 2.5 cm^3 extraction medium containing 50 mM Tris-HCl (pH 7.5), 1 mM $MgCl_2$, 5 mM DTT, and 2 % insoluble PVP. After mixing the filtrate thoroughly, 40 mm^3 aliquots were used for determination of soluble protein and chlorophyll contents. The homogenate was clarified by centrifugation at 10 000×*g* for 10 min; the clear supernatant was decanted slowly and used for enzyme activity. All these steps were carried out at 4 ºC. Subsequently, the net photosynthetic rate (P_N) of attached leaves under high irradiance of 1 200 $\mu mol \cdot m^{-2} \cdot s^{-1}$ and high temperature of 35 ºC in air was measured using a portable photosynthetic gas analyzer (model *TPS-1*, *PP Systems*, Hitchin, UK). In one evening during a cloudless day, 4 μM ATP was sprayed onto the upper surfaces of the leaves of the rice. The following day, a 1.5 mM photo-oxidation agent methylviologen solution, prepared with 1 % (v/v) *Tween-80* solution, was also coated onto the upper surfaces of the leaves. After which, the strains were placed under weak irradiance (20−30 $\mu mol \cdot m^{-2} \cdot s^{-1}$) in a room for an hour to allow the inhibitors to penetrate into the leaves. In addition, superoxide dismutase (SOD) activity was assayed by the method of Giannopolitis and Ries (1977). One unit of SOD activity is defined as the amount of enzyme that caused 50 % inhibition of the initial rate of NBT reduction. Peroxidase (POD) activity was assayed by the method of Kochba *et al.* (1992). One unit of POD activity is defined as the increase of 0.1 A per min. Measurement of malondiadehyde (MDA) content was according to the method of Heath and Packer (1968), which was calculated by the using $\varepsilon_{532-600nm} = 1.55 \times 10^5\ M^{-1}\ cm^{-1}$, and superoxide anion radical ($O_2^{\cdot -}$) generation rate was measured according to the method of Wang and Luo (1990), which was calculated by the following equation: $O_2^{\cdot -}$ production/reaction time × the amount of protein.

Using the maize genome as positive control, all materials were amplified through a PCR according to the characteristic primers of maize C_4-specific PEPC and PPDK genomes. The 1 800-base pair amplified product of the PEPC gene, and the 971-base pair amplified

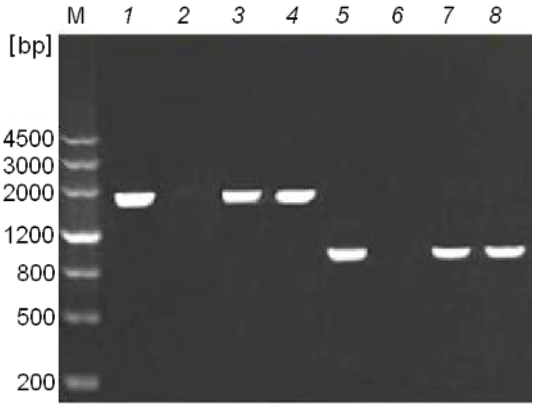

Fig. 1. The PCR analysis of transgenic rice with the PEPC and PPDK genes (lanes *3*, *4* and lanes *7*, *8*, respectively), wild type (lane *2*; lane *6*), and maize (lane *1*; lane *5*). M = markers, *1*−*4* signifies the 1.8 kb-amplified product of the PEPC gene, and *5*−*8* signifies the 0.971 kb-amplified product of the PPDK gene.

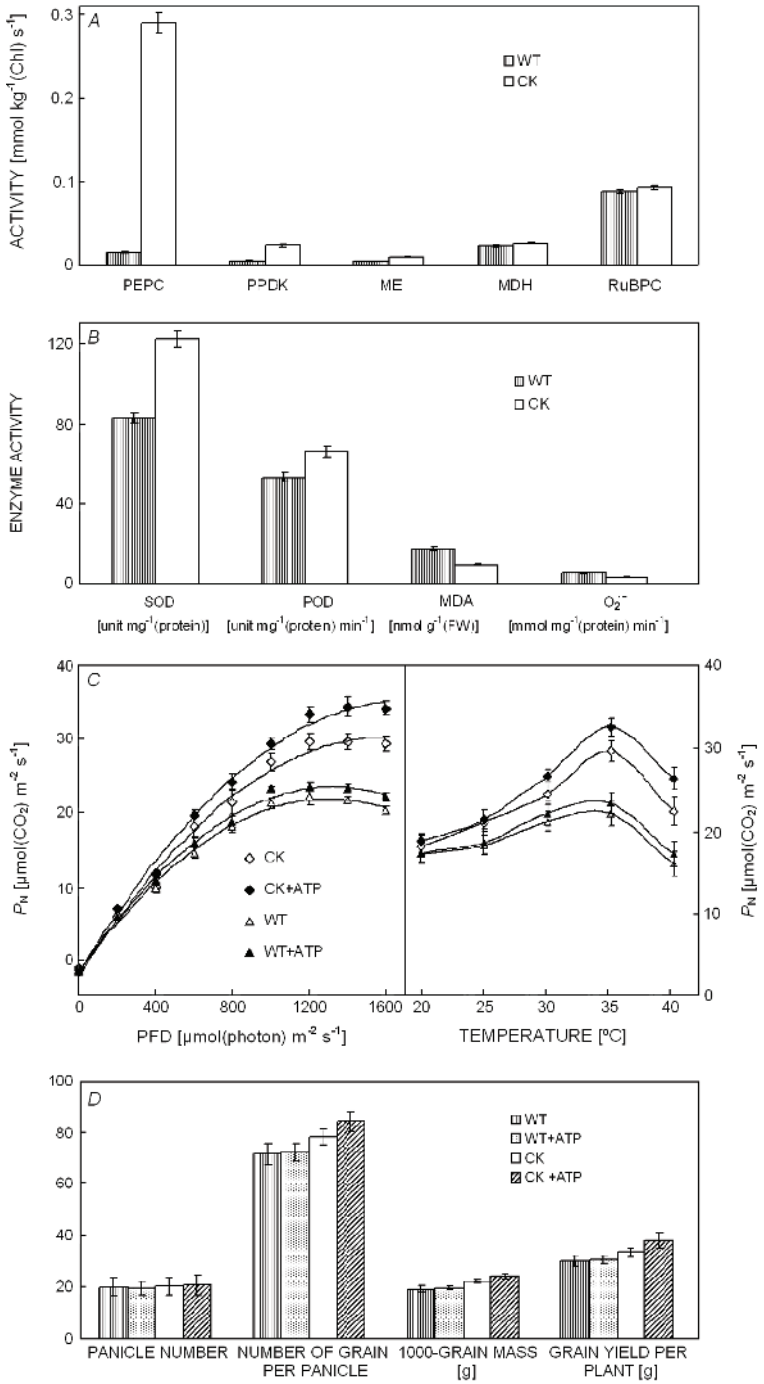

Fig. 2. Comparison of untransformed rice Kitaake (WT) with transgenic rice containing the PEPC and PPDK genes (CK). (*A, B*) Activities of phospho-*enol*pyruvate carboxylase (PEPC), pyruvate orthophosphate dikinase (PPDK), NADP-malic enzyme (ME), NADP-malate dehydrogenase (MDH), ribulose-1,5-bisphosphate carboxylase (RuBPC), superoxide dismutase (SOD), and peroxidase (POD), malonyldialdehyde (MDA) content, and generation rate of superoxide anion radical ($O_2^{\cdot-}$). (*C*) Dependence of net photosynthetic rate (P_N) on photon flux density (PFD), temperature, and effect of added ATP. (*D*) Yield component factors. Means±SE from 5 replicates.

product of the PPDK gene were observed in the maize and the transgenic rice with the PEPC and PPDK genes (CK), respectively, excluding WT (Fig. 1). This suggested that the transgenic rice expressing the maize C_4-specific PEPC and PPDK genes could be precisely selected through PCR.

The activity of the enzyme encoded by the transgene was evidently enhanced in CK, while other enzyme activities were similar to those of the WT rice (Fig. 2*A*). Under high photon flux density (PFD) and high temperature, P_N of CK after ATP treatment increased by 17 and 12 %, respectively, compared to that without ATP treatment (Fig. 2C). The MDA content and the $O_2^{\cdot-}$ generation rate of the CK were lower than those of the WT (Fig. 2*B*). This may be related to the increase in the photo-oxidation-tolerant enzymes SOD and POD. More importantly, after ATP treatment both the number of grain per panicle and 1 000-grain mass of CK were increased by 7.9 and 9.1 %, respectively, as compared with that without ATP. Thus, the grain yield per plan of CK increased cor-respondingly by 15.2 % (Fig. 2*D*).

Our results indicate that the C_4-specific PEPC and

PPDK genes from maize can be highly expressed in the transgenic rice. Under high PFD and high temperature, CK exhibited high P_N after ATP treatment (Fig. 2*C*) and became more tolerant against photo-oxidation (Fig. 2*B*). Moreover, all of these characters allowed the transgenic rice with PEPC and PPDK genes to maintain a higher photosynthetic productivity and produce more grains on a per plant basis (Fig. 2*D*), demonstrating that ATP is the key factor in enhancing photosynthetic capacity in transgenic rice with the C_4 gene.

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