African Journal of Biotechnology Vol. 9 (2), pp. 234-242, 11 January, 2010
Available online at http://www.academicjournals.org/AJB
ISSN 1684–5315 © 2010 Academic Journals

Full Length Research Paper

Characterization of a C_4 maize pyruvate orthophosphate dikinase expressed in C_3 transgenic rice plants

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Accepted 26 May, 2009

Pyruvate orthophosphate dikinase (PPDK) is a key enzyme in plants that utilize the C_4 photosynthetic pathway to fix CO_2. The enzymatic reaction catalyzed by PPDK is critically controlled by light and is one of the rate-limiting steps of the C4 pathway. The intact maize (*Zea mays*) C_4-*PPDK* gene, containing its own promoter, terminator sequences and exon/intron structure was introduced into rice (*Oryza sativa* L. *Indica* "IR64"), a C_3 plant. Expression of C_4-*PPDK* in most transgenic rice lines resulted in increased CO_2 assimilation rates compared to untransformed control plants. Most of the transformants showed higher photosynthetic activities than that of wild-type plant. Total nitrogen in the flag leaves of C_4-*PPDK* transgenics was analyzed. Results showed an increase in total nitrogen compared to untransformed control plants suggesting that C_4-*PPDK* expression in rice promoted nitrogen absorption from the soil. In addition, the photosynthesis rate of some transgenic IR64 lines was also increased in the greenhouse. Molecular analysis revealed that the intact *PPDK* gene integrated in the rice genome and affected the phenotypes of plants particularly tillers and enhanced yield of transgenic IR64 rice plants in the greenhouse.

Key words: *Indica* rice IR64, Maize (*Zea mays*), photosynthesis, PPDK (pyruvate orthophosphate dikinase), C4 rice.

INTRODUCTION

In order to meet the demand for food from the growing world population, there will have to be significant increases in yield of the major crops grown in developing countries. In rice, for example, an estimated 50% yield increase is needed by 2030. Increasing the maximum yield potential is viewed as an important, if not vital, part of any strategy for achieving this increase in yield (Khush and Peng, 1996). Since the harvest index for many crops, such as rice, is approaching a ceiling value, an increase in yield potential will have to involve an increase in crop biomass, that is, there will have to be more photosynthesis (Cassman, 1994; Ying et al., 1998; Mann, 1999a, b). This could be achieved by an increase in leaf area index (LAI) or an increase in net photosynthesis per unit leaf area. Since LAI is generally already high in most crops, the increase in assimilate production should come from improved photosynthesis.

Terrestrial plants are classified into three major photosynthetic types—C_3, C_4, and Crassulacean acid metabolism (CAM) plants—according to the mechanism of their photosynthetic carbon assimilation. About 90% of terrestrial plant species, including major crops such as rice (*Oryza sativa*), wheat (*Triticum aestivum*), soybean

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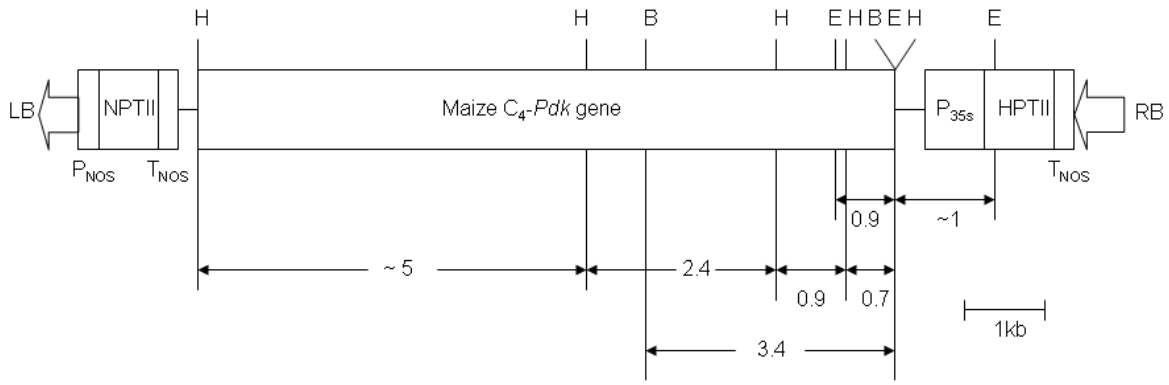


Figure 1. Schematic represetation of the intact *PPDK* gene, containing its own promoter and terminator sequences and intron/exon structure, and the selective antibiotic resistance gene (hygromycin phosphotransferase, *HPT II*) used for rice transformation. LB, left border; RB, right border, hpt, hygromycin phosphotransferase; nptII, neomycin phosphotransferase II gene; PNos, promoter nopaline synthasel 35S, CaMV 35S RNA promoter, TNos, terminator nopaline synthase; H, HindIIIsite; B, BamHIsite; E, EcorR1site.

(*Glycine max*), and potato (*Solanum tuberosum*), are classified as having a C_3 photosynthetic pathway, also called the Calvin cycle or photosynthetic carbon reduction (PCR) cycle. C_4 and CAM plants possess a unique photosynthetic pathway, in addition to the C_3 pathway, that allows them to adapt to specific environments. Although C_3 plants grow well in temperate climates, C_4 plants such as maize (*Zea mays*) and sugarcane (*Saccharum officinarum*) adapt to high-light, arid, and warm environments and achieve higher photosynthetic capacity and higher water- and nitrogen-use efficiencies than C_3 plants by means of the C_4 photosynthetic pathway, which acts to concentrate CO_2 at the site of the reactions of the C_3 pathway (Hatch, 1987). The transfer of C_4 traits to C_3 plants is one strategy being adopted for improving the photosynthetic performance of C_3 plants. In C_3 plants, low PPDK enzyme activity was reported for a number of different tissue types and, depending on the species, this activity is found only in chloroplasts or in both chloroplasts and the cytosol (Aoyagi and Bassham, 1985; Nomura et al., 2000). The function of *PPDK* in C_3 plants is less clear. However, for guard cells of *Vicia faba*, Schnabl (1981) proposed that chloroplastic *PPDK* in concert with cytosolic NADP-malic enzymes (NADP-ME) could play a role in gluconeogenetic phosphoenolpyruvate (PEP) generation from pyruvate during stomatal closure. The recent application of recombinant DNA technology to plant metabolism has considerably advanced our understanding of the regulation of photosynthesis (Furbank and Taylor, 1995). Transgenic japonica Kitaake with the *PPDK* gene was obtained and it contained a significant accumulation of pyruvate orthophosphate dikinase (PPDK) (Fukayama et al., 2001).

In the present study, transgenic IR64 plants with intact *PPDK* gene of maize were obtained by biolistic transformation with the PDS-1000/He Particle Delivery System (Bio Rad). We measured the CO_2 assimilation rate at the beginning of heading stage in the greenhouse and evaluated some characteristics in relation to photosynthesis rate.

MATERIALS AND METHODS

Plasmid constructs, plant materials, transformation and *in vitro* culture

The plasmid pIG121, which contains the intact maize ppdk gene derived by the maize's own promoter and *hph* as marker was used for bombardment (Figure 1). Ten to 12 days old immature embryos of indica rice IR64 were sterilized, isolated, plated and bombarded by PDS-1000He particle gun as described in Christou et al. (1991) and Datta et al. (2001). The bombarded embryos and selected embryogenic cali were cultured on MS medium (Table 1) with 50 mg/L hygromycin for 3 selection cycles with 14-day duration each cycle. Embryogenic cali were selected for regeneration and plantlets were first grown in the culture solution (Datta et al., 1997) before transfer to 14 cm diameter pots. The putative primary transgenics and subsequent seed progenies were grown in the containment transgenic green house following a day night temperature regime of 31/21±2°C and 70-90% relative humidity.

Polymerase chain reaction (PCR) and DNA gel blotting analysis

Genomic DNA was isolated from 1-month old plants using microprep method and 50-100 ng of template DNA was used for PCR analysis with hygromycin phosphotransferase gene (*hph*) specific primer set of the following sequence:

hph F ACTTCTACACAgCCATC
hph R TATgTCCTgCgggTA AAT

PCR volume 25 µL, 10 mmol/L Tris/HCl, 50 mmol/L KCl, pH 8.3, 1.8-1.9 mmol/L MgCl2, 0.16 mmol/L dNTP, 2.0 ng/µL 194 primer, 25-30 ng DNA, 94˚C denaturing for 5 min, 94˚C for 30 s, 60˚C for 30 s, 72˚C for 1 min, 36 cycles, 72˚C extension for 10 min, 4˚C overnight.

Plant genomic DNA was extracted from freshly harvested leaves

Table 1. The segregation of progenies of transgenic lines.

Transgenic line	Total plants	Positive for PCR	Negative for PCR	Ratio	χ^2
ZHI2-7	52	37	15	37:15	0.23
ZHI2-8	49	31	18	31:18	3.00
ZHI2-9	48	35	13	35:13	0.028
ZHI2-10	50	33	17	33:17	1.71

$\chi^2 = \sum(|O-E|-0.5)^2/E$; O, observation value; E, theoritical value; $\chi^2_{0.05,1} = 3.84$.

of transgenic and non-transgenic control plants for southern analysis, following the modified CTAB method (Dellaporta et al., 1983; Datta et al., 1997). Ten micrograms of DNA, digested with restrictton endonuclease *Bam*H1 at 37˚C overnight, were separated by electrophoresis on a 1% (w/v) TAE agarose gel and transferred to a Hybond N^+ NYLON MEMBRANE (Amersham, Arlington Heights, IL) according to the manufacturer's instructions. The *ppdk* coding sequence isolated from plasmid pIG121 Hm by digestion with *BamH1*, was labeled with α-[^{32}P] dCTP using the Rediprime labeling kit (Amersham, Arlington Heights, IL) and used as a hybridization probe.

Total RNA isolation and reverse transcription polymerase chain reaction (RT-PCR)

Total RNA was isolated from green leaves of transgenic and control plants at the heading stage using RNAeasy extraction kit (QIAGEN, Germany). RT-PCR was performed on 2 ug of total RNA using the specific another primers for ppdk following the previously described method (Datta et al., 2002).

ppdk F ACTggTgAggTgATCCTTgg
ppdk R CACCgTgTTCTCCACAAATg

The PCR products were resolved on 1.2% TAE-agarose gel.

Extraction of soluble protein and PPDK activity analysis

The green leaves segments of about 5 cm were harvested from the midsection of the uppermost fully expanded leaf and were immediately frozen in liquid nitrogen until use. Unless stated otherwise, the leaf samples were harvested at 11:00 am on sunny days. Soluble protein was extracted from the leaf segment: Samples were ground in extraction buffer containing: 50 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid)-KOH (pH7.4), 5 mM pyruvate, 2 mM KH_2PO_4, 10 mM $MgCl_2$, 1 mM EDTA, 5 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 10 μm leupeptin, 5% (w/v) insoluble polyvinylpolypyrrolidone, and 10% (w/v) glycerol, with a small amount of sea sand. The homogenate was centrifuged at 15,000 *g* for 10 min at 4℃ and the resultant supernatant was collected as a total soluble protein extract. Protein was determined by the method of Bradford (1976) with bovine serum albumin as the standard. The supernatant after centrifugation of the homogenate was incubated at 25℃ for 1 h to activate PPDK. PPDK activity was assayed in the forward direction at 30℃, essentially by the method of Ashton et al. (1990). The assay mixture contained 50 mM HEPES-KOH (pH 8.0), 10 mM $MgCl_2$, 0.1 mM EDTA, 5 mM Glc 6-P, 10 mm $NaHCO_3$, 2 mM pyruvate, 1.25 mM ATP, 10 mM dithiothreitol, 2.5 mM KH_2PO_4, 5 mM NH_4Cl, 0.2 mM NADH, 12 units malate dehydrogenase (from pig heart; Roche Diagnostics, Basel), and 0.5 unit PEPC (from maize; Biozyme, South Wales, UK), and the reaction was started by adding ATP. To examine the activation state of PPDK *in vivo*, the leaf segment was ground in the extraction buffer without phosphate. The homogenate was centrifuged at 15,000 g for 1 min and the resultant supernatant was assayed immediately. The PPDK activity of maize and wild-type rice leaves, calculated on a protein basis, were 0.50 to 0.80 and 0.01 to 0.03 μmol mg^{-1} protein min^{-1}, respectively.

Slice and microstructure of transgenic plants

Thin (15–20 µm) transverse sections of leaf of transgenic IR64 with the *PPDK* gene and non-transformed control were obtained using Vibratome (Vibratome Series 3000 sectioning system, Technical Product International, USA). The sections were then stained with 0.1% acridine orange for 15 min, washed thoroughly with distilled water, and mounted with dilute glycerin (Krishnan et al., 2001; Pearse, 1972, 1980). Observations and photography were carried out using a Carl Zeiss Axioplan-2 microscope fitted with a digital camera under blue-light excitation.

Evaluation of total nitrogen of flag leaves of transgenic lines

The flag leaves of transgenic lines and control were harvested and the dry weight of flag leaves was determined after oven drying to a constant weight at 70°C. 0.1 g sample was used to determine total nitrogen content by the microkjeldhal method (Humphries, 1956).

Measurement of photosynthesis of transgenic plants

Flag leaf CO_2 assimilation rate, stomatal conductance and internal CO_2 concentration (Ci) were measured using a Li-6200 portable photosynthesis system (Li-Cor Inc., Lincoln, NE) between 10:00 AM and noon under saturating PPFD (Photosynthetic Photon Flux Density) (1500 μmol m^{-2}s^{-1}). T-air (air temperature) measured by the LI6200 was on average 35-41°C (as LiCor 6200 photosynthesis meter requires sufficient quantum of light) in the greenhouse with temperature and humidity being controlled automatically. Three fully expended flag leaves of each transgenic line and control were selected and measured at the beginning of heading stage. Flow rate was maintained at 200-250 µmol s^{-1} so that the relative humidity inside the chamber should be similar to ambient condition. Measurements were made for 20 s immediately after stable decrease in CO_2 concentration inside the chamber was achieved.

RESULTS

Cloning of transgenic IR64 plants with intact *PPDK* of maize

The embryogenic calli of IR64 grew better in modified MS2D medium supplemented with 0.2 mg/L NAA and 0.2

Figure 2. Transgenic IR64 plant showing normal phenotype and good seed setting like control.

mg/L 6-BA and improved differentiation of embryogenic calli. The transformation efficiency of embryogenic calli was also increased by 2.64%. The maize *PPDK* gene construct (Figure 1) was introduced into *Indica* IR64 rice. More than 260 putative transgenic hygromycin resistant plants (T_0) were obtained. About 8% of the transformants showed abnormal morphological and physiological characteristics such as bears, narrow leaves, infertility and decreased plant height, etc. All other plants exhibited normal phenotype with a normal life cycle, grew to maturity, flowered and set seeds (Figure 2). Genomic DNA from the PCR analysis indicated the integration of *hph* gene evident by 0.8 kb band (Figure 3). Gel DNA blot from PCR positive plants confirmed the integration of the *ppdk* gene into rice gneome (Figure 4).

Expression of transgenic plants with intact *PPDK* of maize with RT-PCR

The enzyme of the *ppdk* gene showed high activity in mesophyll cells, with little or no activity in bundle sheath cells (Edwards et al., 2001). The results showed that there was a 1.0-kb fragments in different transgenic lines with intact *PPDK* gene of maize. But some transgenic lines had no 1.0-kb fragment. These results indicated that the intact *PPDK* gene of maize was expressed in some transgenic lines. Some transgenic IR64 plants had no expression (Figure 5).

PPDK activities of leaves in the transgenic IR64 lines

Transformants introduced with intact maize *ppdk* gene exhibited a wide range of activities (Figure 6). The transformants showed higher activities than that of control plants. The activity of transgenic lines, ZHI2-7, ZHI2-8, ZHI2-9 and ZHI2-10 with respect to control was significant increase (P value<0.01) at level of significance. The highest activity was 4.1 fold more than that of control. In addition, the activity of maize was 15.9 fold more than that of control.

Total nitrogen of transgenic IR64 lines with intact *PPDK* of maize

Data of total nitrogen in the flag leaves of transgenic line ZHI2-7 with respect to control was significantly increase (P=0.048<0.05) at 5% level of significance. But total nitrogen of other transgenic lines, ZHI2-8, ZHI2-9 and ZHI2-10 was not significantly increase with respect to control. These results indicated that transgenic lines promote efficient utilization of nitrogen (Figure 7).

Inheritance

The T1 seeds of transgenic lines ZHI2-7, ZHI2-8, ZHI2-9, ZHI2-10 and control were sown in trays in the transgenic greenhouse. There were about 50 seeds for being sown for each transgenic IR64 line. When there were five leaves per plant, these plants were transferred to a 14-cm-diam pot. In addition, leaves were extracted for PCR analysis with hygromycin phosphotransferase gene (*hph*) specific primer. The results showed that the transgenic lines ZHI2-7, ZHI2-8, ZHI2-9, and ZHI2-10 were inserted with single copy intact *PPDK* gene of maize to IR64 genome according to Mendelian segregation (Table 1).

Slice and microstructure of leaves of transgenic IR64 with intact *PPDK* of maize

Leaves of C_4 plants have two types of photosynthetic cells, mesophyll cells (MC) and bundle sheath cells (BSC). Although all the photosynthetic enzymes are confined in MCs in C_3 plants, they are localized in MCs and/or BSCs in C_4 plants. In addition, C_4 plants show extensive venation, with a ring of BSCs surrounding each vein and an outer ring of MCs surrounding the bundle sheath. This unique leaf structure, known as Kranz

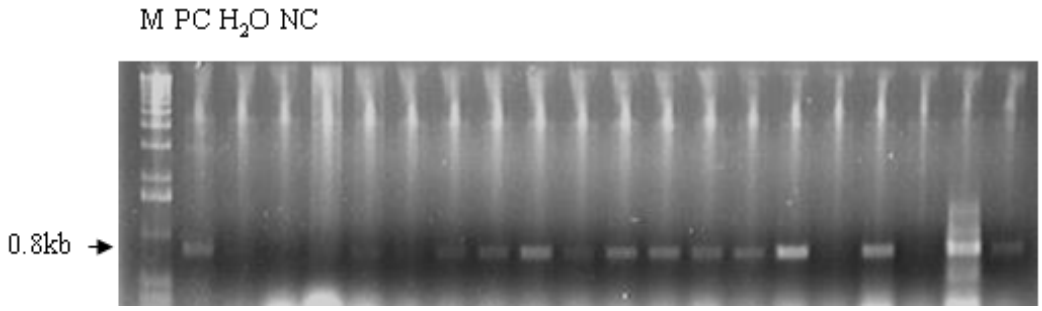


Figure 3. Putative transgenic plants with intact *PPDK* gene of maize were assayed with PCR. From left to right, lane 1: 1-kb ladder; lane 2: positive control (PC); lane 3: H_20; lane 4: negative control (NC); other lanes: putative transgenic IR64 plants.

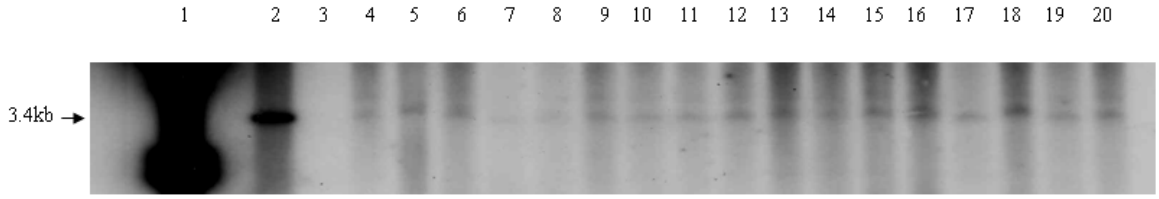


Figure 4. DNA gel analysis for putative transgenic IR64 with intact *PPDK* gene of maize. Lane 1: λDNA/HindIII DNA marker; lane 2: positive control (plasmid DNA); lane 3: negative control; lane 4: ZH12-1; lane 5: ZH12-2; lane 6: ZH12-3; lane 7: ZH12-4; lane 8: ZH12-5; lane 9: ZH12-6; lane 10: ZH12-7; lane 11 ZH12-8; lane 12: ZH12-9; lane 13: ZH12-10; lane 14: ZH12-11; lane 15: ZH12- 12; lane 16: ZH12-13; lane 17: ZH12-14; lane 18: ZH12-17; lane 19: ZH12-26; lane 20: ZH12-39.

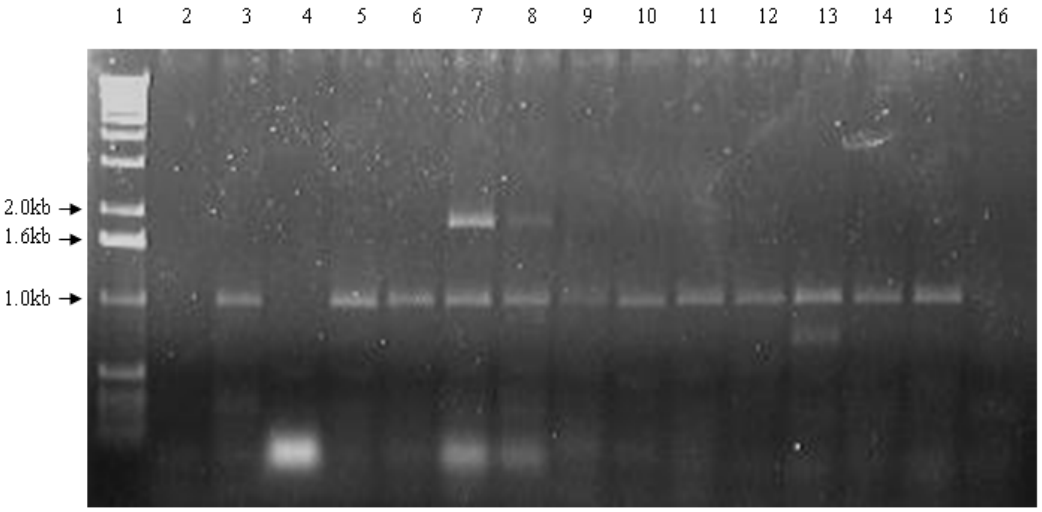


Figure 5. RT-PCR analysis of transgenic IR64 with intact *PPDK* gene. Lane 1: 1-kb ladder; lane 2: negative control; lanes 3 to 16: transgenic IR64 with intact *PPDK* gene of maize (3: ZH12-1; 4: ZH12-2; 5: ZH12-3; 6: ZH12-4; 7: ZH12-5; 8: ZH12-6; 9: ZH12-7; 10: ZH12-8; 11: ZH12-9; 12 ZH12-10; 13 ZH12-11; 14: ZH12-12; 15: ZH12-13; 16: ZH12-14).

anatomy, and the cell-specific compartmentalization of enzymes are essential for operation of the C_4 pathway (Matsuoka, 2001). There was much difference in the leaf structure of both transgenic IR64 with intact *PPDK* and control IR64. The *PPDK* gene was expressed in mesophyll cells in the C_4 pathway. The enzyme of *PPDK* showed high activity in mesophyll cells, with little or no activity in bundle sheath cells (Edwards et al., 2001).

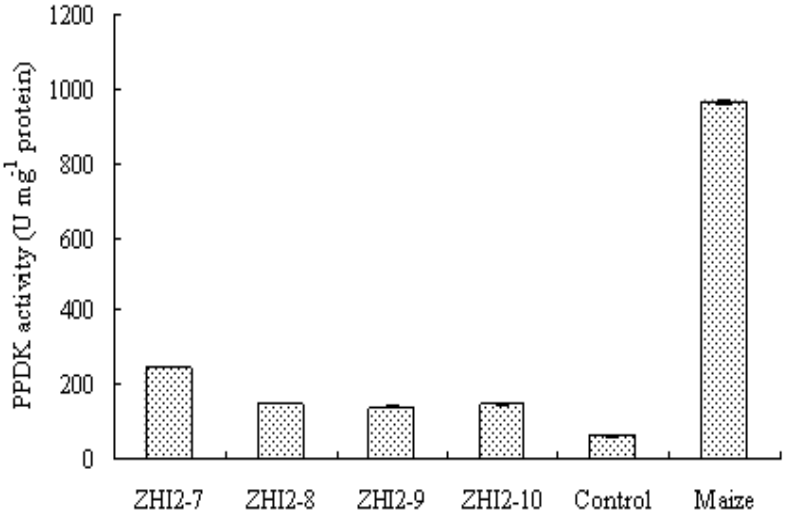


Figure 6. The *PPDK* activities of flag leaves in transgenic lines.

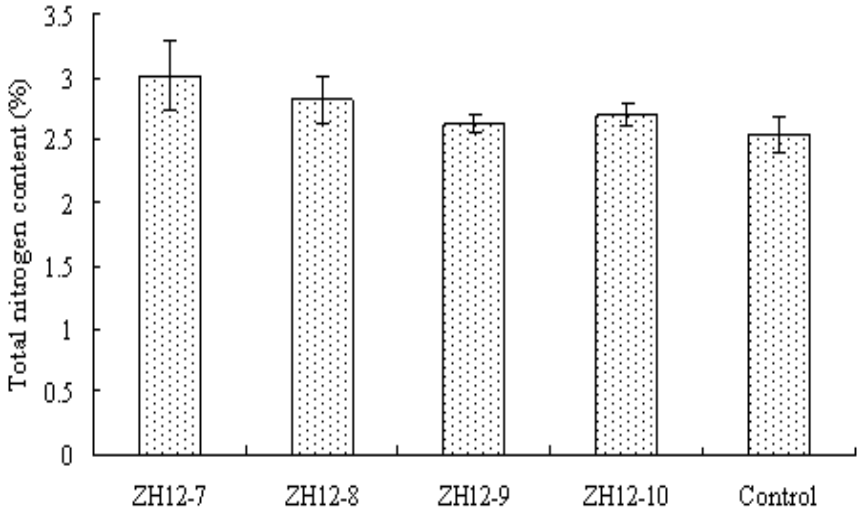
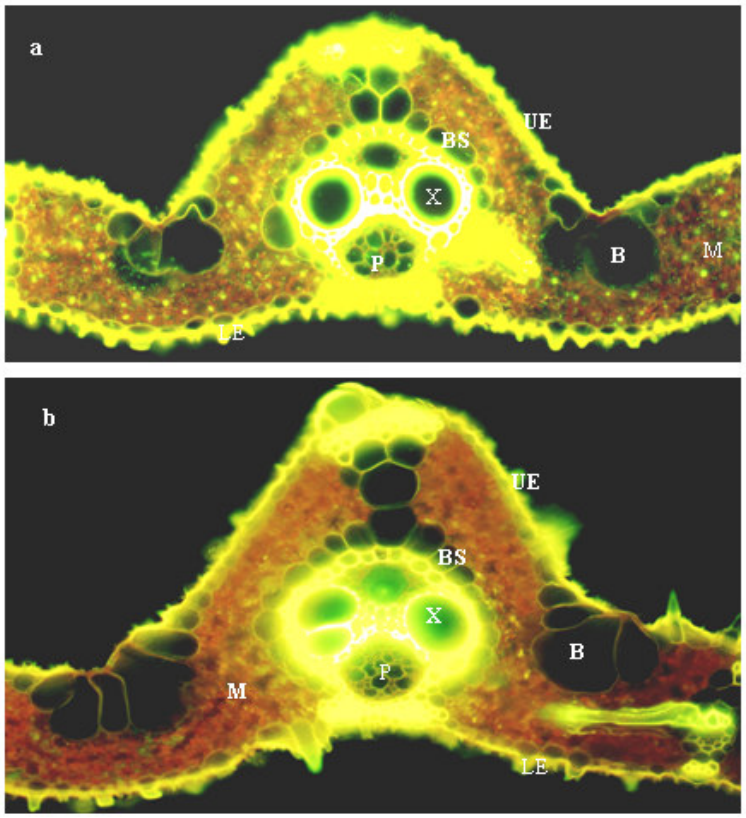


Figure 7. The total nitrogen of flag leaves of transgeniclines with intact *PPDK* gene of maize.

Transverse leaf sections of intact *PPDK* transgenic lines and non-transformed control were compared (Figure 8). It was observed that in many transgenic lines, the leaves were much thicker and longer and showed a high photosynthetic rate. It was observed that *PPDK* does not cause any major change in the anatomy, as control chloroplast is absent in the bundle sheath cells. However, there was an increase in the number of mesophyll cell layers and the cell size of bundle sheath cells, and bulliform cells were more abundant in comparison with the control. It can be concluded that the increase in leaf thickness in transgenic IR64 lines with intact *PPDK* may be due to the increase in number of mesophyll cell layers and overall cell size in the leaves.

Photosynthetic rate of transgenic IR64 lines with intact maize *PPDK*

Plant breeders are concerned about improving the photosynthetic efficiency of both conventional and transgenic plant varieties (Dunwell, 2000). Therefore, the photosynthetic rates of greenhouse grown transgenic rice plants were measured at the heading stage. The results showed that the photosynthetic rate of transgenic lines ZHI2-7, ZHI2-8, ZHI2-9 and ZHI2-10 with respect to control was not significantly increased (P=0.064, 0.15, 0.99, 0.23, respectively <0.05) at 5% level of significance, only the photosynthetic rate of transgenic line ZHI2-12 with respect to control was significantly increase



Non-transformed control (×200)

PPDK transgenic line(×200)

Figure 8. Transverse section of trasgenic IR64 with the *PPDK* gene (b) and non-transformed control IR64 (A) rice leaf stained with acridine orange, both with similar magnification, x200. B, bulliform cells; BS, bundle sheat; LE, lower epidermis; M, mesophyll cells; P, phloem; UE, upper epidermis; X, xylem.

Table 2. Photosynthesis rate, CO_2 concentration and stomatal conductance of transgenic and control plants.

Material	(Photosynthesis rate) PS [μmolm^{-2}s^{-1}]	Ci (internal CO_2 concentration) [μmol mol^{-1}]	SC (Stomatal conductance) [cm s^{-1}]
ZHI2-7	26.80±3.97	2.03±0.15	0.017±0.001
ZHI2-8	25.66±3.37	2.44±0.73	0.018±0.00
ZHI2-9	21.26±1.26	1.53±0.48	0.017±0.001
ZHI2-10	24.86±1.54	1.73±0.39	0.016±0.000
Control	21.22±1.41	2.14±0.18	0.018±0.000

(P=0.04<0.05) at 5% level of significance (Data not shown). But the photosynthetic rate of transgenic lines ZHI2-7, ZHI2-8 and ZHI2-10 was higher than that of control. These results indicated that the photosynthetic rate of most of the transgenic lines with intact *PPDK* gene of maize had been improved (Table 2).

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

Three plants for each transgenic line was harvested respectively when they were mature in the greenhouse. The yield characteristics of each transgenic IR64 lines were observed after all plants were dried in the 65ºC oven. The results showed that 1000-grain weight of transgenic line ZHI2-7 with respect to control was not significantly increase (P=0.145>0.01) at 1% level of significance. A similar observation was found in case of other transgenic lines, ZHI2-8, ZHI2-9 and ZHI2-10 (P=0.156, 0.148 and 0.117, respectively, >0.01) at 1% level of significance. Data of harvest index of transgenic line ZHI2-8, ZHI2-9 with respect to control showed significant increase (P=0.0031, 0.0014<0.01) at 1% level of

Table 3. The yield characteristics of transgenic lines with intact *PPDK* of maize.

Transgenic Lines	Plant dried weight (g)	Panicle length (cm)	Total filled grain	Total filled grain weight (g)	Total unfilled grain	Unfilled grain weight (g)	1000-grain weight (g)	Grain filling (%)	Harvest index (%)
ZHI2-7	35.2±1.2	19.0±0.2	739±2	15.4±1.4	98±4	0.7±0.2	20.8±1.9	88.3±0.5	43.8±2.5
ZHI2-8	28.8±0.6	21.0±0.8	632±6	13.4±2.4	99±3	0.7±0.2	21.2±3.6	86.5±0.5	46.5±7.4
ZHI2-9	17.7±0.9	17.0±0.6	424±4	8.9±0.9	80±8	0.6±0.1	21.0±2.0	84.1±1.5	50.3±2.6
ZHI2-10	39.5±1.4	20.0±0.8	689±3	14.1±2.1	176±16	1.1±0.3	20.5±3.0	79.7±1.6	35.7±4.1
Control	37.3±1.3	19.0±1.8	507±6	12.5±1.7	113±7	0.5±0.3	24.7±3.1	81.8±1.1	33.5±3.4

Harvest index = filled grain weight/biological yield×100%; grain filling % = filled grains/total grains×100%.

significance. In addition, data of grain filling of transgenic lines ZHI2-7, ZHI2-8 and ZHI2-9 with respect to control showed significant increase (P=0.0001 and 0.0006<0.01 and P=0.0273<0.05 respectively) at 1% level of significance (Table 3).

DISCUSSION

To increase yield potential researchers have tried various methods to improve photosynthetic ability per leaf area with limited success (Dunwell, 2000; Horton, 2000; Ishimaru, 2003; Bandyopadhyay et al., 2007; Taneguchi et al., 2008). In leaves of C4 plants, 266 the activity of PPDK is rapidly modulated in response to changes in light intensity by reversible protein phosphorylation, which is mediated by a bifunctional regulatory protein (Burnell and Hatch, 1985). In transgenic plants over-expressing *PPDK* derived from higher plants, no change in photosynthetic characteristics were observed; including transgenic *japonica* rice that showed a 40-fold increase in PPDK activity compared to the wild type (Fukayama et al., 1999). In general, the reaction of *PPDK* is freely reversible, depending on the concentration of substrates, activators, and inactivators (Burnell and Hatch, 1985). Therefore, the over expression of *PPDK* did not result in significant effects on carbon metabolism in the leaves (Miyao, 2003). In this study, the photosynthetic rate of some transgenic IR64 plants containing the intact maize *PPDK* gene was significantly increased, in transgenic line, ZHI2-12 (27.99 μmol m^{-2} s^{-1}). However, some transgenic plants with higher photosynthetic rates could be infertile, such as transgenic line ZHI2-21 (21.77 μmol m^{-2} s^{-1}). In the greenhouse, most grains of transgenic line, ZHI2-21 were unfilled (data not shown). The adequate sunlight and other conditions in the greenhouse might play some important roles in determining the sufficient grain filling of the transgenic seeds.

Nitrogen is one of the most important nutrients necessary for plant growth (frequently it is the major limiting nutrient) and its incorporation from the environment onto biomolecules determines crop productivity and yield (Lglesias et al., 2004). 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).

In the atmosphere, nitrogenase action on N_2 provides metabolically useful nitrogen to the environment. Ammonia, the product of this reaction, is converted to glutamate and glutamine and then to other nitrogen-containing compounds required for the growth and maintenance of plants and animals (Oaks and Hirel, 1985). In rice grain yield is mainly determined by the sum of carbohydrate accumulated before heading and produced after heading. It is the flag leaf that contributes the most carbohydrate after heading (Cook and Evands, 1983). The total nitrogen in the flag leaves of transgenic lines was higher than those of the untransformed control, indicating that the photosynthetic capacities of transgenic IR64 plants have been increased. Introduction of the intact maize C4-*PPDK* gene into japonica rice led to significant accumulation of the PPDK protein in the chloroplasts of leaves homozygous lines (Fukayama et al., 2001). The accumulation of PPDK affected the contents of other components in the leaves (chlorophyll content, Rubisco and so on) and total leaf nitrogen (Fukayama et al., 2001).

In a previous study, a transgenic line PD25 showed an increase of approximately 7% in leaf nitrogen. The Rubsico content on a leaf area basis was increased slightly, although on a total nitrogen basis it remained unchanged, and the chlorophyll content was decreased slightly (Fukayama et al., 2001).

In the present study, total nitrogen of flag leaves from transgenic IR64 plants was higher than that of the untransformed control. The harvest index of most transgenic plants was higher than that of control plants, indicating that the yield of transgenic IR64 plants with intact PPDK gene of maize could be improved. Investigation of the main agronomic traits for three types of transgenic rice containing the maize C4 photosynthesis genes *pepc, ppdk,* or *pepc+ppdk*) indicated that the transgenics containing the maize *pepc* and/or *ppdk* genes had more panicles and higher yield than the untransformed control (Wang et al., 2004).

This present work might be an indication to enhance yield by utilizing higher photosynthesis and translating to

yield. Further research is needed to evaluate the materials and to see the advantage of yield in the open field conditions.

ACKNOWLEDGEMENTS

We are grateful to Prof. M. Matsuoka of Nagoya University, Japan and Dr Mitsue Miyao from NIAS, Japan for valuable suggestions and providing the construct containing *ppdk* gene. Thanks are also due to Dr G.S. Khush, Dr S Peng of IRRI for necessary help. Research support to SKD from DBT, Govt. of India is gratefully acknowledged.

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