Photosynthetic Characteristics and Heterosis in Transgenic Hybrid Rice with Maize Phosphoenolpyruvate Carboxylase (*pepc*) Gene

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Abstract: Three F_1 hybrids derived from the sterile rice lines Gang 46A, 776A and 2480A and the improved restorer line Shuhui 881 containing maize phosphoenolpyruvate carboxylase (*pepc*) gene were used to analyze the effect of *pepc* gene on the heterosis and photosynthetic characteristics, while the F_1 obtained by crossing Shuhui 881 with the above three sterile lines served as controls. The dynamics of photosynthetic characteristics in leaves of three F_1 with *pepc* gene and their controls were determined at the initial-tillering, maxium-tillering, elongation, initial-heading, heading, maturity stages, and other different times after flag leaf fully expanded. The PEPCase activities of the three F_1 with *pepc* gene increased significantly as compared with control plants during the whole developmental stages. Moreover, the net photosynthesis rate (Pn) also increased to certain extent. The data showed that PEPCase activity was significantly correlated to Pn with a correlation coefficient of 0.6081**. The photosynthetic indexes of the three F_1 with *pepc* gene were obviously superior to respective controls in apparent quantum efficiency, light compensation point and carboxylation efficiency, while the CO_2 compensation point was lower than that of corresponding control. The Pn of the three F_1 with *pepc* gene at light saturation point and CO_2 saturation point was also higher than that of control plants. In addition, the three F_1 with *pepc* gene had an average increase of 37.10% in grain yields per plant in comparison with control plants. The results indicated that the photosynthetic characteristics of hybrid rice containing *pepc* gene had been improved to some extent due to the introduction of *pepc* gene.

Key words: hybrid rice; phosphoenolpyruvate carboxylase gene; photosynthetic characteristic; high photosynthetic efficiency breeding; heterosis

The plants can be classified into C_4, C_3 and CAM categories based on the photosynthetic pathways. Under high light intensity and high oxygen content the C_4 species have relatively higher photosynthetic rate than C_3 plants. Plant physiologists and breeders had done a lot of work to improve the photosynthetic productivity of C_3 plants by increasing C_4 photosynthetic characteristics in C_3 plants [1-2]. Previous studies [3] revealed that though C_4 photosynthetic characteristics might shuttle within the same C_3 genus by hybridization, but the results were not as good as expected when C_4 maize were crossed with C_3 rice within the same class. However, the key factor for high photosynthetic efficiency in C_4 plants did not lie in its Kranz anatomic structure, but in the

enzymatic system controlling the C_4 pathway [4]. Recently, numerous key enzymatic genes involved in C_4 pathway, such as *PEPC*, *NADP-ME*, *PPDK*, had been cloned in maize, sorghum, and amaranth [5]. Ku et al [6] had successfully introduced phosphoenolpyruvate carboxylase (PEPCase) gene (a key enzyme stimulating the CO_2 fixing in C_4 pathway of maize) into a C_3 japonica rice variety, Kitaake, which resulted in an equivalent or higher expression of PEPCase by using *Agrobacterium*-mediated transformation. However, the utilization of agronomic traits of original transgenic varieties in rice had been limited due to certain pitfalls, such as short growth duration. Li et al [7] and Wang et al [8-9] got F_1 generation with high PEPCase activities and high photosynthetic rate after crossing the highly-expressed transgenic rice with some Chinese hybrid rice parents. Thus, it confirmed that the highly expressed *pepc* gene can be

inherited by utilizing the combination of conventional breeding and biotechnologies, suggesting a new way to realize physiological breeding with high photosynthetic efficiency in rice.

After years of backcrossing by using a widely used indica restorer line Shuhui 881 as receptor and the *pepc*-transgenic line as donor, the improved Shuhui 881 was obtained via molecular marker-assisted selection (MAS) with self-designed specific primer for *pepc* gene by the Rice Research Institute of Sichuan Agricultural University, in which *pepc* gene had been transformed, showing 5.15-99.03% similarity to the shared gene of Shuhui 811. The results indicated that *pepc* gene could be inherited and well expressed under a new genetic background [10]. In this study, we crossed the offspring derived from an individual with 99.03% similarity to its backcross parents and three male sterile lines to study the photosynthetic characteristics and heterosis at different stages after flag leaf fully expanded and the whole life cycle in hybrid rice, aiming to provide valuable information for the high photosynthetic efficiency breeding in hybrid rice.

MATERIALS AND METHODS

Rice materials

In the summer of 2000, rice variety Kitaake containing *pepc* gene (donor) transformed from C$_4$ maize (provided by Professor JIAO De-mao in Jiangsu Academy of Agricultural Sciences) was crossed with indica restorer line Shuhui 881 (receptor, abbreviated as 881R). We obtained a series of lines of the improved Shuhui 881 (abbreviated as 881P) containing *pepc* gene by MAS and consecutive backcross in Wenjiang, Sichuan Province and Linshui,

Hainan Province. One of 881P lines showing 99.03% similarity of genetic background to 881R was crossed with three male sterile lines, Gang 46A (abbreviated as G46A), 776A, and 2480A to make F$_1$ hybrids. At the same time, the F$_1$ generations obtained by crossing 881R with the three sterile lines mentioned above were treated as controls, namely G46A/881R (CK1), 776A/881R (CK2), and 2480A/881R (CK3). On 15 April 2005, the materials were planted at Rice Research Institute of Sichuan Agricultural University. The *pepc*-positive plants verified by *pepc* gene-specific primer and their controls were transplanted into fields at the 3-leaf stage. Each material was planted in 4 lines with 3 replications; each line contained 12 hills. The row-to-row spacing was 27 cm and the hill-spacing was 16 cm. The management adopted was similar to routine practice in paddy field.

Determination of target gene

DNA extraction was performed according to the modified SDS mini-extraction method with some modification by Wang et al [11]. The sequences of C$_4$ *pepc* gene-specific primers designed by BLAST in our laboratory, were 5'-AAGCAGGGA AGCGAGACG-3' (forward), and 5'-GATTGCCGCCAGCAGTAG-3' (backward); the position of amplified fragments was depicted in Fig. 1. The PCR reaction system was 25 µL containing 2.5 µL 10×reaction buffer, 2.0 µL dNTP (2.5 mmol/L), 2.0 µL primer (approximately 1 µmol/L), 2.0 µL template DNA (about 50 ng), 0.2 µL *Taq* polymerase (5 U/µL), and 16.3 µL ddH$_2$O. The PCR reaction procedures were as follows: pre-denaturalization at 94℃ for 240 s; followed by 35 cylces of denaturalization at 94℃ for 30 s, annealing at 55℃ for 30 s, elongation at 72℃ for 30 s; and
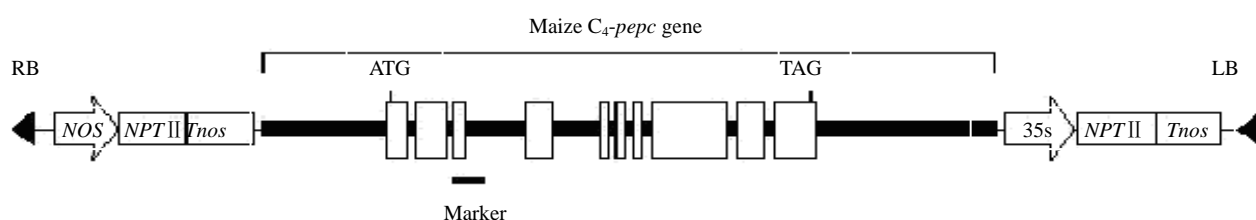

Fig. 1.   Structure of intact maize *pepc* gene and the antibiotic resistance gene for rice transformation.

Marker indicated the interest fragment by amplifying the maize *pepc* gene-specific primer.

finally extended at 72℃ for 10 min. Agarose gel electrophoresis with EB was then performed to detect the amplification product, and the final result was obtained by observation on Gel Imaging System, BIO-RAD Quantity One.

Determination of PEPCase activity

The second leaf from the top at the initial-tillering, maximum-tillering, elongation, initial-heading, heading, maturity stages, and flag leaves at different times after fully expanded were collected for determination of PEPCase activity with 2 replications, and for each F_1 hybrid five individual plants were sampled. The detection was performed every 7 d at 0 to 35 days after flag leaf fully expanded. According to the methods described by Kung et al[12] and Gonzalez et al[13], 0.25 g of rice leaves (vein discarded) were collected, moderate extraction medium (50 mmol/L Tris-HCl pH 7.5, 1 mmol/L $MgCl_2$, 5 mmol/L DTT, 1 mmol/L EDTA, 5% glycerin) was added, and then the mixture was subjected to grinding quickly and completely. The grinding liquids were centrifuged for 10 min at 13 000 × *g* and the supernatants were collected for analysis of enzymatic activity. The reaction solution was 1 mL in volume, containing 50 mmol/L Hepes-KOH (pH 8.0), 10 mmol/L $NaHCO_3$, 5 mmol/L $MgCl_2$, 0.2 mmol/L NADH, 2 mmol/L phosphoenoipyruvate carboxy-kinase (PFP), and 1.5 U maletedehydrogena (MDH). The initial time was clocked once adding phos-phoenolpyruvate (PEP), the change of light densities at 340 nm was recorded five times within 1 min, and the PEPCase activity was calculated finally. The whole processes were performed under freezing conditions.

Determination of photosynthetic indexes

Net photosynthetic rate (Pn) was measured at 10:00-11:30 a.m. with LI-6400 type infra-red gas analyzer (IRGA). The concentration of CO_2 in leaf cells was 400 µmol/mol, the photosynthetic flux density (PFD) was 1200 µmol/(m²·s), and the temperature used was 30℃. The effect curve of Pn against PFD was determined at the heading stage at 30℃; the gradients of PFD in leaf cells were 50, 150, 200, 400, 600, 800, 1000, 1200, and 1500 µmol/(m²·s),

and the CO_2 concentration was 400 µmol/mol. As a result, the apparent quantum efficiency, light compensation point, light saturation point, and photosynthetic rate were determined according to the curve effect in question. After completion of the above processes, the gradients of CO_2 concentration were changed to 400, 300, 200, 150, 100, 50, 400, 400, 600, 800, 1000 and 1200 µmol/mol, and the PFD to 1200 µmol/(m²·s). Carboxylation efficiency, CO_2 compensation point, CO_2 saturation point and its Pn were calculated in the same way mentioned above. The whole processes were automatically performed in the closed-path air system.

Observation and data processing of agronomic traits

Five individual samples for each F_1 hybrid were collected at the maturity stage and their agronomic traits, including plant height, panicle length, spikelet number per panicle, seed setting rate, 1000-grain weight, and grain yield, were determined. The variance of analysis and data were then processed using the software, Data Processing System (DPS).

RESULTS

Determination of target gene

The total genomic DNA of the entire materials was amplified with maize *pepc* gene-specific primer and maize genome was treated as positive control. According to Fig. 2, all hybrid rice with *pepc* gene produced a coincident fragment with a length of 311 bp from Lanes 1 to 15, being consistent with the result of maize genome in Lane 19. On the contrary, the three controls (Lanes 16-18) showed no such pattern, indicating transgenic rice plants with *pepc* gene could be precisely screened via the maize *pepc* gene-specific primer.

PEPCase activity and performance of Pn at different growth stages

It is clear from Fig. 3 that the three F_1 generations containing *pepc* gene exhibited a PEPCase activity higher than control at the six major stages, indicating that *pepc* gene can be expressed stably and effectively under new genetic backgrounds by hybridization.
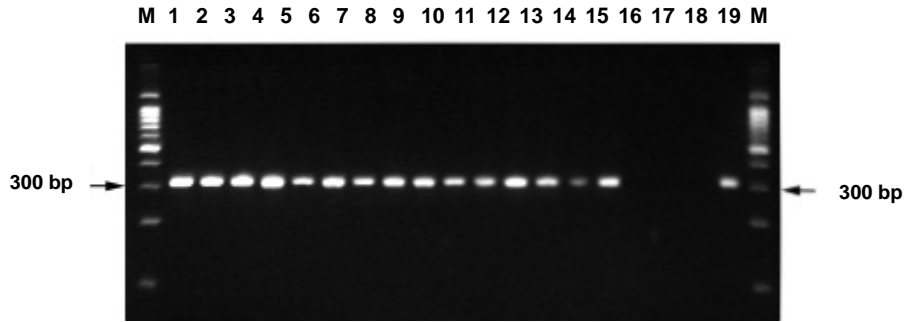
Fig. 2.   PCR analysis of hybrid rice with maize C4-type *pepc* gene.

Lanes 1-15, hybrid rice with maize C4-type *pepc* gene; Lanes 1-5, G46A/881P; Lanes 6-10, 776A/881P; Lanes 11-15, 2480A/881P; Lanes 16-18, CK1, CK2, CK3; Lane 19, Maize DNA; M, 100 bp DNA marker.

However, the three F_1 generations showed a distinctive expression. The PEPCase activity of G46A/881P at the six stages was 1.93-6.03 times as high as that of CK1, while the difference peaked at the maxium-tillering stage, followed by the initial-heading stage (5.74 times) when the PEPCase activity reached the peak. On the other hand, the PEPCase activity of 776A/881P increased by 1.34-7.72 times compared with CK2, with the greatest increase at the heading stage when the PEPCase activity was the highest at the same time. The PEPCase activity of 2480A/881P was 3.93-7.94 times as high as that of CK3, with the maximum activity at elongation stage. Thus PEPCase activity of the 3 F_1 generations containing *pepc* gene kept on rising to a certain stage till reached the peak. After gaining the
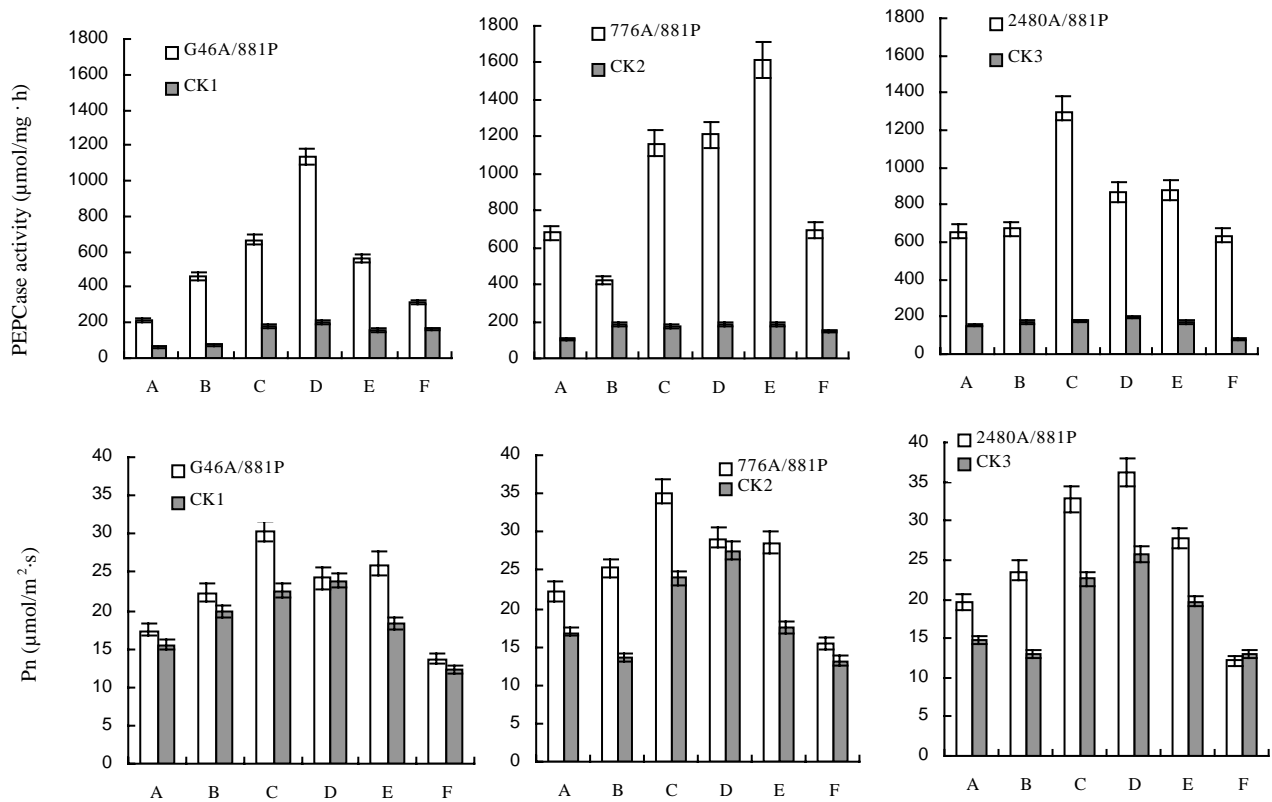

Fig. 3. Dynamics of Pn and PEPCase activities in the second leaf from the top for three F_1 with *pepc* gene and their CKs at different stages.

A, Initial-tillering stage; B, Maximum-tillering stage; C, Elongation stage; D, Initial-heading stage; E, Heading stage; F, Maturity stage.

peak value, the PEPCase activity plummeted. In contrast, PEPCase activity of the three controls was significantly lower than that of the three F_1 generations with the *pepc* gene throughout the growth duration without obvious fluctuations.

At the six major stages, Pn performance of the three F_1 generations containing *pepc* gene was mostly superior to their corresponding controls and kept increasing till reached the highest point at a certain stage (Fig. 3). The only difference existed in the time reaching the highest point, i.e. the elongation stage for G46A/881P and 776A/881P, while the initial-heading stage for 2480A/881P. After the peak point, except for Pn of G46A/881P increased a little at the heading stage the other two F_1 containing *pepc*-gene showed a decreasing trend.

PEPCase activity and Pn performance after full expansion of flag leaves

The last three function leaves of rice play a vital role in accumulation of dry matter into seeds, especially the flag leaves. We can see from Fig. 4 that PEPCase activity of the three F_1 generations with *pepc* gene gradually increased as a whole since the full expansion of flag leaves, and began to decline after reached the peak. The only difference lied in the fact that the highest points varied at 21 d, 14 d, 7 d after full expansion of flag leaves for G46A/881P, 776A/881P, and 2480A/881P, respectively. In addition, the increase in every F_1 generation containing *pepc* gene was huge against controls 35 d after full expansion of flag leaves, due to the fast drop of PEPCase activity in controls. Therefore, it can be concluded that when PEPCase activity of the three F_1 with *pepc* gene was at the highest point and the increase in the activity against controls also reached the highest at the same time.

During the whole detection period after full expansion of flag leaves, Pn of the three F_1 generations containing *pepc* gene was higher than that
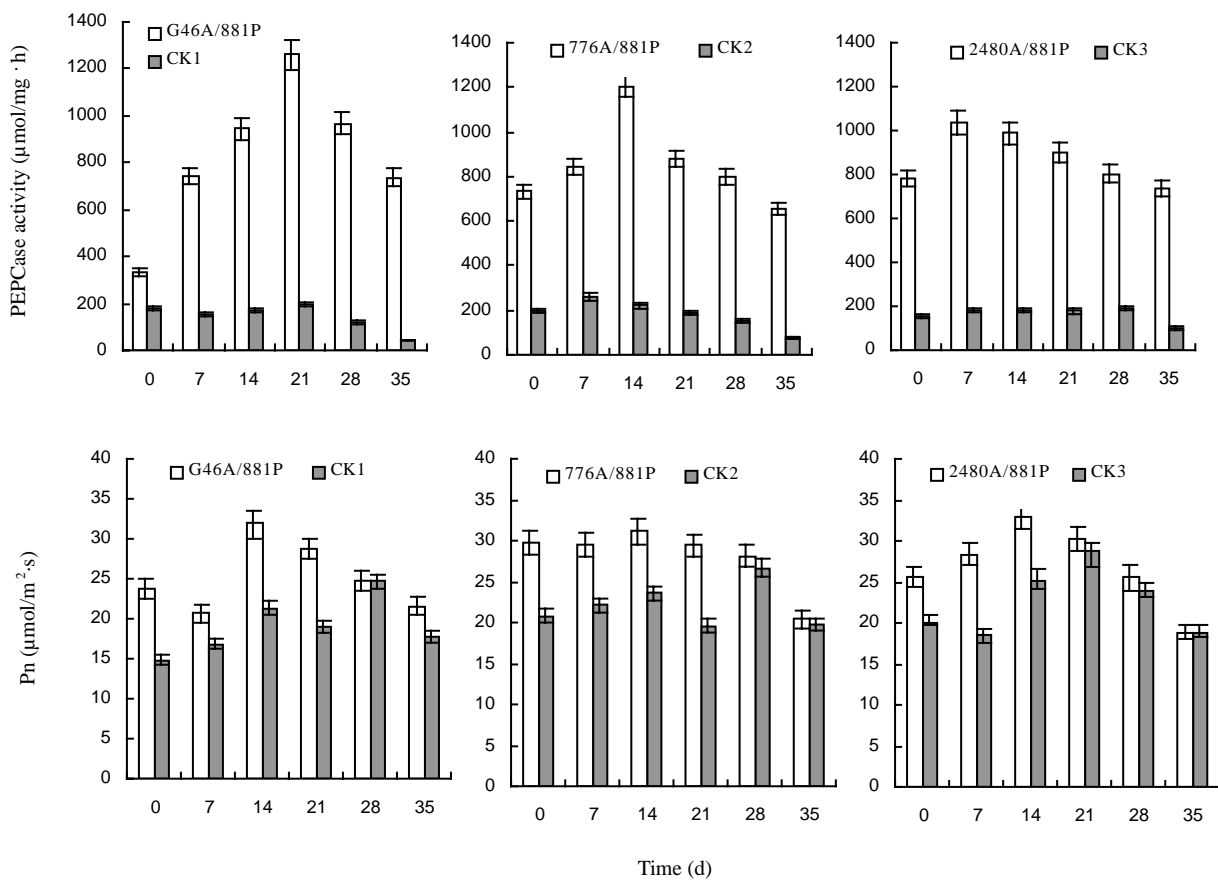

Fig. 4. Dynamics of Pn and PEPCase activities in flag leaves for three F_1 with *pepc* gene and their CKs.

of controls with distinctive features. After the full expansion of flag leaves, the Pn of G46A/881P declined slightly, while Pn of 2480A/881P gradually increased, and that of 776A/881P was relatively stable during the whole detection period. However, the Pn of the three F_1 containing *pepc* gene was peaked 14 d after full expansion of flag leaves.

Determination of photo-physiological indexes

The data in Table 1 show that the apparent quantum efficiency of the three F_1 with *pepc* gene was 30.6%, 54.3%, and 59.7% higher than that of the controls, respectively. Meanwhile, the light compensation points was elevated by 29.8%, 31.2%, and 36.9%, respectively, indicating that hybrid rice was more photophilous after being introduced into *pepc* gene. The Pn at light saturation points in the three F_1 with *pepc* gene increased within a range of 11.7% to 12.4% compared with controls.

In comparison with controls, the carboxylation efficiency of G46A/881P, 776A/881P, and 2480A/881P was enhanced by 34.3%, 38.7%, and 40.0%, respectively, while the CO_2 compensation point was decreased by 30.7%, 33.3%, and 23.5% accordingly, suggesting that the introduction of *pepc*

gene can improve the utilization efficiency of low CO_2 concentration. Further determination of the Pn at CO_2 saturation point in the three F_1 containing *pepc* gene resulted in an increase of 14.1-15.1%.

Analysis for agronomic traits

The agronomic traits among F_1 containing *pepc* gene and their controls are presented in Table 2. There were no significant difference in plant height and panicle length except for 2480A/881P and G46A/881P with a competitive advantage of -6.95% in plant height and 7.10% in panicle length, respectively, but in 2480A/881P, G46A/881P, and 776A/881P a positive competitive advantage of 62.2%, 9.30%, and 17.25% in panicle number per plant, spikelet number per panicle, and seed setting rate was observed respectively. The 1000-grain weight of the three F_1 with *pepc* gene was significantly higher than that of controls, confirming the contribution of *pepc* gene to the seed setting rate. In the final individual productivity, the three transgenic F_1 (G46A/881P, 2480A/881P, and 776A/881P) significantly higher than the controls, and increased by 26.31%, 34.70%, and 50.30%, respectively. The genetic background similarity between 881P and 881R was 99.03%,

Table 1. Photosynthetic indexes of the three F_1 hybrids with *pepc* gene and CKs.

Material	Apparent quantum efficiency	Light compensation point (μmol / m².s)	Pn at light saturation point (μmol / m².s)	Carboxylation efficiency	CO_2 compensation point (μmol/mol)	Pn at CO_2 saturation point (μmol / m².s)
G46A/881P	0.052±0.008**	36.70±2.37**	28.0±1.29**	0.094±0.005**	40.68±2.34**	27.4±2.25*
G46A/881R(CK1)	0.040±0.004	28.27±1.87	24.9±0.93	0.070±0.008	58.62±4.37	24.0±0.77
776A/881P	0.071±0.005**	30.06±1.82**	28.6±1.01**	0.104±0.008**	41.51±2.27**	30.5±1.39*
776A/881R (CK2)	0.046±0.003	22.92±1.73	24.7±0.82	0.075±0.004	62.22±4.75	26.5±1.04
2480A/881P	0.075±0.004**	26.19±2.37**	29.5±1.47*	0.110±0.006**	45.05±4.28**	29.1±2.11*
2480A/881R (CK3)	0.047±0.004	19.13±1.32	26.4±0.22	0.078±0.010	58.83±3.56	25.5±1.12

*, ** Significant at $P < 0.05$ and $P < 0.01$ levels, respectively.

Table 2.   Agronomic traits of the three F_1 hybrids with *pepc* gene and CKs.

Material	Plant height (cm)	Panicle length (cm)	Panicle no. per plant	Spikelet no. per panicle	Seed setting rate (%)	1000-grain weight (g)	Grain yield per plant (g)
G46A/881P	122.22±2.41	26.11±0.53**	6.89±0.39	231.07±4.82**	79.3±2.4	27.13±0.97**	34.23±3.46**
G46A/881R(CK1)	119.55±2.04	24.38±0.06	7.00±0.26	211.40±7.97	74.8±3.9	23.92±0.51	27.10±2.22
776A/881P	123.44±1.83	27.74±0.79	10.33±1.52	271.81±14.22	69.0±7.9*	26.35±1.13*	50.50±7.01**
776A/881R (CK2)	126.56±0.20	27.38±0.20	0.00±1.46	296.42±28.64	58.9±3.4	25.10±0.94	37.49±3.92
2480A/881P	113.56±3.18*	26.46±0.23	8.11±1.07**	217.73±7.91**	81.4±4.3	26.04±0.23**	37.68±2.66**
2480A/881R (CK3)	121.45±1.95	27.83±0.98	5.00±0.67	273.33±18.53	79.4±7.2	23.26±1.25	25.07±2.10

*, ** Significant at $P < 0.05$ and $P < 0.01$ levels, respectively.

indicating they were near-isogenetic lines in terms of inheritance. It can be concluded that the increase of 1000-grain weight, and grain yield per plant etc., was mainly attributed to the introduction of *pepc* gene. Thus, the improvement in yield is possible through plant breeding by transferring *pepc* gene.

DISCUSSION

Recently, integration of conventional breeding, physiological breeding and biotechnological breeding is becoming an important strategy for crop varietal development. Among them, high light efficiency is a common focus both for physiologists and breeders. Creation and application of C_4 photosynthetic transgenic germplasms indicated a promising prospect for photosynthetic efficiency improvement in C_3 crops. By crossing *pepc* gene-improved Shuhui 881 with the three sterile lines, we obtained the hybrid rice that can be directly used in rice production. It was more practical and valuable in rice production than those described by Li et al [7] and Wang et al [8-9]. Our preliminary results revealed that *pepc* gene showed high expression in F_1 containing *pepc* gene. At the six major stages and different times after full expansion of flag leaves, the PEPCase activity of F_1 containing *pepc* gene is significantly higher than that of controls (Figs. 3-4), and Pn is significantly correlated with PEPCase activity (0.6081[**]) according to the correlation analysis (Fig. 5). In terms of inheritance, 881P and 881R are near-isogenic lines with a homology as high as 99.03%. Compared with controls, F_1 containing *pepc* gene showed an advantage of individual productivity and photosynthetic physiology. This is mainly attributed to the introduction of *pepc* gene, making it possible to utilize C_4 high photosynthetic gene in practice.

In this study, we noted that PEPCase activities of the three F_1 hybrids with *pepc* gene were much higher than those of the control during their entire life cycle. Actually, *pepc* gene is a key gene responsible for photosynthesis in C_4 plants, but since C_4 plant has been evolved from C_3 plants, which shares great sequence homology in *pepc* gene encoded [14]. Generally, *pepc* gene will be less likely expressed or completely silent in C_3 plants [14-15]. Previous reports
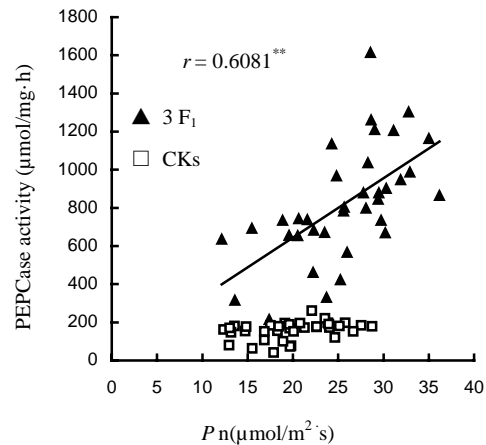

Fig. 5. The correlation between PEPCase activities and net photosynthetic rate (Pn) for the three F_1 hybrids with *pepc* gene and their CKs

assumed that some system of C_4 photosynthesis in C_3 crops such as wheat [16] and rice [17] has limited due to the relatively low enzymatic activity. Moreover, the present results on photosynthetic indexes (Table 1) revealed that apparent quantum efficiency, light compensation point, and carboxylation efficiency of the three F_1 containing *pepc* gene increased by 30.6-59.7%, 29.8-36.9%, and 34.3-40.0%, respectively, while the CO_2 compensation point decreased by 23.5%-33.3%. In terms of CO_2 saturation point and Pn performance, the three F_1 containing *pepc* gene were apparently higher than controls. Thus, it can be suggested that the introduction of *pepc* gene might improve the C_4 micro photosynthetic cycle, which brings about the decrease of light respiration rate and the increase of Pn thereby. However, further investigation is needed to confirm that rice hybrids have evolved a physiological performance similar to the primary osculant type of C_3-C_4 [18]. It is also clear from the study that PEPCase activity and Pn are not following a synchronous change at some growth stages. Therefore, to understand the mechanism(s) of expression and operation after introduction of *pepc* gene into hybrid rice, further experiment should be carried out.

ACKNOWLEDGEMENTS

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