Physiological characteristics of the primitive CO_2 concentrating mechanism in PEPC transgenic rice

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Abstract The relationship between carbon assimilation and high-level expression of the maize PEPC in PEPC transgenic rice was studied by comparison to that in the untransformed rice, *japonica kitaake.* Stomatal conductance and photosynthetic rates in PEPC transgenic rice were higher than those of untransformed rice, but the increase of stomatal conductance had no statistical correlation with that of photosynthetic rate. Under high levels of light intensity, the protein contents of PEPC and CA were increased significantly. Therefore the photosynthetic capacity was increased greatly (50%) with atmospheric CO_2 supply. While CO_2 release in leaf was reduced and the compensation point was lowered correspondingly under CO_2 free conditions. Treatment of the rice with the PEPC-specific inhibitor DCDP showed that overexpression of PEPC and enhancement of carbon assimilation were related to the stability of Fv/Fm. Labeling with $^{14}CO_2$ for 20 s showed more ^{14}C was distributed to C_4 primary photosynthate asperate in PEPC transgenic rice, suggesting that there exists a limiting C_4 photosynthetic mechanism in leaves. These results suggest that the primitive CO_2 concentrating mechanism found in rice could be reproduced through metabolic engineering, and shed light on the physiological basis for transgenic breeding with high photosynthetic efficiency.

Keywords: PEPC transgenic rice, phosphoenopyruvate carboxylase (PEPC), carbonic anhydrase (CA), stomatal conductance, CO_2 concentrating mechanism.

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C_4 plants such as maize have CO_2 concentrating mechanism and higher photosynthetic efficiency than C_3 plants, especially under high light intensity, high temperature and drought conditions. In recent years, due to the rapid development of transgenic technique, different transgenic rice plants with high-level expression of C_4 genes have been created by the successful introduction of genes encoding the key C_4 photosynthetic path enzymes PEPC, PPDK and NADP-ME through agrobacteria-mediated transformation[1−3]. In addition, a PEPC+PPDK transgenic rice plant was created through hybridization[4]. PEPC transgenic rice has exhibited the enhancement of photosynthetic capacity in comparison with untransformed rice [5], and the grain yield per plant has been

shown to be 14%—22%[6] higher, which indicates broad prospects for improving the productivity of rice crops through gene engineering. In this paper, the relationship between the expression of PEPC and photosynthetic metabolism was studied with a view to understand whether a primitive CO_2 concentrating mechanism exists in PEPC transgenic rice. Such a mechanism would provide an experimental basis for the improvement of photosynthetic characteristics of rice crops through biotechnology.

1 Materials and methods

1.1 Plant materials

The 3rd generation PEPC transgenic rice plants were obtained by Ku et al.[1] through introduction of an intact maize gene encoding C_4-specific PEPC into Japanese rice *cultivar kitaake*. The 7th generation of stable germplasms[5] used in this study was procured from Nanjing City and Sanya City, China, and grown in a net room at the Jiangsu Academy of Agricultural Sciences. The seeds were sown in the first ten days of May. There were five hills in each pot and one seedling per hill. Plants were watered and fertilized conventionally.

1.2 Measurement of CO_2 exchange in leaves

1.2.1 Photosynthetic CO_2 exchange rates in leaves. These were monitored with the LI-6200 portable photosynthesis system, a CO_2 analyzer. The light source was provided by the metal halogen lamp (24 V, 250 W) of the epidiascope. The light intensity was regulated by the distance between the lamp and the chamber, and a water trough was maintained between the lamp and the chamber for heat insulation. Different CO_2 concentrations were created and regulated by a gas dilution instrument. The light/CO_2 curve of photosynthesis was drawn from experimental data. According to the data of initial stage of CO_2 response curves, linear regression analysis was performed in which the slope was equal to carboxylation efficiency. Stomatal conductance and intercellular CO_2 concentration in leaves were recorded directly from the photosynthesis system.

1.2.2 Measurement of CO_2 exchange under high light intensity and free-CO_2 (N_2 flushing). CO_2 concentration in leaf chamber and intercellular was chased, recorded and measured using an infrared gas analyzer (S-225, UK) under 1000 μmol/(m^2 • s) at 28℃.

1.2.3 Measurement of photosynthetic O_2 evolution in leaves. Flag leaves were cut into 1 cm^2 segments and put into solution (100 mmol/L Hepes-KOH, pH 7.8 and 20 mmol/L $NaHCO_3$), and kept in an environment of reduced air pressure. The rate of O_2 evolution was measured with an SP-2 oxygen electrode (Chinese Academy of Sciences, Shanghai) under PAR 800 μmol/(m^2 •s) at 28℃.

1.3 Measurement of enzyme protein content

1.3.1 Protein extraction and electrophoresis. Rice leaves (0.5 g) were harvested and ground

into extraction medium (50 mmol/L Tris-HCl, pH 7.5 containing 1 mmol/L $MgCl_2$, 5 mmol/L DTT and 2% (*w*/*v*) insoluble PVP). After complete maceration, the crude extract was centrifuged at 13000 *g* for 10 min, and proteins were extracted from the supernatant. SDS-PAGE electrophoresis was conducted with 10 mg protein sample per well at room temperature and constant voltage (80 V) for 2 h.

1.3.2 Western blot analysis. The gel was soaked in transfer buffer (150 mmol/L glycine, 20 mmol/L Tris-HCl, 0.01% SDS, 4% methanol, pH 8.8). Then a Nitrocellulose membrane (NC) was applied and the setup was electrophoresed at 250 mA for 1.5 h, in order to transfer the protein onto the NC. The transferred NC was soaked in blocking buffer (1% nonfat milk powder, 20 mmol/L Tris-HCl, pH 8.0, 100 mmol/L NaCl) at room temperature for 2 h before addition of the antibody (anti- PEPC, -CA, -Rubisco LSU or Rubisco SSU) and incubated at room temperature for 1 h. Following this incubation, the NC was washed by PBS (3X, 10 min each), and goat anti-rabbit Ig G (1:1500) was added to blocking buffer for 1 h. Then the NC was washed with TBS (20 mmol/L Tris-HCl pH 8.0, 100 mol/L NaCl; 3X for 10 min each). Finally, the NC was developed using AP (100 mmol/L Tris, 100 mol/L NaCl, 5 mmol/L $MgCl_2$, BCIP/ NBT).

1.4 Application of inhibitor DCDP

20 mmol/L of the PEPC-specific inhibitor 3, 3-dichloro-2-(dihydroxyphosphinoylmethy1) propenoate (DCDP) in 1% (*v*/*v*) Tween-80 was smeared on the leaf surface. The control was 1% (*v*/*v*) Tween-80 alone. The plants were then put in the dark or under a weak light of 20—30 μmol/(m^2 • s) for 2 h, so the application could penetrate into the leaves.

1.5 $^{14}CO_2$ fixation and identification of intermediary metabolites

$^{14}CO_2$ fixation experiments were conducted on attached leaves according to the methods of Chapman et al. (1974)[7] and Winter et al. (1982)[8]. Leaves were enclosed in a transparent chamber and pre-illuminated at 1200 μmol/(m^2 • s) and 30℃ for 5 min. The chamber was then sealed and $^{14}CO_2$ (400 μci) was injected into the chamber for a 20 s lighted pulse, then the tissue was frozen in liquid N_2. Intermediary metabolites were extracted from frozen leaves by boiling in 80% (*v*/*v*) ethanol for 15 min, followed by extractions (2X) with 20% (*v*/*v*) ethanol. The ethanolic extraction was then dried under reduced pressure at 30—35℃ and resuspended in 1 mL of 50% ethanol (*v*/*v*). After extraction, an aliquot was taken to determine total fixed $^{14}CO_2$. Standard products were 3-phosphoglyceric acid (3-PGA), malate, asperate and sucrose. According to the method of Wang[9], the labeled products were separated by two-dimensional chromatography on Whatman 3 mm filter paper and autoradiograms were prepared to identify the ^{14}C-labeled products. The radioactivity in the various products was determined by liquid scintillation spectroscopy after placing excised labeled areas in vials containing 50 mg PPO in 10 mL toluene. This experiment was conducted at the Institute of Applied Atomic Energy at the Chinese Academy of Agricultural Science.

1.6 PSII photochemical efficiency

Attached leaves were dark adapted for 30 min and PSII photochemical efficiency (Fv/Fm) was measured by an FMS-2 portable fluorescence meter.

2 Results

2.1 The expression of main photosynthetic enzymes in PEPC transgenic rice and untransformed rice

Fig. 1 shows that the contents of PEPC and CA protein were low in untransformed rice (control) under moderate light intensity (0 h) and increased somewhat under high light intensity for 2—10 h. After the maize gene was introduced into rice, the PEPC transgenic rice exhibited a higher content of PEPC under moderate light intensity, which was induced to increase further under high light intensity. The CA protein showed a similar trend. The protein expression in both genotypes was similar to previously reported changes in an activity[5].

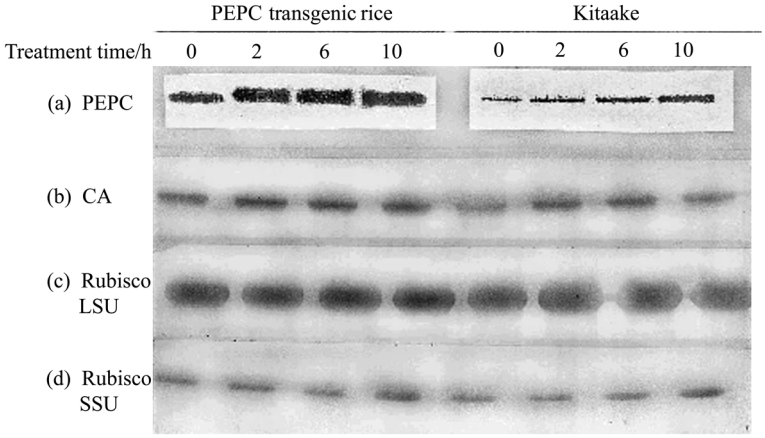

Fig. 1. The protein content of PEPC, CA and Rubisco (Western blot analysis) in PEPC transgenic rice and untransformed rice. Moderate light intensity was 600 μmol • m^{-2} • s^{-1} before treatment (0 h); high light intensity was 1400 μmol • m^{-2} • s^{-1} after treatment of 2, 6, 10 h.

In addition, Rubisco LSU (a key enzyme in C_3 pathway) was different, and Rubisco SSU did not change appreciably. These results demonstrated that the enzymes related to CO_2 concentrating in PEPC transgenic rice were activated to high-level expression under high light intensity.

2.2 The relationship between stomatal conductance and photosynthetic rate in PEPC transgenic rice and untransformed rice

Fig. 2 shows that stomatal conductance and photosynthetic rates in both transgenic and control rice enhanced with the increase of light intensity (PAR). Therefore stomatal conductance correlated with photosynthetic rate in untransformed rice under different light intensity with a correlation coefficient r^2 was equal to 0.800**. The coefficient in PEPC transgenic rice was 0.606**, which indicates that a significant positive correlation exists between stomatal conductance and

photosynthetic rate. In order to elucidate whether this relationship was parallel or causal, the correlation coefficient between the increase of stomatal conductance and the increase of photosynthetic rate was tested statistically, and found to carry a coefficient of 0.06, which indicates no correlation. Although PEPC transgenic rice showed 80% more stomatal conductance (fig. 2(a)) under the light intensity of 700—1000 μmol/(m^2 • s) as compared with untransformed rice, the photosynthetic rate did not increase correspondingly (fig. 2(b)). When the light intensity was higher than 1000 μmol/(m^2 • s), stomatal conductance decreased in both genotypes. Indeed, while the photosynthetic rate in PEPC transgenic rice increased to high level under PAR of 1200—1400 μmol/(m^2 • s), the photosynthetic rate increased by about 50 percent. The above results demonstrated that the increment of photosynthetic capacity in PEPC transgenic rice under high light intensity might not be due to the increment of stomatal conductance supplying more CO_2, but the enhancement of C_4 metabolism to utilize CO_2 more effectively.

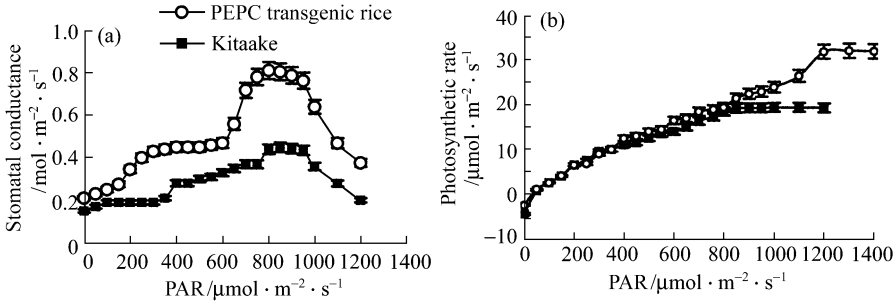

Fig. 2. The change of stomatal conductance and photosynthetic rate in untransformed rice Kitaake and PEPC transgenic rice under different light intensities (PAR). The correlation coefficient between stomatal conductance and photosynthetic rate in untransformed rice was 0.800** , n = 23. The correlation coefficient between stomatal conductance and photosynthetic rate in PEPC transgenic rice was 0.606**, n = 25. Compared with untransformed rice, the correlation coefficient between the increase of stomatal conductance and photosynthetic rate in transgenic rice was 0.06, which indicates no correlation, n = 25.

2.3 The CO_2 exchange characteristic in PEPC transgenic rice and untransformed rice

The rice leaves were treated under high light intensity and at different CO_2 concentrations (fig. 3). Under atmospheric CO_2 (350 μmol/mol), the carboxylation efficiency of untransformed rice was 0.077, while that of PEPC transgenic rice was 0.115 (an increase of 50%). This increased carboxylation efficiency may be related to the expression of PEPC and CA, which are key enzymes for concentrating CO_2 (fig. 1). Fig. 3(b) shows the performance of CO_2 exchange under CO_2-free or low-CO_2 conditions. CO_2 release in untransformed rice Kitaake (control) was 62 μmol/mol, while that in PEPC transgenic rice was 50 μmol/mol. The results indicate that the introduction of the C_4 photosynthesis enzyme PEPC allows more fixation of CO_2 released in leaves under high light intensity, causing the CO_2 compensation point to decrease by 20%.

In order to examine whether the enhancement of photosynthesis was related to the introduced PEPC gene, transgenic rice leaves were treated with DCDP, a specific inhibitor of PEPC. The results in fig. 4 show that the photosynthetic rate in untransformed rice did not vary while that in

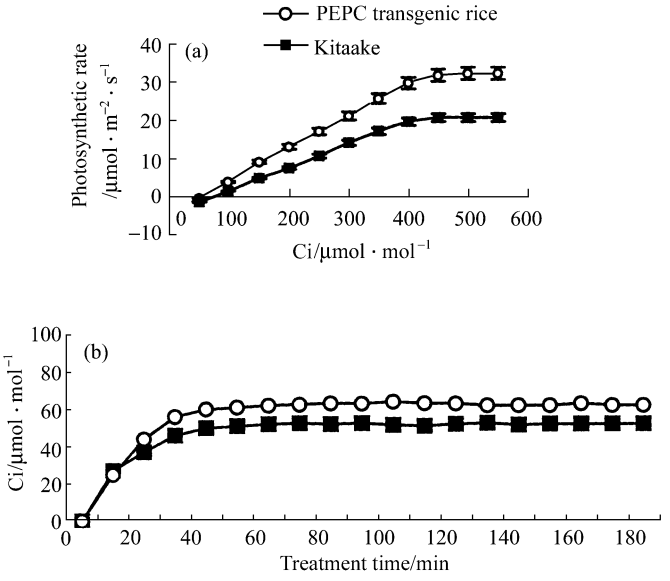

Fig. 3. The CO_2 exchange of PEPC transgenic rice and untransformed rice Kitaake under different CO_2 concentrations. (a) Light intensity 1200 μmol. • m^{-2} • s^{-1}; (b) light intensity 1000 μmol • m^{-2} • s^{-1}. Ci, intercellular CO_2 Concentration.

PEPC transgenic rice decreased until it was close to that of the untransformed rice. This indicated that the increase of photosynthetic capacity in PEPC transgenic rice was due to the action of the maize PEPC gene. Fig. 5 shows the results of the ^{14}C pulse-chase experiment. The proportions of the C_4 photosynthetic primary product, and the C_3 photosynthetic primary products 3-PGA in transgenic rice were close to those in untransformed rice. However, in PEPC transgenic rice, more label was distributed in asperate, indicating that although the C_4 pathway of NADP-ME type in maize[10,11] might not be integrated into the transgenic rice, the metabolic capacity of some

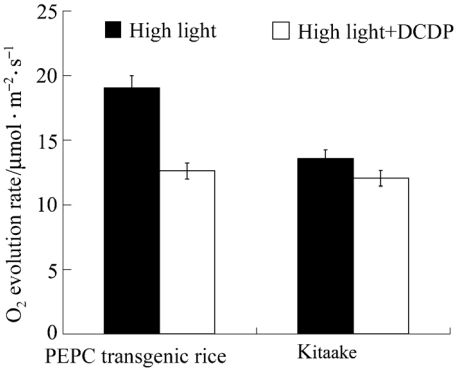

Fig. 4. The effects of DCDP on oxygen evolution rate in different genotype rice under high light intensity. The intensity of high light (treatment) was 1400 μmol • m^{-2} • s^{-1}. The oxygen evolution rate was measured under a light intensity of 1000 μmol • m^{-2} • s^{-1}.

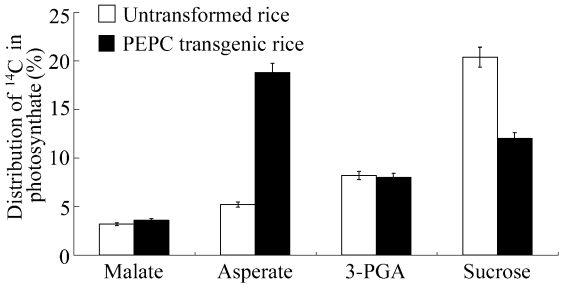

Fig. 5. The distribution of ^{14}C in photosynthate of PEPC transgenic rice and untransformed rice Kitaake with labeling for 20 s.

C_4 photosynthate is enhanced by the introduction of maize PEPC gene.

2.4 Effects of DCDP, a specific inhibitor of PEPC, on PSII photochemical efficiency in PEPC transgenic rice

Fig. 6(a) shows that the transgenic and untransformed rice plants exhibited photoinhibition and reduced PSII photochemical efficiency (Fv/Fm) under high light intensity similar to that of noon in summer. However, PEPC transgenic rice showed less photoinhibition under these conditions. After the treatment with DCDP, the decrease of Fv/Fm in PEPC transgenic rice was close to that observed in untransformed rice, indicating that PEPC overexpression accelerates CO_2 assimilation and maintains stable efficiency of the energy conversion in PSII.

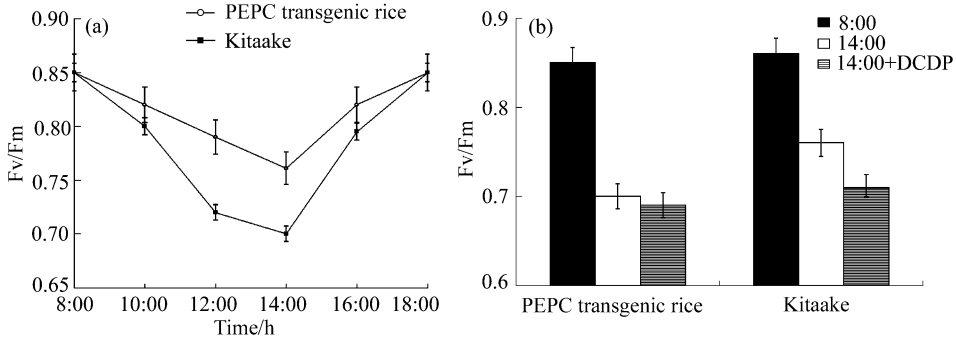

Fig. 6. (a) Diurnal change of Fv/Fm in PEPC transgenic rice and untransformed rice; (b) change of Fv/Fm after the treatment of inhibitor DCDP.

3 Discussion

In the last decade, due to the development of recombinant DNA technology, more attention has been paid to the introduction of C_4 photosynthetic gene into C_3 plant, and a systematic review has recently been published[12]. Transgenic C_3 plants with different kinds of C_4 photosynthetic enzymes have been obtained using the agrobacterium-mediated transforming system [1,13—17]. The PEPC transgenic rice used in this study has been well characterized in some regards, but there have been varying reports about their photosynthetic physiology. For example, Japanese scientists did not observe the improvement of photosynthetic characteristics when PEPC transgenic rice was grown indoors under moderate light intensity[18]. In contrast, the high light intensity and high temperature conditions of summer in the middle and lower reaches of the Yangzi River were used to study the comparative photosynthetic characteristics of rice plants transgenic for PEPC, PPDK, NADP-ME and PEPC+PPDK[5]. Results indicated that the activity of PEPC in PEPC transgenic rice was increased to 20-fold higher than that of untransformed rice, and that the photosynthetic rate and carboxylation efficiencies were increased by 55% and 50% respectively, while the CO_2 compensation point decreased by 27% and the grain yield per plant increased by 14%—22%[6]. At the same time, PEPC+PPDK transgenic rice did not exhibit more obvious photosynthetic vigor than PEPC transgenic rice[5].

This leads to the question of why overexpression of PEPC (a key enzyme in C_4 photosynthesis) leads to increased photosynthetic efficiency in transgenic rice whose leaves lack the necessary C_4 Kranz anatomy. It has been proposed that high expression of PEPC in the guard cells would lead to an increase in stomatal conductance, resulting in increased photosynthetic capacity[4]. Here, we show that although the stomatal conductance in PEPC transgenic rice increased (fig. 2), this was not correlated with the increase of photosynthetic rate. It has been recently reported that the aquatic angiosperm *Hydrilla verticillate*[19] and the terrestrial plant *Borszczowia aralocaspia*[20] possess primitive types of C_4 photosynthesis without Kranz anatomy, suggesting that a primitive types of C_4 photosynthesis can run in single mesophyll cells. Recent experiments[21] showing that extraneous C_4 acids accelerate the photosynthetic rate in spinach leaves and chloroplasts also support this viewpoint. It has been reported by Hibberd and Quick (2001)[22] that the tobacco (a typical C_3 plant) can decarbonize to four-carbon acids without Kranz anatomy. Our previous paper[5] reported that the activities of other C_4 photosynthetic enzymes such as PPDK, NADP-MDH, as well as NADP-ME were highly expressed in PEPC transgenic rice under high light intensity[5], the protein contents of PEPC and CA, which relate to the concentration and fixation of CO_2, were induced to increase syntonically (fig. 1). After ^{14}C was pulsed into rice for 20 s, the label of ^{14}C in PEPC transgenic rice appeared more in asperate (fig. 5), which showed that the C_4 pathway in rice leaves differed from the NADP-ME pathway in maize[10,11]. This indicates that the metabolic capacity of some photosynthates in rice can be accelerated in the presence of a key C_4 gene (PEPC). This could be the physiological and biochemical basis of the increase of carboxylase efficiency and the stability of the photochemical efficiency of PSII (figs. 3 and 6). These results demonstrate that a primitive CO_2 concentrating mechanism exists in the leaves of PEPC transgenic rice. However, further investigations will be necessary to fully elucidate this model of photosynthetic carbon metabolism pathway in transgenic rice with C4 photosynthetic gene.

New discoveries in basic research can lead to revolutionary innovations, and the introduction of photosynthetic C_4 genes has been applied in rice breeding, the parent line of the hybrid plant with high photosynthetic efficiency has been bred in Jiangsu and Anhui Academy of Agricultural Sciences of China[23]. Our research suggests that transgenic biotechnology with C_4 photosynthetic genes may be a valuable method for the super-high yield rice breeding in China.

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