

Breeding of Marker-free *Indica* Three-lines Restorer with *ZmC₄Ppc* by Marker-assisted Selection

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Abstract

Enhancing photosynthetic productivity of rice is always the key goal of researchers by to use exogenous C_4 genes. The full-length of intact maize gene of C_4 -specific PEPCase (*ZmC₄Ppc*) is 6781 bp that makes it difficult for marker-assisted selection (MAS) in rice. The specific marker of intact *ZmC₄Ppc* was designed and termed as MRpc. MRpc was verified that it was a gene marker because *ZmC₄Ppc* could be overexpressed at every developmental stage and it was used for MAS of *indica* restorer Shuhui881 with *ZmC₄Ppc* and FPM881 was gotten, which was tested for genetic background, PEPCase activity, net photosynthetic rate (P_n), general combining ability (GCA) and specific combining ability (SCA). The results indicated that FPM881, which similarity of genetic background had arrived at above 95%, showed a higher PEPCase activity and net photosynthetic rate than those of control. Analysis of yield components showed that some of the progenies carrying *ZmC₄Ppc* had better GCA and SCA than those of the control. Then, six lines of FPM881 were planted in larger population by selfing and gotten selectable-marker free plants with target gene using MRpc and *hpt* primers. Our results indicated that introduction of *ZmC₄Ppc* via MAS and its stable expression could increase grain yield of hybrid rice.

Keywords: Hybrid rice (*Oryza sativa* L.), *ZmC₄Ppc*, MRpc, Marker-assisted selection, Marker-free

1. Introduction

Photosynthesis is main source power of crop production. Most of crops, such as rice and wheat, are classified as C_3 plants because the first product fixed atmospheric CO_2 is 3-PGA. However, up to 50% carbon fixed is lost during photorespiration because of competitive inhibition of O_2 and CO_2 in active site of Rubisco. Because of possessing the C_4 photosynthetic pathway as CO_2 -concentrating mechanisms (CCM), C_4 plants can achieve high photosynthetic performance and high water- and nitrogen-use efficiencies. In terrestrial C_4 plants, CO_2 release from C_4 acids and the resulting elevation of cellular CO_2 levels take place at a site that is physically separated from the site of initial carboxylation. This separation occurs through the bundle sheath and mesophyll cells in typical/classical C_4 plants (Hatch, 1987; Leegood, 2002), and through two distant subcellular compartments in the discovered single-cell C_4 plants (Edwards *et al.*, 2004). By contrast, in some aquatic plants (*H. Verticillata*) C_4 photosynthesis is accomplished in a single cell without any compartmentation and chloroplast differentiation (Bowes *et al.*, 2002; Leegood, 2002). Therefore, the key of C_4 pathway activation is enzymes relative to CCM.

The key enzymes of C_4 pathway consist of phosphoenolpyruvate carboxylase (PEPCase), pyruvate phosphate dikinase (PPDK), NADP-dependent malic enzyme (NADP-Me) etc. The function of PEPCase, namely, control

coefficients (C_j), is the largest (0.35) than the other key enzymes during photosynthesis in C_4 plant (Furbank *et al.*, 1997; Dever *et al.*, 1998; Bailey *et al.*, 2000). PEPCase catalyzes irreversibly PEP to yield OAA and Pi using HCO_3^- as the inorganic carbon substrate, which plays a key role while atmospheric CO_2 is fixed in photosynthesis of C_4 and CAM plants. The genetic structures of key enzymes of C_4 pathway such as C_4 -specific PEPCase etc have been decoded and *ZmC4Ppc* has 10 exons and 9 introns with 6781 bp (Matsuoka *et al.*, 1989). C_4 pathway isn't the privilege of C_4 plants. There are a lot of experimental evidences that C_3 plants have C_4 photosynthetic biochemical characteristics (Hibberd *et al.*, 2002). The pathway with C_4 photosynthetic characteristics in C_3 plants is named as C_4 -like pathway because of the absence of the Kranz anatomy. It has been proved that Kranz anatomy is not essential for terrestrial C_4 plant photosynthesis (Voznesenskaya *et al.*, 2001). There is C_4 -like pathway in rice lemma because it has been proved that $^{14}\text{CO}_2$ is fixed by PEPCase using $^{14}\text{CO}_2$ tracer and LED technology (Imaizumi *et al.*, 1997).

Introducing a C_4 -like pathway into C_3 plants is one of the proposed strategies for the enhancement of photosynthetic productivity (Endo *et al.*, 2008). The intact genes of key enzymes of C_4 pathway, *ZmC4Ppc*, *ZmC4Pdk* etc have been transformed to Kitaake, respectively by Ku *et al.* (1999) and Fukayama *et al.* (2001) by using the *Agrobacterium*-mediated transformation and they were over expressed. Ku *et al.* (2000) reported that transgenic rice plants expressing the maize PEPCase had higher photosynthetic capacities (up to 30% higher) than untransformed plants. Besides improving photosynthetic capacity and yield, introduction of key enzymes genes in C_4 pathway into rice had a good potential to enhance its tolerance to stress of photoinhibition and photooxidation under field conditions. Jiao *et al.* (2001) reported that the light saturated photosynthetic rate could be greatly increased (55%) by overproduction of the maize PEPCase in transgenic rice plants and PEPCase activity can be greatly increased up to 20-fold than that of non-transformants. Ku *et al.* (2001) found that the grain yield was about 10~20% higher in Kitaake with *ZmC4Ppc* transgenic rice lines under small-scale field trial, as compared with the untransformed plants. Bandyopadhyay *et al.* (2007) reported that overproduction of the maize PEPCase enhanced photosynthesis and increased crop yield of transgenic rice plants. PEPCase was overexpressed in the same intracellular compartment, cytosol, in C_3 plants as in C_4 plants (Miyao, 2003). It will be an important biotechnology pathway to transfer key enzyme genes of C_4 pathway from transgenic rice materials to elite rice varieties by marker-assisted selection (MAS). However, its band is not clear and repetition is poor if the intact genes are amplified. We designed the specific marker of *ZmC4Ppc* and applied in the breeding of *indica* restorer with *ZmC4Ppc* by MAS. On the other hand, transgenic rice, namely trans-*ZmC4Ppc* Kitaake, has selectable marker hygromycin phosphotransferase gene (*hpt*), which serves no useful purpose once transgenic plants being regenerated and continues to express its gene products. Furthermore, it results in more and more environmental and consumer concerns. Therefore, breeding of marker-free *indica* restorers with target gene is crucial.

2. Materials and methods

2.1 Plant materials and DNA extraction

Rice varieties, Shuhui 881 was afforded by Rice Research Institute of Sichuan Agricultural University, Kitaake, a extremely early *Japonica* variety from Hokkaido of Japan by Mr. Fujimoto Kan and Kitaake transformed with *ZmC4Ppc* by Mr. DM Jiao. Maize varieties, Yayu 10, Yuyu 22 and Linao 4 were afforded by High Technology Agricultural Company of Sichuan Agricultural University. Their DNAs were extracted according to the method of Xiang *et al.* (2007).

2.2 BLAST (Basic local alignment search tool) (Altschul *et al.*, 1990)

BLASTs were carried out between the nucleotide sequences of *ZmC4Ppc*, and *indica* 9311, respectively. (<http://btn.genomics.org.cn:8080/blast.php?name=rice>). Meanwhile, BLASTs were also carried out between the nucleotide sequences of *ZmC4Ppc*, and all GenBank+EMBL+DDBJ+PDB rice sequences (but no EST, STS, GSS, or phase 0, 1, or 2 HTGS sequences), respectively (<http://www.ncbi.nlm.nih.gov/blast/Blast.cgi>). All the jobs were done at the server of Sichuan Agricultural Biotechnology Center.

2.3 Design of specific primers

The software of designing primer — Primer Premier 5.0 was applied.

The synthesis reaction of primers was done by Shanghai Sangon.

2.4 The PCR reaction system and the reaction conditions

Reaction system (25 μL): $10\times$ reaction buffer 2.5 μL , dNTP 2.0 μL (2.5 mmol/L), primers 2.0 μL (about 1 $\mu\text{mol/L}$), DNA template 2.0 μL (template about 50 ng). Taq polymerase (5 U/ μL) 0.2 μL , ddH₂O 16.3 μL .

The reaction condition of *ZmC₄Ppc* amplified: first stage: 94°C for 4 min, one cycle. Second stage: (1) denaturation, 94°C for 30 s, (2) annealing, 53°C for 30 s, (3) extension: 72°C for 30 s, 35 cycles. Third stage: to be kept warm at 72°C for 10 min, one cycle.

The DNA marker was bought from TaKaRa Biotechnology Co., Ltd (Cat. No: D3405A. Description: ϕ X174-Hae III digest). The amplified products of PCR were loaded on 1.7% agarose gel. Samples were electrophoresed at 120 V for 2 h in 0.5×TBE buffer. After electrophoresis, the gels were stained with Bio-rad Gel Doc 2000.

The reaction condition of simple sequence repeats (SSR) was carried out according to Chen *et al.* (1997). The difference of electrophoresis was agarose gel concentration of 3% at 160 V for 3 h as compared with the electrophoresis of PCR production of *ZmC₄Ppc*. The aim of SSR reaction was to screen differential primers and confirmed similarity degree of genetic background for FPM881, as compared with Shuhui881.

2.5 Determination of PEPCase activity

The assay of PEPCase activity was carried out according to the method of Huang *et al.* (2002). The developmental stages of determination were initial-tillering, tillering, jointing, initial-heading, full-heading and maturing. Five plants were used at each stage and the determination of each plant was repeated for 3 times.

2.6 Measurements of net photosynthetic rate (P_n)

The portable photosynthesis system LI-6400 was used. The last second leaves from main stem were used for the measurement of P_n at c. 10: 00 AM under controlled condition of a CO₂ concentration of 400 $\mu\text{mol}\cdot\text{mol}^{-1}$, 30°C, and a light intensity of 1200 $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$. The dates of P_n measurements were the same as those for the determination of PEPCase activity. Five **plants with 3 repetitions** were used in the assay.

2.7 PCR analysis of *hpt* gene

PCR analysis was carried out using primers specific for the *hpt* as previously described (Ma *et al.*, 2004), Forward: 5' TAC ACA GCC ATC GGT CCA GA 3', Reverse: 5' TAG GAG GGC GTG GAT ATG TC 3'.

2.8 Measurements of general combining ability (GCA) and Special combining ability (SCA) for improved Shuhui 881 with *ZmC₄Ppc* (FPM881) gotten by MAS

3×7 incomplete diallel cross design was used (there were 3 male sterility lines and 7 FPM881 restorer lines). All of the F₁ was sowed in experiment farmland of Rice Research Institute of Sichuan Agricultural University. The plantlets contained *ZmC₄Ppc* gene, which were analysed by PCR in third leaf stage, were preserved and transplanted to field when seedling-age arrived at 40 days. Random block, 1.43 m² of every plot area with three times replication. Standard of transplanting: Row spacing, 0.27 m, socket spacing, 0.16 m, having same management compared with field. At maturity, five plants were harvested for every plot and measuring number of panicles per plant (No. of panicles per plant, panicles), number of spikelets per panicle (No. of spikelets per panicle, grains), seed setting rate (%), thousand-grain weight (1000-grain weight, gram) and grain yield per plant (gram). Their results were analyzed by analysis of variance (ANOVA) and calculating of combining ability. All of the data were operated in DPS software.

3. Results

3.1 The specific primer sequences

By BLAST, the fragments of *ZmC₄Ppc* existed only in maize rather than in rice were 1-1330 bp, 2180-3065 bp and 6320-6781 bp. Using software Primer Premier 5.0, the 1-1330 bp of *ZmC₄Ppc* was used for primer design. Its primer sequences were as follows and named as MRpc: Forward: 5' AAG CAG GGA AGC GAG ACG 3', Reverse: 5' GAT TGC CGC CAG CAG TAG 3'. The site of amplification by the primers was 970-1280 bp in *ZmC₄Ppc*, and the size of the product was 311 bp, $T_m=58.0^\circ\text{C}$. Its site of fragment amplified was showed in Fig.1.

3.2 Verification

The genomic DNAs of the materials Yuyu 10, Yuyu 22, Linao 4, Kitaake with *ZmC₄Ppc*, Kitaake, Shuhui 881 and FPM 881 were amplified with MRpc by PCR (Fig.2). The amplified bands could be seen for Yuyu 10, Yuyu 22, Linao 4, Kitaake with *ZmC₄Ppc* and FPM 881, with the same size of 310 bp as compared with DNA marker (Aim in showing FPM881, the later being bred line, on figure2 was to compared it with maize having same band).

MRpc was used to screen transgenic material, Kitaake with *ZmC₄Ppc* and the transgenic plants with specific band were reserved. Then, their PEPCase activities and net photosynthetic rate were determined, together with their wild-type Kitaake in next year (Fig.3A). The PEPCase activity values of Kitaake with *ZmC₄Ppc* were

significantly higher than that of Kitaake at 6 different stages. The PEPCase activity was the highest at elongation stage and the lowest at maturing. The PEPCase activities of Kitaake with *ZmC₄Ppc* were 4.47~25.26 times as much as Kitaake at 6 different stages.

The net photosynthetic rate (P_n) of Kitaake and Kitaake with *ZmC₄Ppc* were shown in Fig.3B. The P_n of Kitaake with *ZmC₄Ppc* began to increase gradually from initial-tillering and reached its maximum at initial-heading, then began to decrease gradually. The P_n of Kitaake began to increase from tillering and reached its maximum at elongation, then began to decrease. The P_n of Kitaake with *ZmC₄Ppc* at different stages was higher than that of Kitaake except initial-tillering. The result was similar to the report of Li *et al.*(2001), who determined P_n of Kitaake with *ZmC₄Ppc* and Kitaake when the transgenic material were selected by determining PEPCase activity.

In a word, MRpc is a specific marker, which means band could be amplified uniquely from rice variety that has been transformed with *ZmC₄Ppc*, and has included the target gene in rice genome. The *ZmC₄Ppc* in transgenic materials, which was selected by MRpc, could be over expressed. The fact indicates that MRpc is gene marker of *ZmC₄Ppc*.

3.3 Performance of improved restorer bred by MAS

MRpc was applied in MAS. Shuhui 881 (recurrent parent) and Kitaake with *ZmC₄Ppc* (donor parent) were crossed in Nanjing. The hybrid was backcrossed and subjected to MAS continuously in Wenjiang, Sichuan (summer), and Lingshui, Hainan (spring), and improved Shuhui 881 with *ZmC₄Ppc* (FPM 881, BC₆F₂) was bred successfully. The breeding procedure for FPM 881 by MAS was shown in Fig.4. To analyze its genetic background, the polymorphism was analyzed between Kitaake with *ZmC₄Ppc* and Shuhui 881 by using SSR primers of 530 pairs distributed in the rice genome. One hundred and three pairs of them had polymorphism. The *F* values (Nei *et al.*, 1979) of similarity of 10 plants of FPM 881 were between 95.15%~99.03% as compared with Shuhui 881, the recurrent parent (Table 1).

To detect the photosynthetic characteristics of FPM 881, the plants with *F* value 99.03% were selected. Their PEPCase activity and P_n were measured at same developmental stage above. Their PEPCase activities of FPM 881 was the highest (1264.2 $\mu\text{mol}/(\text{mg}\cdot\text{h})$) at the elongation stage, and was the smallest (355.0 $\mu\text{mol}/(\text{mg}\cdot\text{h})$) at the initial-tillering stage (Fig.5A). They were increased significantly, and reached 23.3, 4.4, 4.4, 3.7, 1.3 and 3.7-fold at 6 different stages, respectively. The fact indicated that *ZmC₄Ppc* could be over expressed during MAS by using MRpc.

The P_n values of FPM 881 at 6 different stages were shown in Fig.5B. They were higher than that of Shuhui881 at initial-tillering, tillering, elongation, initial-heading, increased by 18.5%, 1.4%, 22.3% and 0.3%, respectively. The value of both PEPCase activity and P_n at elongation stage was the highest. It means that a higher PEPCase activity could be transformed to improve P_n . The P_n of FPM 881 at full-heading and maturing stage did not differ significantly from Shuhui 881, which might be due to the overcast and rainy weather at the time of measurement.

3.4 Analysis of GCA and SCA of FPM 881

The GCAs of 6 lines of FPM 881 and Shuhui 881 had different performances in different traits (Table 2). To number of panicles per plant, number of spikelets per panicle, seed setting rate and thousand-grain weight, the highest was line 5, Shuhui 881, line 3 and line 2, respectively. Grain yield per plant of line 1 had the highest GCA, secondly line 6, which had not significant difference, however, compared with line 1. Therefore, line 1 and line 6 could be further examined and utilized for GCA. SCA of each combination was listed in Table 3. The highest combination of number of spikelets per panicle, seed setting rate and thousand-grain weight was 2480A/Shuhui 881, 776A/line 3 and 776A/line 3, respectively. SCA of seed setting rate and thousand-grain weight of 776A/line 3 was the highest, respectively, the reason existing in higher GCA of line 3 in seed setting rate and thousand-grain weight. However, line 3 had lower GCA in grain yield per plant, its reason existing in too low GCA of line 3 in number of spikelets per panicle (Table 2). To grain yield per plant, SCA of 776A/line 6 was the highest, arrived at 18.01.

3.5 Identification and elimination of *hpt* gene

In BC₆F₄ of MAS, *ZmC₄Ppc* and *hpt* genes were amplified together in plants of above 6 lines, which began to selfing with size of 72 plants for each line, but not backcross again, and the elite plant with *ZmC₄Ppc* was reserved. In BC₆F₆, there were 3 plants in line 6, which had *ZmC₄Ppc* but not *hpt* (Fig.6). It indicated that target gene and marker-gene had segregated in this generation and the plants with *ZmC₄Ppc* gene but not *hpt* gene had gained. Therefore, marker- free *indica* three-line restorer FPM881 has been bred successfully.

4. Discussion

The existence of the biochemical characteristics of C_4 photosynthesis in the stems and petioles of C_3 plants indicates that the essential biochemical components and regulatory elements control the cell specific gene expression required for C_4 photosynthesis are already present in C_3 plants (Jeanneau *et al.*, 2002). C_3 plants have necessary genetic mechanism of high level expressing C_4 -specific genes. Therefore, to transfer the key enzyme genes in C_4 pathway into C_3 crops will be an efficient way to enhance its biomass production and grain yield. Wang *et al.* (2004) reported that the light saturated photosynthetic rate of F_1 hybrids of Kitaake with *ZmC₄Ppc* and photo-sensitive genetic sterile rice lines were 50% higher than that of the receptor parents. Huang *et al.* (2002) observed that the carboxylation efficiency of Kitaake transformed with *ZmC₄Ppc* increased by 49.3% and the CO_2 compensation point decreased by 26.2% than that of untransformed rice, Kitaake. Our results also proved that the introduction of *ZmC₄Ppc* into rice can enhance its photosynthetic capacity and efficiency.

MRpc is a gene marker of *ZmC₄Ppc* because its PCR results showed that MRpc only amplifies specific band from the materials that contain *ZmC₄Ppc* gene and its size of product is about 311 bp. The results of PEPCase activity and P_n of Kitaake with *ZmC₄Ppc* and FPM881 indicated that *ZmC₄Ppc* could be overexpressed. The highest PEPCase activity and P_n of FPM 881 were at elongation during 6 different developmental stages. PEPCase activity and P_n of FPM 881 were increased by 4.4-fold and 22.3% over control, Shuhui 881 at elongation, respectively. It showed that the increase of PEPCase activity could be transformed into elevating of P_n . However, the main increase of PEPCase activity was not at elongation, but at initial-tilling. By comparison with Shuhui 881 at same stage, PEPCase activity of FPM 881 increased 23.3-fold. The fact indicated that the expression of *ZmC₄Ppc* was constitutive, and highly efficient.

Besides MRpc reported in this study, there is no another report about specific marker of *ZmC₄Ppc* gene in rice. Related reports that tried to breed of restorers with *ZmC₄Ppc* by MAS are little at present; Wang *et al.* (2002) studied on 5129 and Wanjing 97 of two-line restorers by using Kitaake with *ZmC₄Ppc* and the MAS markers, p_1 GTTCCAACCACGTCTTCAAAGC; p_2 ATTTACCCGCAGGACATATCC. However, there was not any introduction about their origin, its specificity, background screening and combining ability in his report. Therefore, it was not known that similarity degree of genetic background and combining ability of improved restorers compared with recurrent parent. The studies have revealed that C_3 plants have at least two different type of genes; one type encode enzymes with 'housekeeping' function, and the other type are very similar to the C_4 genes of C_4 plants, though expression of the latter is very low or even undetectable in C_3 plants. These C_4 -like enzymes have high homology compared with C_4 -specific enzymes, and C_4 -like enzyme genes are progenitor of C_4 -specific enzyme genes in evolution (Ku *et al.*, 1996; Miyao, 2003). Besides these, there is C_3 -specific PEPCase in rice. Specificity of amplification by marker designed will not be assured if specific fragments are not found by nucleotide BLAST.

Six lines of FPM 881 and their control, Shuhui 881 were analyzed by GCA and SCA, and the better improved restorers could be selected out from offspring lines of MAS, which similarity of genetic background had arrived at 95% and more. Line 1 and line 6 had higher GCA in grain yield per plant and 776A/line 6 had the highest SCA in grain yield per plant of all F_1 , which also had the highest grain yield per plant, and its competing heterosis was 34.7%. Therefore, line 1 and line 6 can be further test-crossed with other sterile lines. These results proved that *ZmC₄Ppc* exerts gene effect in rice genetic background. The specific marker MRpc should be applied to breed elite *indica* restorers, maintainers and male sterile lines with *ZmC₄Ppc*, because they likely have higher net photosynthetic efficiency and grain yield.

Along with the continuous increase of commercial cultivation of trans-genetic plants, its safety problem in environment and food has been concerned widely, especially, the presence of marker genes of antibiotics or herbicide resistance in trans-genetic crops. Nowadays, without selectable-marker genes is an important prerequisite of breeding trans-genetic crops. It is a simple method for getting selectable-marker free plants with target gene by sexual crossing (Kai, *et al.*, 2002). Yu *et al.* (2005) obtained selectable-marker free transgenic rice plants with improved disease resistance from the selfing progeny of the transgenic plants. We have also gained success by this method.

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Table 1. Result of genetic background analysis of FPM 881

Plant no.	No. of primers	No. of differential loci	Differential SSR	Chr. of differential loci distributing	F%
1	103	2	RM307, RM413	4,5	98.06
2	103	3	RM71, RM307, RM413	2,4,5	97.09
3	103	3	RM71, RM307, RM413	2,4,5	97.09
4	103	2	RM71, RM307	2,4	98.06
5	103	2	RM71, RM307	2,4	98.06
6	103	5	RM71, RM220, RM283, RM307, RM413	2,1,4,5	95.15
7	103	2	RM71, RM307	2,4	98.06
8	103	3	RM220, RM283, RM307	1,4	97.09
9	103	1	RM307	4	99.03
10	103	1	RM307	4	99.03

Chr. means chromosome.

Table 2. Relative values of general combining ability of 5 grain yield components for 7 restorer lines

Restorer line	No. of panicles per plant	No. of spikelets per panicle	Seed setting rate	1000-grain weight	Grain yield per plant
Line1	-0.7792	-0.0219	3.548	3.0599	9.6303
Line2	-2.6343	-0.1364	-0.6481	3.2431	0.5719
Line3	2.9033	-10.8391	4.2766	3.1598	-0.2594
Line4	-4.0326	6.6098	2.0745	0.1629	0.8796
Line5	7.9564	-1.9768	-6.7326	0.8331	0.9604
Line6	5.2153	-0.9773	2.4655	-0.7112	8.6427
Shuhui881	-8.6288	7.3417	-4.984	-9.7476	-20.4255

Table 3. Relative values of specific combining ability of 5 grain yield components for 21 combinations

Male sterile line	Restorer line								
	Line1	Line2	Line3	Line4	Line5	Line6	Shuhui881		
No. of panicles per plant	G46A	5.7394	-2.0826	0.6447	3.4274	10.7925	-16.8681	-1.6534	
	776A	-12.0958	-8.8286	-10.213	-1.9065	-1.4358	12.3944	22.0853	
	2480A	6.3564	10.9111	9.5683	-1.5209	-9.3566	4.4736	-20.4319	
No. of spikelets per panicle	G46A	5.9211	0.807	7.8778	-5.4019	2.7669	2.2278	-14.1987	
	776A	-0.5875	3.3252	-14.896	1.3171	0.8975	4.0603	5.8838	
	2480A	-5.3336	-4.1321	7.0186	4.0848	-3.6645	-6.2882	8.3149	
Seed setting Rate	G46A	-3.209	2.9275	-1.502	3.4614	-2.8715	-0.1102	1.3038	
	776A	2.8277	0.1185	6.948	-1.0338	-0.6889	-1.019	-7.1525	
	2480A	0.3813	-3.0461	-5.446	-2.4275	3.5603	1.1292	5.8487	
1000-grain weight	G46A	3.3388	0.4085	-0.4323	-2.2429	1.1078	0.4043	-2.5842	
	776A	-1.1494	-1.9819	3.8704	-1.549	-1.6822	-0.9246	3.4167	
	2480A	-2.1894	1.5734	-3.4381	3.7919	0.5744	0.5203	-0.8325	
Grain yield per plant	G46A	9.6957	-0.2485	5.1431	0.6358	10.5365	-17.9146	-7.848	
	776A	-8.1477	-4.0738	-16.329	0.0332	-1.9301	18.009	12.4379	
	2480A	-1.548	4.3223	11.1854	-0.669	-8.6064	-0.0945	-4.5899	

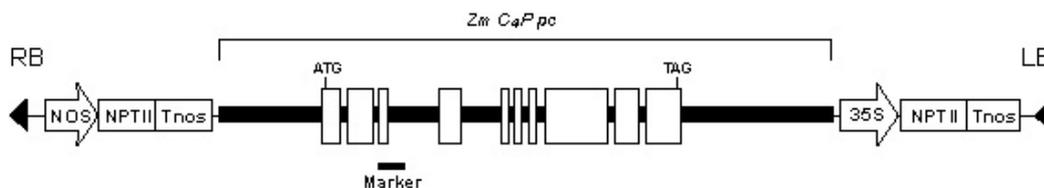


Figure 1. Structure of intact *ZmCaPpc* and the selective antibiotic resistance gene used for rice transformation. Marker showed the site of band amplified on the intact *ZmCaPpc*.

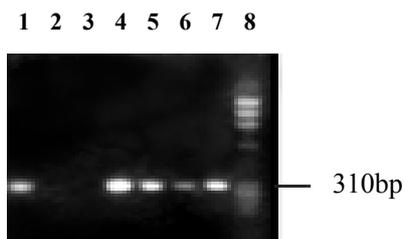


Figure 2. Bands of *ZmCaPpc* amplified by PCR with MRpc

Lane 1, FPM881 (rice); Lane 2, Shuhui881 (rice); Lane 3, Kitaake (rice); Lane 4, Kitaake with *ZmCaPpc* (rice); Lane 5, Linao4 (maize); Lane 6, Yuyu22 (maize); Lane 7, Yayu10 (maize); Lane 8, DNA marker.

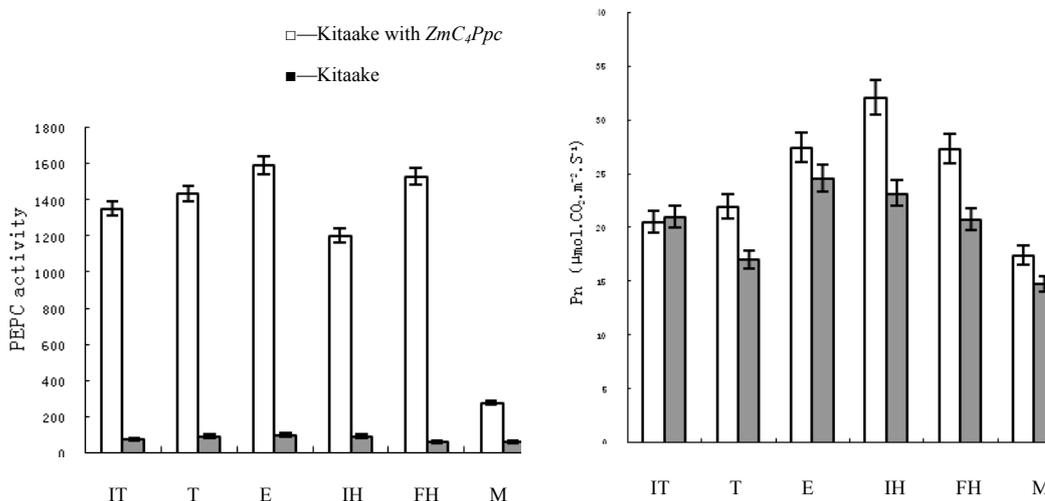


Figure 3. The PEPCase activities and net photosynthetic rate of Kitaake with *ZmC4Ppc* and Kitaake at different developmental stages. IT, initial-tillering; T, tillering; E, elongation; IH, Initial-heading; FH, Full-heading; M, maturing.

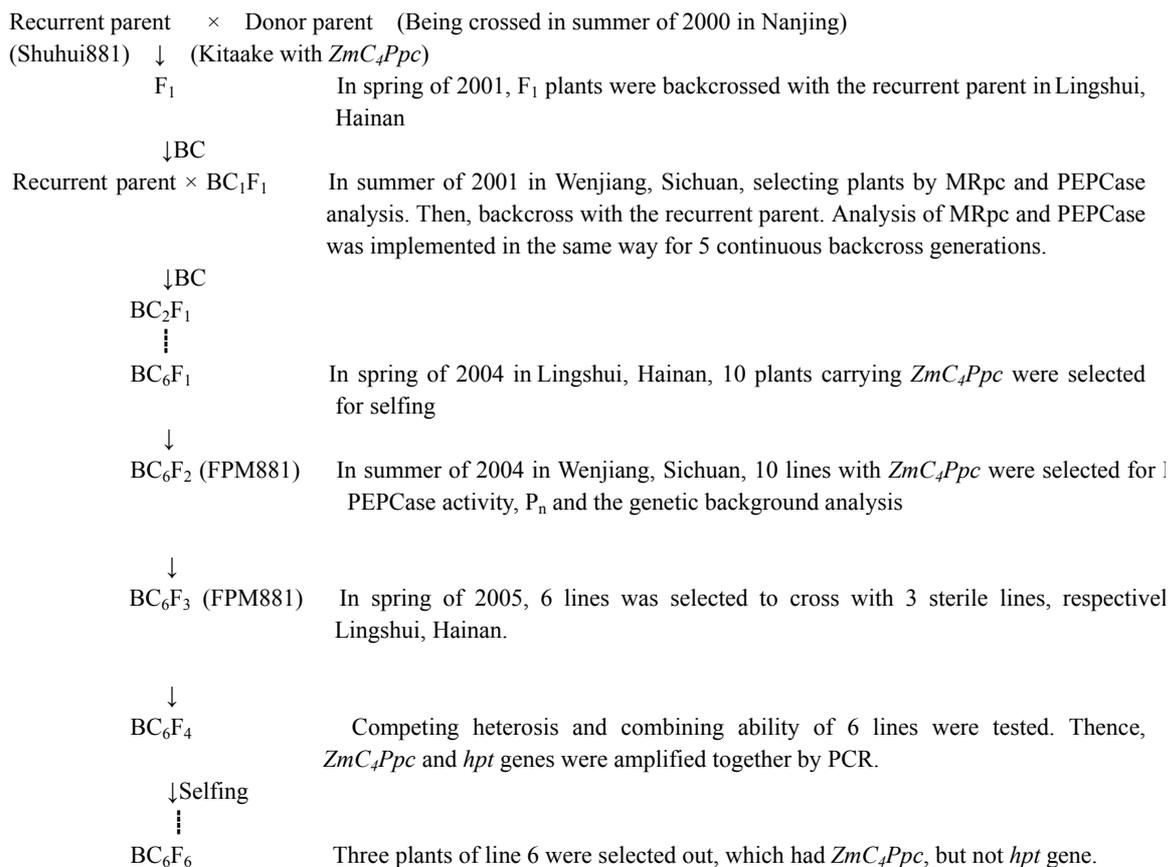


Figure 4. Breeding procedure for improved Shuhui 881 with *ZmC4Ppc* (FPM 881) and not *hpt* gene by marker-assisted selection (MAS)

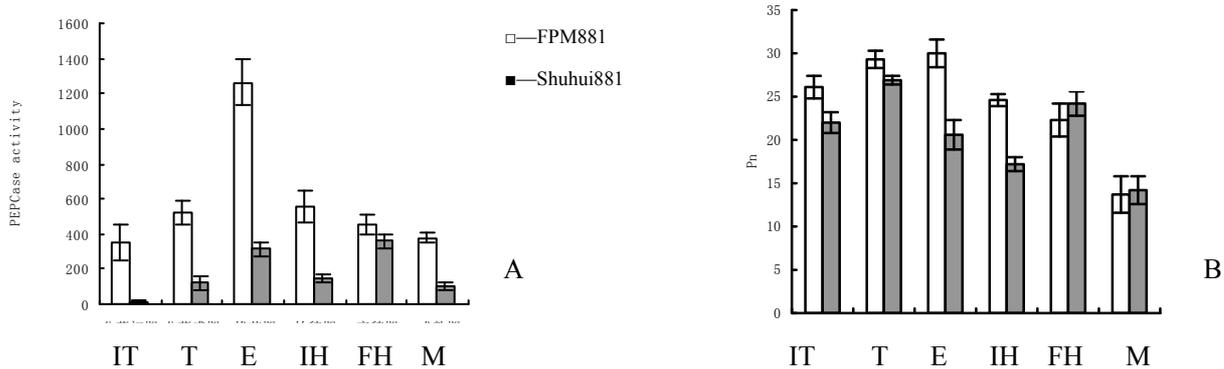


Figure 5. PEPCase activities and net photosynthetic rate of FPM 881 and Shuhui 881 at different developmental stages. IT, initial-tillering; T, tillering; E, elongation; IH, initial-heading; FH, full-heading; M, maturing

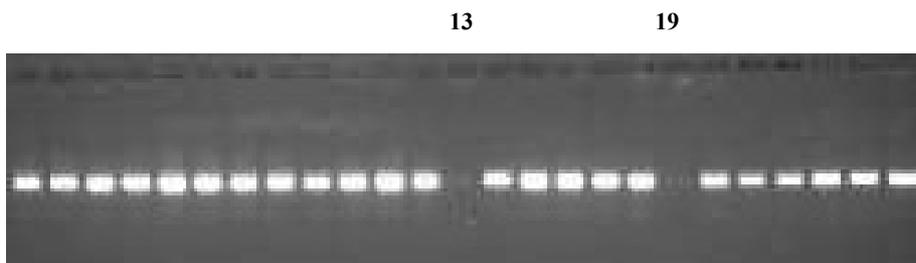


Figure 6. Amplified bands of FPM881 lines with *ZmC₄Ppc* by the *hpt* primers. Lane 13 and lane 19 were the target plants, which had *ZmC₄Ppc* but not *hpt* gene