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RESEARCH ARTICLE

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Integration of C_4-specific *PPDK* gene of maize to C_3 rice and its characteristics in relation to photosynthesis

Abstract Pyruvate orthophosphate dikinase (PPDK) is a key enzyme in photosynthesis in some plants that exploit the C_4 photosynthetic pathway for the fixation of CO_2. The C_4-specific *PPDK* encoding pyruvate orthophosphate dikinase was introduced into C_3 plant, a rice (*Oryza sativa* L. cv. *indica* IR64) mediated by biolistic and *Agrobacterium* transformation. The C_4-*PPDK* gene of maize was integrated to *indica* IR64 with polymerase chain reaction (PCR)-Southern blotting. The total nitrogen of flag leaves of transgenic IR64 was analyzed with Kjeldahl method for quantitative determination of nitrogen, indicating that the total nitrogen of flag leaves of most transgenic IR64 was higher than that of non-transgenic control IR64 formants in the greenhouse. The maximum value of total nitrogen of flag leaves was 3.61% among transgenic IR64 plants, 1.07% higher than that of non-transgenic control IR64 formants. The total nitrogen of flag leaves of transgenic IR64 was increased by 42.1%. The factors for yield of transgenic IR64 plants were analyzed, indicating there was a greater difference in yield-forming factors among transgenic IR64 plants in the greenhouse, i.e. dried plant weight, harvested index and so on. Thus, it could help rice breeders select different materials for breeding.

Keywords characteristics, integration, maize (*Zea mays*), photosynthesis, pyruvate orthophosphate dikinase (*PPDK*) gene, rice (*Oryza sativa* L.)

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1 Introduction

Photosynthesis is the basis of life on earth and yet its efficiency in most plants is compromised because ribulose-bisphosphate carboxylase (Rubisco), the primary carboxylating enzyme, is promiscuous (Bowes et al., 1971; Brown et al., 2005). The control of photosynthetic gene expression in plants is often complex, with regulation occurring at many levels and in response to numerous developmental, metabolic, or environmental signals (Tobin and Silverthorne, 1985; McCormac et al., 1997). The photosynthetic C_4 cycle acts as a pump that concentrates CO_2 at the site of Rubisco. Photorespiration is thereby largely abolished, and the net photosynthesis rate is increased. This is achieved by the metabolic interaction of mesophyll and bundle-sheath cells, and is dependent on the correct compartmentalization of the enzymes in the C_4 photosynthetic carbon assimilation pathway (Hatch, 1978, 1987). It has been shown that this compartmentalization is due to differential gene expression (Nelson and Langdale, 1992). Thus, phospho*enol*pyruvate carboxylase (PEPCase), NADP-malate dehydrogenase (MDH) and pyruvate orthophosphate dikinase (PPDK) are highly expressed in mesophyll cells, whereas malic enzyme (ME) and Rubisco are localized preferentially to bundle-sheath cells (Hatch, 1987).

Pyruvate orthophosphate dikinase (PPDK; EC 2.7.9.1) plays an important role in C_4 photosynthetic carbon metabolism and in crassulacean acid metabolism (CAM). The enzyme catalyzes the synthesis of the primary acceptor of CO_2, namely, phospho*enol*pyruvate (Kluge and Osmond, 1971; Hatch and Slack, 1986). This reaction is considered to be the rate-limiting step in these metabolic pathways and it has been studied extensively not only from an enzymological perspective (Edwards et al., 1985) but also from a molecular biological perspective (Matsuoka, 1995). In contrast to the high activities of PPDK in the green leaves of C_4 and CAM plants, very low activity has been detected in the green leaves of C_3 plants (Edwards et al., 1982; Meyer et al., 1982). Therefore, not much attention has been paid to the importance of this enzyme in C_3 plants. The primary aim in the paper was to develop *indica* IR64 with intact *PEPC* and *PPDK* genes of maize and carried out characteristics in relation to photosynthesis.

2 Materials and methods

2.1 Plasmid, transformation of rice, and media

The spikelets of IR64 were collected at 10–12 days after anthesis. The immature embryos were dehulled and sterilized in 70% ethyl alcohol for 1 min and then in 50% chlorox (5%–25% Na-hypochlorite solution) for 30 min.

The immature embryos of IR64 were isolated and inoculated to the induction media (Table 1). After one day, the immature embryos were collected in a new induction media and bombarded with the intact *PPDK* gene of maize mediated by biolistic transformation. After 16 h, the bombarded immature embryos were transferred to the selective media for three cycles of selection every 14 days. After selection, the surviving calli were transferred to the pre-differentiation media for 14 days. The transformation mediated with *Agrobacterium tumefaciens* accords to the procedures (Datta et al., 1997).

Table 1 Media used in the experiments

Induction media	MS basal media + 2,4-D 2 mg/L + 300 mg/L CH + 0.2 mg/L NAA + 8 g/L agar, pH 5.8
Selective media	MS basal media + 2,4-D 2 mg/L + 300 mg/L CH + 0.2 mg/L NAA + 50 mg/L hygromycin + 8 g/L agar, pH 5.8
Pre-differentiation media	MS basal media + maltose 30 g/L + sorbitol 20 g/L + kinetin 30 g/L + NAA 0.5 mg/L + 8 g/L agar, pH 5.8
Differentiation media	MS basal media + 1 mg/L NAA + 2 mg/L kinetin + 2.5 g/L gelrite, pH 5.8
Rooting media	MS basal media + 2.5 g/L gelrite, pH 5.8

The embryogenic calli were then transferred to the regeneration media and incubated at 25°C under a 16-h photoperiod (Datta et al., 1992). Regenerated plants with well-developing roots were eventually transplanted to 14-cm-diameter pots and grown to maturity in a greenhouse for transgenic plants (Figs. 1 and 2).

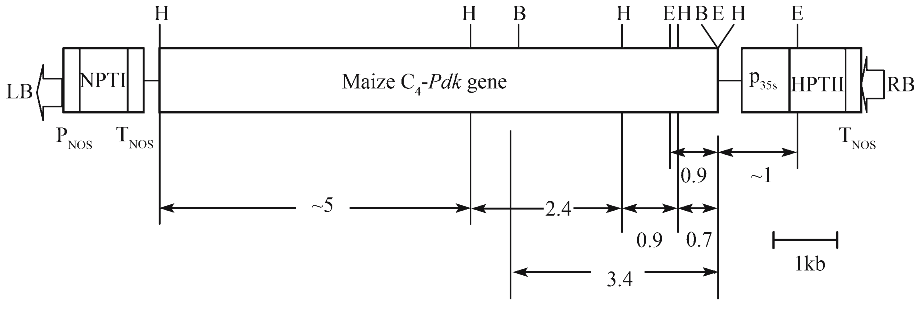
To confirm the putative transgenic IR64 plants with polymerase chain reaction (PCR) for the antibiotic resistance (hygromycin phosphotransferase) gene, the forward primer used was 5′AC TTC TAC ACA GCC ATC 3′ and the reverse primer was 5′ TAT GTC CTG CGG GTA AAT 3′, with PCR volume of 25 µL, 10 mmol/L Tris/HCl, 50 mmol/L KCl, pH 8.3, 1.8–1.9 mmol/L MgCl$_2$, 0.16 mmol/L dNTP, 2.0 ng/µL primer, 25–30 ng DNA, 94°C denaturing for 5 min, 94°C for 30 sec, 60°C for 30 sec, 72°C for 1 min, 36 cycles, 72°C extension for 10 min, and stored at 4°C overnight.

2.2 Extraction of total DNA and PCR-Southern blotting

Leaf tissues of putative transgenic IR64 plants were chopped and ground to a fine powder in a pre-chilled mortar and pestle. The process of DNA extraction was the same as Dellaporta's method (Dellaporta et al., 1983). Polymerase chain reaction volume of 25 µL, 10 mmol/L Tris/HCl, 50 mmol/L KCl, pH 8.3, 1.8–1.9 mmol/L MgCl$_2$, 0.16 mmol/L dNTP, 2.0 ng/µL primer, 25–30 ng DNA, 94°C denaturing for 5 min, 94°C for 30 sec, 55°C for 30 sec, 72°C for 1 min, 36 cycles, 72°C extension for 5 min, and stored at 4°C overnight.

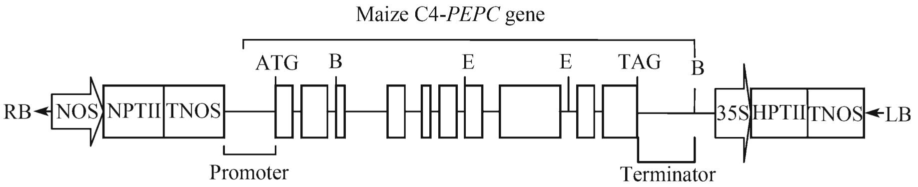
For intact *PPDK* gene of PCR-Southern blotting, the forward primer was 5′CTC TGT TAC GCA AGG AAG 3′ and the reverse primer was 5′CTC TCG TCT GAA GCA AAG 3′. The PCR samples were separated by electrophoresis on a 1% (w/v) TAE agarose gel and transferred under alkaline denaturing conditions to a Hybond N$^+$ nylon membrane (Amersham). The radioactive probe was prepared with PCR products under the template of the plasmid containing intact *PEPC* gene of maize by the primer of intact *PPDK* gene method using α-[^{32}P] dCTP and the Rediprime labeling system (Amersham). Hybridization with the probe was conducted according to the manufacturer's instructions. Following hybridization, the membrane was washed and exposed to X-ray Hyperfilm™ MP (Amersham).

For intact *PEPC* gene, the PCR samples were separated by electrophoresis on a 1% (w/v) TAE agarose gel and transferred under alkaline denaturing conditions to Hybond N$^+$ nylon membrane (Amersham). The radioactive probe was



Note: LB and RB stand for left border and right border, respectively. *hpt* and *nptII* stand for hygromycin phosphotransferase and neomycin phosphotransferase II gene, respectively. PNos and TNos stand for promoter nopaline synthase and terminator nopaline synthase, respectively. 35S stands for CaMV 35S RNA promoter, and H, B and E stand for *Hin*dIII site, *Bam*HI site and *Eco*RI site, respectively.

Fig. 1 Schematic representation of the intact maize gene and the selective antibiotic resistance gene (hygromycin phosphotransferase, *HPT II*) used for rice transformation, containing its own promoter and terminator sequences and intron/exon structure



Note: The intact *PEPC* gene of maize is an 8.8 kb fragment containing all exons, introns and the promoter (from 1212) and terminator (2.5 kb) sequences; ATG and TAG stand for initiation codon and termination codon, respectively; B and E stand for *Bam*HIsite and *Eco*RIsite, respectively; LB and RB stand for left border and right border, respectively.

Fig. 2 Schematic diagram of the intact *PEPC* gene of maize and the selective antibiotic resistant gene hygromycin phosphotransferase (*HPTII*) used for rice transformation

prepared with PCR products under the template of the plasmid containing intact *PEPC* gene of maize by the primer of intact *PEPC* gene method using α-[^{32}P] dCTP and the Rediprime labeling system (Amersham). *PEPC* gene forward primer was CAC ACC CTC AAT TAG CTA GG, *PEPC* gene reverse primer was TGA GAG TTC TGG TAT GGA CC. Hybridization with the probe was conducted according to the manufacturer's instruction. Following hybridization, the membrane was washed and exposed to X-ray Hyperfilm™ MP (Amersham).

2.3 Measurement of the total nitrogen of flag leaves of transgenic IR64 plants

The total nitrogen of flag leaves between transgenic IR64 plants and non-transgenic IR64 plants was analyzed with Kjeldahl nitrogen quantity method.

3 Results

3.1 The putative transgenic IR64 plants obtained

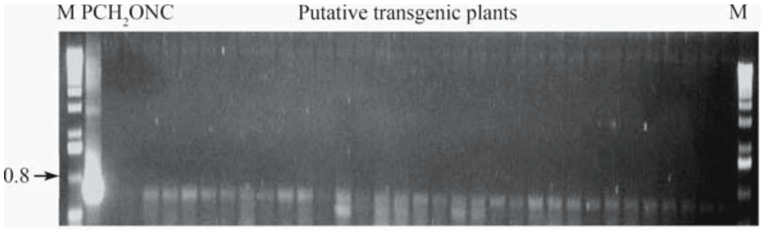
Figures 1 and 2 describe the construct of intact *PPDK* and *PEPC* genes of maize, containing an engineered *hph* gene. The gene constructs were introduced into *indica* IR64 to express the maize *PPDK* and *PEPC* genes in the chloroplasts of the mesophyll cells. The immature embryos of IR64 were bombarded with plasmid pIG121 Hm with intact *PPDK* gene of maize mediated with agrobacterium and by biolistic. The putative transgenic plants of IR64 were confirmed with hygromycin primer by PCR. The results showed that some putative transgenic IR64 plants had a 0.8 kb fragment, which was resistant to hygromycin phosphotransferase, indicating that the intact *PPDK* or *PEPC* gene of maize was integrated to the genome of *indica* IR64 and in the meantime, the fertile transgenic IR64 plants with intact *PPDK* gene of maize were obtained (Fig. 3).

3.2 Confirmation of putative transgenic plants of IR64 with PCR-Southern blotting

The putative transgenic plants were positively confirmed with hygromycin gene primer by PCR. The positive transgenic plants were confirmed with *PEPC* or *PPDK* gene primer by PCR-Southern blotting. The results showed that there was a 0.56 kb fragment for *PPDK* gene of transgenic plants, but no 0.56 kb fragment for *PPDK* gene of non-transgenic plants, indicating that the intact *PPDK* gene from maize was integrated to *indica* rice IR64 genome. For *PEPC* gene of maize, there was no 1.0 kb fragment by PCR-Southern blotting, indicating that the intact *PEPC* gene of maize was not integrated to the genome of *indica* rice IR64 (Figs. 4 and 5).

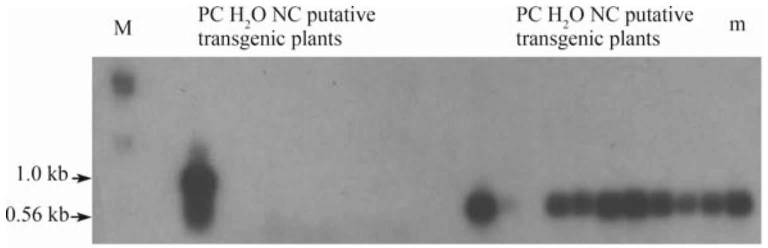
3.3 Measurement of height of transgenic IR64 plants

The height of transgenic plants of IR64 was measured when these plants were at the mature stage in the greenhouse. There were no significant differences in plant height between transgenic plants and non-transgenic plants of IR64 in the greenhouse. The tallest transgenic plant was 102.0 cm, which was 26.5 cm taller than that of non-transgenic plant (control). However, some transgenic plants were lower than the control in the greenhouse, indicating that the height of *indica* rice IR64 was affected by the intact *PPDK* gene of maize (Fig. 6).



Note: From left to right, lane 1: 1 kb ladder; lane 2: positive control (PC); lane 3: H_2O; lane 4: negative control (NC); other lanes: putative transgenic plants; last lane: 1 kb ladder.

Fig. 3 Analysis of putative transgenic plants with *PEPC* and *PPDK* genes with PCR



Note: From left to right, lane1: positive control (PC); lane 2: H_2O; lane 3: negative control (NC); other lanes: putative transgenic plants; last lane: maize.

Fig. 4 PCR-Southern blotting analysis of the putative transgenic plants with *PEPC* and *PPDK* genes of maize

Fig. 5 Transgenic IR64 plants with intact *PPDK* gene of maize

3.4 Tiller of transgenic IR64 plants

The tiller number of transgenic plants of IR64 was counted when these plants entered the mature stage in the greenhouse. The results showed that there were different effects for different transgenic plants of IR64 with intact *PPDK* gene of maize. The most tiller number of transgenic plant IR64 was 26, which was 15 more than that of non-transgenic IR64 (control) in the greenhouse. However, the tiller number of some transgenic IR64 was smaller than that of the control in the greenhouse, indicating that the tiller number of transgenic IR64 was affected with the intact *PPDK* gene of maize (Fig. 7).

3.5 Total nitrogen of flag leaves of transgenic IR64 with intact *PPDK* gene of maize

Photosynthesis supplied by leaves and metabolized in the roots provides (a) carbon and energy for nitrogen assimilation, and (b) substrate in the form of phospho*enol*pyruvate

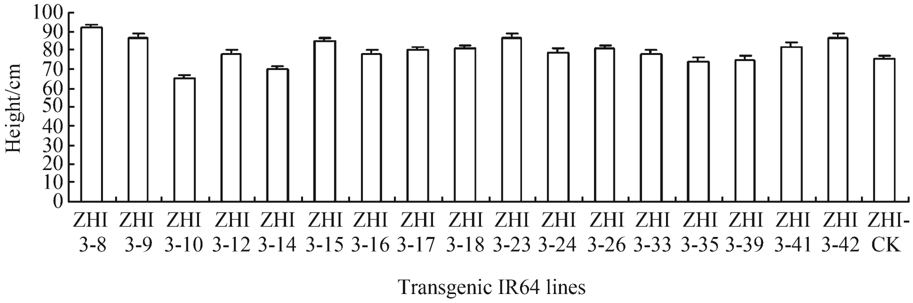


Fig. 6 Height of transgenic IR64 plants with intact *PEPC* and *PPDK* genes of maize

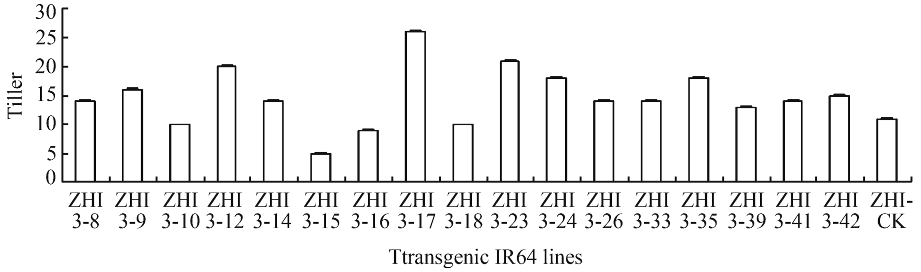


Fig. 7 Tiller number of transgenic IR64 plants with intact *PEPC* and *PPDK* genes of maize

(*PEP*) for *PEPC*. There are also some evidences that malic acid itself may be transported from shoots to roots (Deane-Drummond et al., 1979; Oaks and Hirel, 1985), thereby bypassing a requirement for *PEP* and *PEPC*. Nitrogen is a limiting factor in photosynthetic capacity. C_4 plants not only have higher efficiency than C_3 plants for the utilization of light and water, but also use nitrogen more efficiently, perhaps owing to low photorespiration that affects carbon and nitrogen metabolism (Oaks, 1994; Sugiyama, 1998). The total nitrogen of flag leaves of partial transgenic plants IR64 was measured when the transgenic plants and non-transgenic IR64 plants were at heading stage. The results showed that the total nitrogen in the flag leaves of most transgenic plants was higher than that of the control. The highest nitrogen content of flag leaves of a transgenic plant was 3.61%, which was 1.07 percent higher than that of the control (Fig. 8). However, for transgenic plant ZHI3-9, its total nitrogen content was lower than that of the control, indicating that transgenic plants with intact *PPDK* gene of maize affected the nitrogen assimilation during the growth and development of rice plants in the greenhouse.

3.6 The yield characteristics of transgenic IR64 plants with intact *PPDK* of maize

There is a persistent hope amongst plant breeders, no matter whether conventional or transgenic varieties are used, that the photosynthetic efficiency of crops can be improved. After all, the first three priorities for a breeder are yield, yield, and yield, and it is assumed that the improved solar energy capture can be translated into greater harvestable yield (Dunwell, 2000). The transgenic plants and non-transgenic plants were harvested when they were at dough stage in the greenhouse. The results showed that the 1 000-grain weight of transgenic plants was obviously improved, compared with the control. There was no great difference in the grain filling (%) between transgenic plants and control in the greenhouse. The best grain filling (%) was 98.33%, which was 2.18% higher than that of the control (Table 2). However, there was much difference in the plant dry weight between transgenic plants and control. The most weight of dry plant ZHI3-23 was 52.4 g, which was 15.1 g dry weight more than that of the control, but its harvest index was 16.03%, which was 9.59 % lower than that of the control, which indicated that the source and stock of transgenic plants were not coordinated.

4 Discussion

Global demand for rice as one of the major staple crops has strengthened the need for its increased productivity. The conventional hybridization has failed to successfully transfer C_4 traits to C_3 plants. Due to lack of proper C_4 traits in the rice gene pool, the use of genetic engineering to produce better variety of C_4 *indica* rice is a better solution. Enzymes involved in C_4 photosynthesis may also play important roles in plant defense mechanism to counteract biotic and abiotic stresses.

Physiological impacts were minimal and no changes in photosynthetic characteristics were observed even in

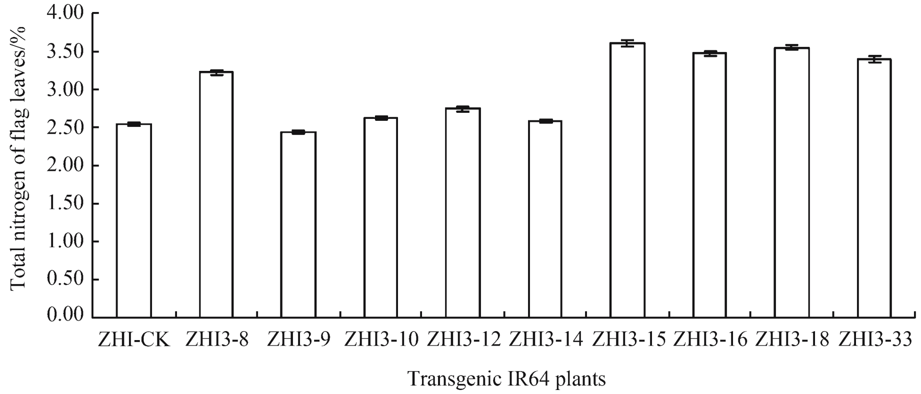


Fig. 8 Total nitrogen concentration in flag leaves of transgenic IR64 plants with intact *PEPC* and *PPDK* genes of maize

Table 2 Yield characteristics of transgenic IR64 plants with intact *PPDK* gene of maize

Line of transgenic IR64 plants	Plant dry weight /g	Panicle length /cm	Filled grain		Unfilled grain		Weight of 1000-grain /g	Grain filling /%	Harvest index /%
			Total number of grain	Weight /g	Total number of grain	Weight /g			
ZHI3-8	39.0	21.1	500	12.5	110	0.5	25.0	96.15	32.05
ZHI3-9	46.6	21.1	624	15.6	116	0.5	25.0	96.89	33.48
ZHI3-10	17.8	20.1	252	6.3	90	0.4	25.0	94.03	35.39
ZHI3-12	46.5	20.1	708	17.7	71	0.3	25.0	98.33	38.06
ZHI3-14	34.1	21.1	532	13.3	68	0.3	25.0	97.79	39.0
ZHI3-15	18.2	19.2	256	6.4	92	0.4	25.0	94.12	35.16
ZHI3-16	24.0	20.2	292	7.3	121	0.5	25.0	93.59	30.42
ZHI3-17	48.7	22.0	736	18.4	165	0.7	25.0	96.34	37.78
ZHI3-23	52.4	19.0	336	8.4	147	0.6	25.0	93.33	16.03
ZHI3-24	47.1	18.0	572	14.3	150	0.6	25.0	95.97	30.36
ZHI3-26	45.0	20.6	578	14.3	86	0.4	24.7	97.28	31.78
ZHI3-35	45.0	20.1	460	11.5	90	0.4	25.0	96.64	25.56
ZHI3-39	36.3	17.2	432	10.8	265	1.3	25.0	89.26	29.75
ZHI3-41	40.6	21.1	416	10.4	84	0.4	25.0	96.30	25.62
ZHI3-42	50.3	21.2	292	7.3	244	1.2	25.0	85.88	14.51
Control	37.3	19.0	507	12.5	113	0.5	24.7	96.15	33.51

Note: Harvest index = filled grain weight/Biological yield × 100%; grain filling (%) = filled grains/total grains × 100%.

transgenic rice plants with a 20-fold increase in activity (Fukayama et al., 2001). In general, the reaction of PPDK is reversible, depending on concentrations of substrates, activators, and inactivators. This may be the reason why the overproduction of PPDK does not result in significant effects on carbon metabolism in rice leaves. Although preliminary, overproduction of PPDK increased the grain number per plant and 1000-grain weight of rice plants (Miyao-Tokutomi and Fukayama, 2005). In our study, the immature embryos of IR64 were bombarded with intact *PEPC* gene and *PPDK* gene of maize mediated by biolistic. Among the transgenic IR64 plants, no *PEPC* gene was detected with PCR-Southern blotting in transgenic IR64 plants. The total nitrogen in the flag leaves of some transgenic IR64 plants with intact *PPDK* gene of maize was increased. The grain number of some transgenic IR64 plants with intact *PPDK* gene of maize was also increased in the greenhouse. In the meantime, the index of harvest of some transgenic IR64 plants was improved in the greenhouse, indicating that photosynthetic rate was not only improved with intact *PPDK* gene of maize in transgenic rice plants, but also would improve yield of rice.

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