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Full Length Research Paper

Integration of C4-specific ppdk gene of Echinochloa to C3 upland rice and its photosynthesis characteristics analysis

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Pyruvate orthophosphate dikinase (PPDK) plays a key role in C_4 photosynthetic pathway. The enzymatic reaction is one of the rate-limiting steps of the C_4 photosynthetic pathway. In this paper, the gene encoding *Echinochloa* pyruvate orthosphate dikinase (GenBank accession number: AB289641) was introduced into H65, a upland rice variety, using an *Agrobacterium*-mediated system. Southern hybridization, Northern hybridization and enzyme activity determination all confirmed the effective expression of *Echinochloa* C_4 type PPDK in upland rice with the enzyme activity being elevated 1 - 11 folds. However, no appreciable change demonstrated in carbon assimilation of the transgenic upland rice though increased photoinhibition was noted under high light intensity.

Key words: Pyrurate orthophosphate dikinase, *Echinochloa*, transgenic upland rice, photosynthesis.

INTRODUCTION

According to differences in carbon assimilation pathways in photosynthesis, three photosynthetic types may be distinguished among land plants: C_3, C_4, and CAM. Compared with C_3 plants, the unique Kranz structure and C_4 pathway endow C_4 plants and CAM plants with higher efficiency in photosynthesis, water utilization and nutrient utilization (Hatch, 1987). These advantages of C_4 plants are especially valuable during stresses caused by high temperature, high light intensity and drought. It has therefore long been a much sought for objective to improve the photosynthetic properties of C_3 plants by introducing the photosynthetic traits of C_4 plants into them.

Pyruvate, orthophosphate dikinase (PPDK) catalyzes the formation of phosphoenol pyruvate, the primary acceptor of CO_2 in C_4 plants (Edwards et al., 1985). The enzymatic reaction is critically controlled by light and is one of the rate-limiting steps of the C_4 photosynthetic pathway (Trevanion et al., 1997; Ap Rees and Hill, 1994). PPDK is regarded as an important target in efforts to improve the productivity of C_3 plants such as rice, wheat, and bean. It thus appears that transfer of PPDK gene into C_3 plants might be an effective way of lowering photorespiration and improving photosynthetic efficiency of C_3 plants.

There are some reports on the successful transfer of PPDK of C_4 plants into C_3 plants and their high level expression in the latter by means of gene engineering techniques (Ishimaru et al., 1997; Ishimaru et al., 1998; Furayama et al., 2001). So far, however, no report has been published on systematic study of the photosynthetic physiology such as CO_2 exchange in transgenic plants expressing PPDK of the C_4 plant. In previous study, the gene of *ppdk* was isolated from maize. In this study, we chose *ppdk* from *Echinochloa* as gene sources. The inheritance distance rice is more closely related to *Echinochloa* than maize. Moreover, *Echinochloa*, as a paddy weed, is in the same environment. In this work of ours, *Echinochloa* PPDK was introduced into upland rice using the transformation system mediated by *Agrobacterium*, and then systematic study on the photosynthetic physiology involved was carried out. It is hoped that better understanding of the physiology might prove helpful to the genetic approach for improving photosynthesis in C_3 plants.

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Figure 1. The schematic diagram of transgenesis vector of p1301-pUbi-PPDK. P35S, cauliflower mosaic virus 35S promoter; Pubi, maize ubiquitin gene promoter; Tnos, nopaline synthase gene terminator; Hpt, hygromycin phosphotransferase gene; Gus, β-glucuronidase gene; Efpdk, coding region of pdk gene from Echinochloa fruma

MATERIALS AND METHODS

Experimental materials

Echinochloa crusgalli var. frumentacea (Roxb.) W. F. Wight was cultivated in green house and, after 20 d of growth, seedling were harvested for further experiments.

The variety of upland rice (Oryza sativa L.) used for transformation experiment was H65. Both the transgenic upland rice (PDK) and the untransformed controls (WT) were cultivated under controlled conditions until the booting stage when specimens were collected for determination of the physiological indices.

Construction of expression vector and genetic transformation of rice

A transgenesis vector was constructed on the basis of pCAMBIA1301: at the polycloing sites were inserted ubiquitin promoter, PPDK cDNA and Nos terminator. Designated as p1301-pUbi-PPDK, the structure is shown in Figure 1.

The expression vector p1301-pUbi-PPDK was then introduced into the *Agrobacterium tumefaciens* strain EHA105 by freezing-thawing. The transformation of upland rice was performed by the method of Hiei et al. (1994).

Southern and Northern hybridization of transgenic upland rice

Genomic DNA was extracted from upland rice using the method of SDS. 20 ug of the extracted DNA, digested with *Kpn* I, were separated by electrophoresis in 0.8% agarose gel before being transferred to Hybond membrane for Southern hybridization. All the procedures, including prehybidization, were performed in routine manner.

Total RNA was extracted from the leaves using guanidine isothiocyanate and was separated with electrophoresis in 1.2% denaturing gel. It was then transferred to Hybond nylon membrane for Northern hybridization.

Extraction of soluble proteins and determination of its enzyme activity

Naught point two gram of upland rice leaves that had been exposed to full light for 2 h was rapidly placed in a pre-cooled mortar and ground with 1.5 mL extracting buffer in ice bath. The buffer was composed of 50 mM Tris-HCl, pH7.5, 10 mM $MgCl_2$, 1 mM EDTA, 2.5 mM PEP, 10 mM DTT, 10 mM DTT with insoluble PVP, and 14 mM mercaptoethanol. After full maceration, the extract was centrifuged at 13 000 g and 4 for 10 min and the supernatant was gathered for activity determination.

The PPDK activity was assayed as described by Hatch and Slack. The total volume of reaction mixture was 1.0 mL, containing 250 mmol/L Tris-HCl (pH 8.3), 50 mmol/L DTT, 0.1 mmol/L $MgSO_4$, 50 mmol/L $NaHCO_3$, 15 mmol/L sodium pyruvate (freshly prepared), 25 mmol/L K_2HPO_4 and 50 mmol/L NH_4Cl, 5 mmol/L NADH, 50 mmol/L ATP, 80 units NAD-malate dehydrogenase, 30 units PEPC and an appropriate amount crude enzyme extract.

Measurement of CO_2 exchange and chlorophyll fluorescence

Photosynthesis rates of upland rice leaves in booting stage under various light intensities were measured using a portable photosynthmeter, LI-6400 (LI-COR., USA). After adaptation to darkness for 30 min, parameters of chlorophyll fluorescence were measured using a portable fluorometer, FMS2 (Hansatech Co.,UK).

RESULTS

Southern blotting and Northern blotting analysis

Southern hybridization of *Kpn* I digested genomic DNA of the transgenic upland rice was carried out using a full-length PPDK cDNA as probe. As expected, a hybridization band was found at the position of 3 kb in each of the transgenic plant while nothing was detected in the corresponding position of the control (Figure 2A). This confirms the successful integration of *Echinochloa* PPDK into upland rice genome.

In order to make clear if there is any relation between the level of expression of PPDK in upland rice and the level of transcription, Northern hybridization was done to analyze the expression of PPDK in upland rice, and correlation is found between PPDK activity and transcription level as can be seen from Figure 2B.

Expression of PPDK from *Echinochloa* in transgenic upland rice plants

Altogether 87 plants of transgenic upland rice transformed with *Echinochloa* C_4 type PPDK were obtained using *A. tumefaciens* as mediator. Determination of PPDK activity in leaves of transgenic upland rice revealed elevation to various extents: 1 - 11 folds increase was noted in comparison with the control. However, most of PPDK activities of the transgenic rice are 2 - 3 folds than that of the control (Figure 3).

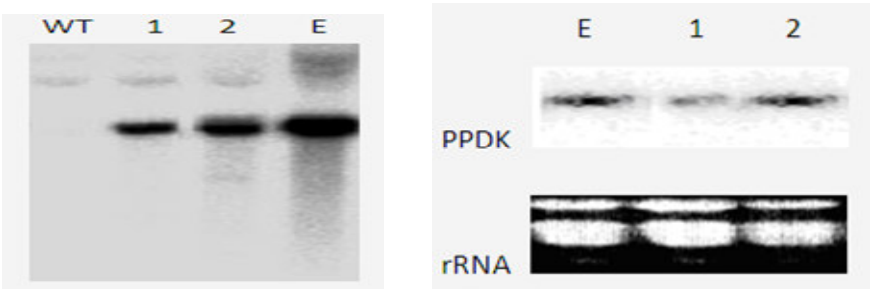

Figure 2. Southern blotting and Northern blotting analysis of transgenic upland rice. **A.** Southern Hybridization. **B.** Northern Hybridization. WT, control; 1, 2, transgenic plants; E, *Echinochloa*.

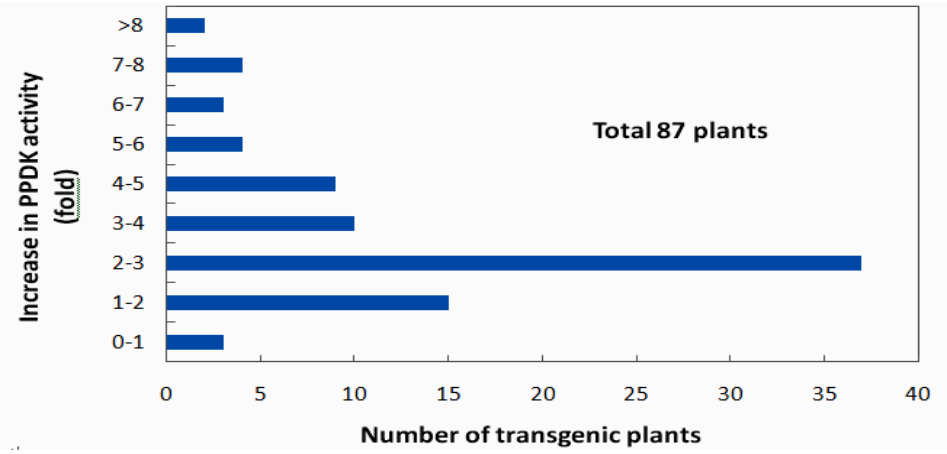

Figure 3. The PPDK activities of leaves in the primary transgenic rice plants. Transgenic plant introduced with the Ubi promoter::cDNA construct. Enzyme activities are expressed as fold increase over the activity in wild-type rice plants.

CO_2 exchange and chlorophyll fluorescence in transgenic upland rice

The introduction of C_4 photosynthetic enzyme into C_3 plants was intended to improve the efficiency of solar energy utilization. We measured the net photosynthetic rates of transgenic upland rice expressing PPDK under various light conditions as shown in Figure 4. Under 200 umol.m^{-2}.s^{-1}, net photosynthetic rates were closed between the transgenic rice and the control. With the increase of light intensity, light saturation was reached at 1200 umol.m^{-2}.s^{-1}. Net photosynthetic rate of the transgenic rice is higher than untransformed rice. They were 24 and 21.5 umol.m^{-2}.s^{-1}, respectively.

PSII photochemical efficiency (*Fv/Fm*) is an important parameter characterizing the status of photochemical reaction. The parameters of chlorophyll fluorescence were good indexes for estimating the activity of PSII. As shown in Figure 5, greater decline of Fv/Fm was found under light intensity at noon for the transgenic upland rice than for the control (WT), suggesting greater susceptibility of the former to photoinhibition.

DISCUSSION

Because C_3 plants do not possess the Kranz anatomy, for a long time it remains doubtful that an effective C_4 cycles could be established in C_3 plants simply by transferring C_4 photosynthetic enzyme genes into C_3 plants. However, it was recently discovered that some aquatic angiosperms, such as *Hydrilla verticillate* (Magnin et al., 1997) and *Egeria densa* (Casati et al., 2000), possess a primitive type of C_4 photosynthesis without Kranz anatomy. Moreover, it was reported lately that the expression of C_4 type PEPC in rice can improve its photosynthetic capacity with enhanced tolerance to photo-oxidation (Chi et al., 2001; Huang et al., 2001; Zhang et al., 2003). The

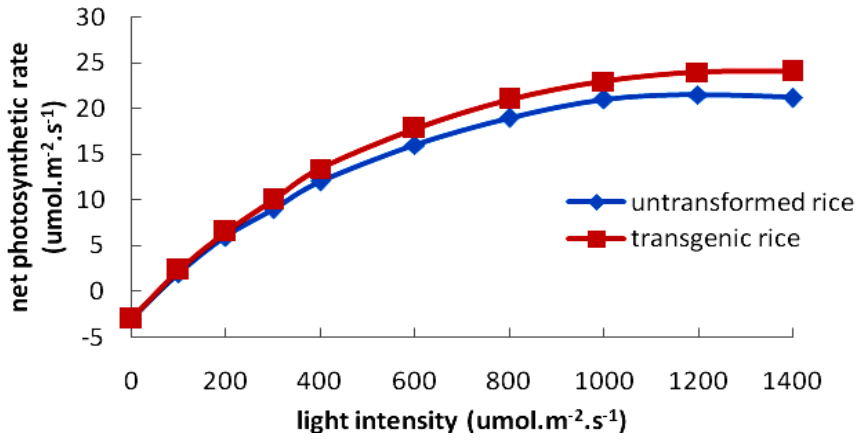

Figure 4. Light-photosynthesis curve of transgenic rice and untransformed rice.

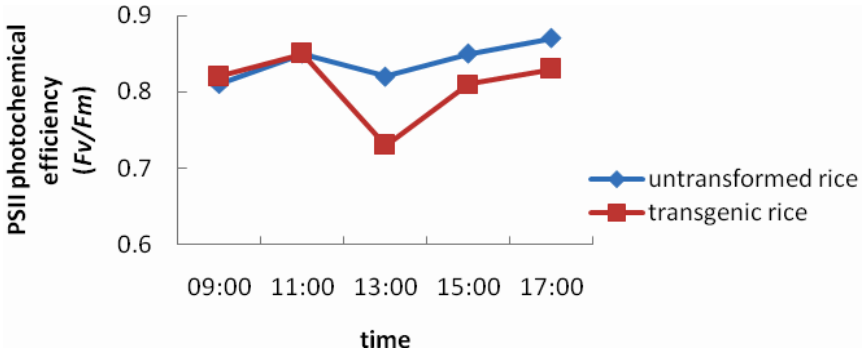

Figure 5. Daily change of chlorophyll fluorescence.

primitive CO_2 concentration mechanisms in transgenic rice expressing PEPC gene and those expressing PEPC gene were also detected (Suzuki et al., 2000; Jiao et al., 2003). These results further strengthened the possibility of improving C_3 photosynthetic performance by gene engineering. In this study, PPDK, another key enzyme involved in C_4 photosynthesis was introduced into a C_3 plants, upland rice and the photosynthetic characteristics of transgenic plants were analyzed in detail. To our surprise, with the elevated PPDK activity was still too small to influence the photosynthetic characteristics of the transgenic plants as a whole. The introduction of C_4 PPDK alone into C_3 plants could contribute little to the improvement of C_3 photosynthetic performance. A report suggests that the optimal activities of decarboxylating enzymes (NAD- and NADP-ME) were lower than those of PPDK in the transgenic potatoes. Thus, in addition to increasing PPDK activity, a high expression of a decarboxylating enzyme such as NADP-ME could be necessary to obtain a greater change in C_3 photosynthetic characteristics (Ishimaru et al., 1998). As to the influence on C_3 photosynthetic characteristics by PPDK and NADP-ME coordination, further experiments are required.

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