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High level expression of C_4 photosynthesis enzymes in transgenic rice

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Introduction

Most terrestrial plants, including many important crops such as rice and wheat, assimilate CO_2 through the C_3 photosynthesis pathway, and are classified as C_3 plants. However, some plants, such as maize and sugarcane, possess the C_4 photosynthesis pathway in addition to the C_3 pathway, and these are classified as C_4 plants. The C_4 pathway acts to concentrate CO_2 at the site of the reactions of the C_3 pathway, and thus inhibits photorespiration (Hatch, 1987). This CO_2-concentrating mechanism, together with modifications of leaf anatomy, enables C_4 plants to achieve high photosynthetic capacity and high water and nitrogen use efficiencies (Hatch, 1987). Consequently, the transfer of C_4 traits to C_3 plants is one strategy being adopted for improving the photosynthetic performance of C_3 plants.

It is likely that any genes for C_4 photosynthesis enzymes introduced into C_3 plants will need to be expressed at high levels to have a significant effect on metabolism. So far, only modest increases in C_4 enzyme activity have been achieved in C_3 plants, well below the activities found in C_4 plants (for review, see Matsuoka et al. 2001). We have designed gene constructs for high level expression of C_4 enzymes in a C_3 plant, rice, based on our previous studies of the expression of promoters of the C_4 photosynthesis genes in C_3 plants (Matsuoka et al. 1993, 1994). We report here that each of three key enzymes of the maize C_4 pathway, namely, phospho*enol*pyruvate carboxylase (PEPC), pyruvate, orthophosphate dikinase (PPDK) and NADP-malic enzyme (NADP-ME), can be expressed in the mesophyll cells of rice leaves at high levels comparable to or even higher than those in maize. We also report physiological impacts of overproduction of C_4 enzymes in rice plants.

Materials and Methods

C_4 genes were cloned into pIG121Hm and introduced into calli derived from rice (cv. Kitaake) using *Agrobacterium*-mediated transformation. Transgenic rice were regenerated from hygromycin-resistant calli, planted in soil, and grown in a naturally illuminated greenhouse maintained at 26 °C/21 °C (day/night).

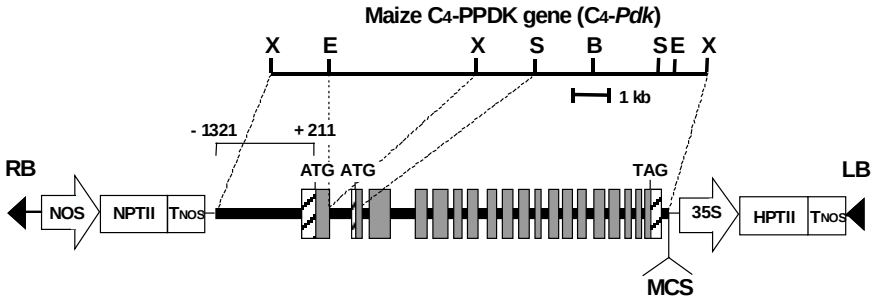

Fig. 1. A construct used for introduction of the maize C_4- *Pdk* gene into rice. A 4 kb part in the first intron of the maize gene was deleted to shorten the length of the introduced gene. Top diagram shows the restriction map of the maize gene. B, E, S and X indicate *Bam*HI, *Eco*RI, *Sal*I and *Xba*I sites, respectively.

Results and Discussion

How to Overproduce C_4 Enzymes in Rice Leaves

Previous studies using a reporter gene demonstrated that the promoters of the maize C_4-specific genes encoding PEPC and the chloroplastic form of PPDK can drive mesophyll cell-specific expression in rice leaves at levels higher than the CaMV 35S promoter does (Matsuoka et al. 1993,1994). Based on these findings, we initially introduced the intact maize gene for C_4-specific PEPC (C_4-*Ppc* gene), containing its own promoter and terminator sequences and exon/intron structure, into rice. As expected, introduction of the intact gene was effective in expressing the maize PEPC at high levels in rice leaves: About 15% of the transformants showed the PEPC activities 30- to 110-fold over that of wild-type rice or 1- to 3-fold the maize activity, and the level of the maize PEPC protein accounted for 12-15% of total leaf soluble protein (Ku et al. 1999).

This strategy could work for another C_4 enzyme, PPDK. When the intact maize C_4-specific PPDK gene (C_4-*Pdk* gene; Fig. 1) was introduced into rice, the PPDK activity in the leaves was increased up to 40-fold over that of wild type rice or more than half of the PPDK activity of maize leaves (Fig. 2). The level of PPDK protein in the leaves increased with increasing activity of PPDK to reach about 20% of total leaf soluble protein (Fig. 3). In a homozygous

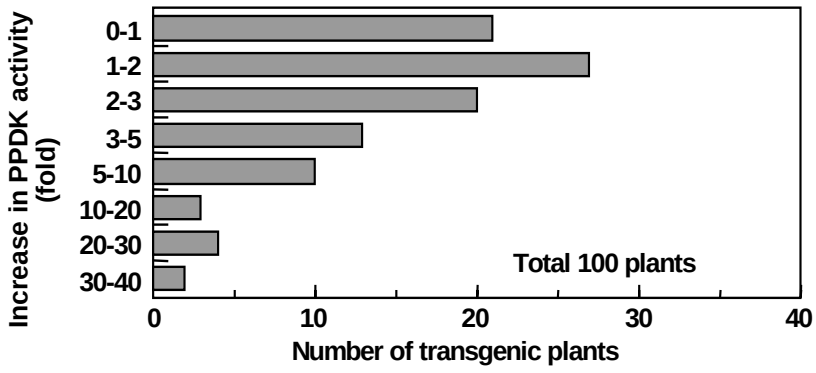

Fig. 2. The PPDK activities of leaves in the primary transgenic rice plants introduced with the intact maize C_4-*Pdk* gene construct. PPDK activities are expressed as fold increases over the activity in wild type rice plants.

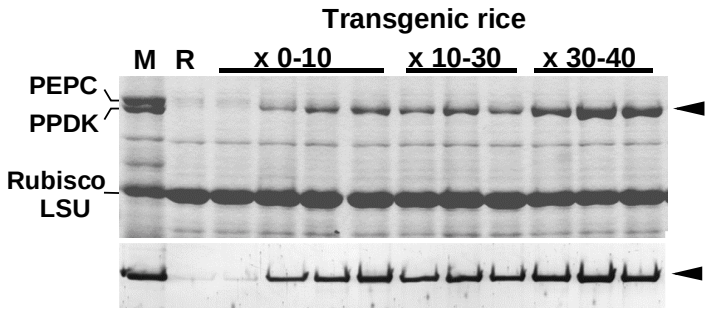

Fig. 3. Accumulation of the maize PPDK protein in leaves of the primary transgenic rice plants introduced with the intact maize C_4-*Pdk* gene construct. Total leaf soluble protein was analyzed by SDS-PAGE. (Upper panel) polypeptide profiles after staining with Coomassie blue; (lower panel) immunoblot profiles with an antiserum raised against the maize C_4-specific PPDK. M, maize; R, wild type rice. Numbers on the top of the panels represent PPDK activities of transgenic rice plants, expressed as fold increases over the activity in wild type rice.

line the PPDK protein accounted for 35% of total leaf soluble protein or 16% of total leaf nitrogen. In contrast, introduction of a chimeric gene containing the full-length cDNA of the maize chloroplastic PPDK, fused to either the maize C_4-*Pdk* promoter or the rice *Cab* promoter, only increased PPDK activity and protein level slightly (Fukayama et al. 2001). These observations suggest that the intron(s) or the terminator sequence of the maize gene, or a combination of both, is necessary for high-level expression.

In high-expressing lines of transgenic rice, the levels of mRNA transcribed from one copy of the intact maize C_4-*Ppc* and C_4-*Pdk* genes were comparable and their expression showed the same organ specificity in maize and transgenic rice (Ku et al. 1999; Fukayama et al. 2001). It is thus likely that the intact maize genes for PEPC and PPDK behave in a quantitatively and qualitatively similar way in both maize and transgenic rice plants.

The above finding, however, raises a question why the majority of the transformants introduced with the intact maize gene failed in overproducing C_4 enzymes (see Fig. 2). It was found that significant rearrangement of the introduced gene had occurred in low-expressing lines. Fig. 4A shows DNA gel blot analysis of the transformants introduced with the intact maize C_4-*Pdk* gene. Probes and restriction enzymes used were designed to examine if the entire maize gene was inserted into the rice genome. In a high-expressing line (lane H), only the bands of the expected sizes were detected. In contrast, all the low-expressing lines tested showed different band patterns. Bands corresponding to the 5´-side of the maize gene was totally absent in a plant of lane 4, and some to several bands were detected in addition to those expected for the intact gene in plants of lanes 1-3 and 5. These observations indicated that partial deletion and/or chimeric linking of the introduced gene had occurred in these plants. It is likely that such rearrangements of the introduced gene, together with the positional effects and gene silencing, are responsible for low expression levels of the maize protein in the majority of transformants.

Similar analysis was carried out with transgenic rice plants introduced with the full-length cDNA of the maize PPDK fused to the maize C_4-*Pdk* promoter (Fig. 4B). The rearrangement of the introduced gene was observed in five out of nine plants (lanes 1,2,4,7,9) while the rest four contained some to multiple copies of the introduced gene in an intact form. These observations suggest that, unlike the intact maize C_4-*Pdk* gene, the presence of the intact

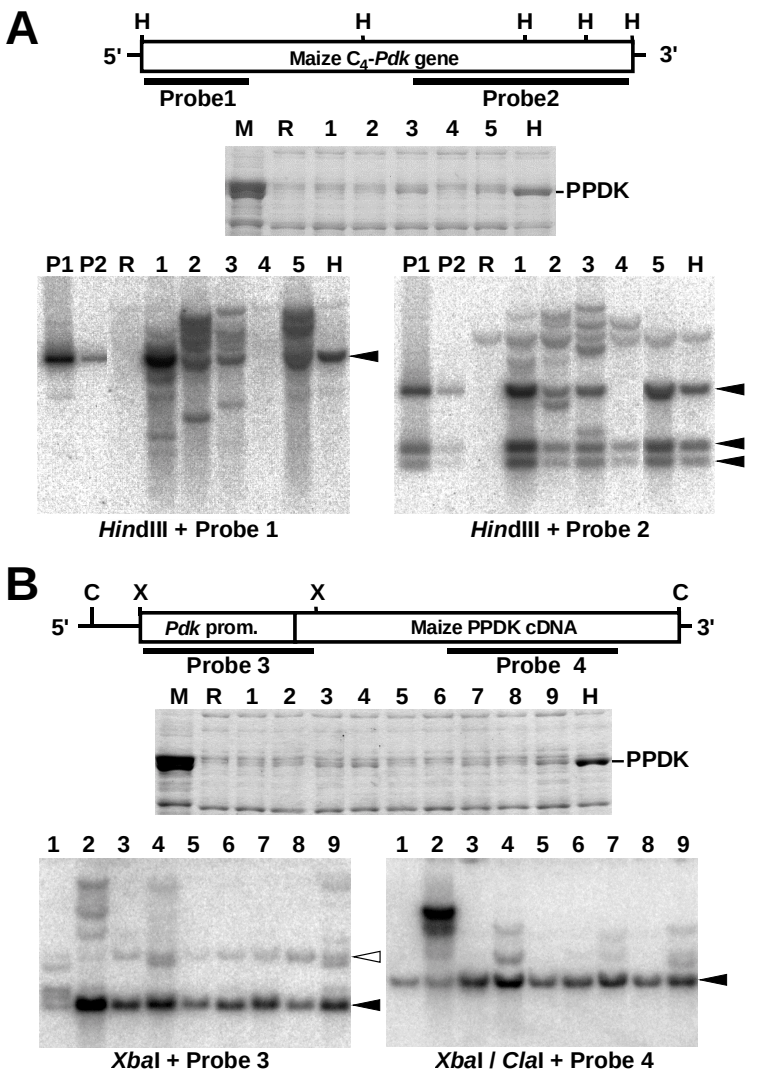

Fig. 4. DNA gel blot analyses of low-expressing lines of transgenic rice plants of T_2 generation. (A) Transformants introduced with the intact maize C_4-*Pdk* gene construct with PPDK activities in leaves less than twofold wild type levels (lanes 1-5), together with a high-expressing line (lane H) for comparison. (B) Transformants introduced with the maize PPDK cDNA fused to the maize C_4-*Pdk* promoter (lanes 1-9). (Top panels) restriction maps of the introduced genes. (Middle panels) polypeptide profiles after staining with Coomassie blue. (Bottom panels) DNA gel blots. Restriction enzymes and probes used were indicated on the lower side of the blots. P1 and P2 represent the plasmid DNA used for transformation, of which amounts corresponded to ten and one copies, respectively, per haploid genome of rice. M, maize; R, wild type rice. Arrowheads indicate the positions of bands expected for intact transgenes. An open arrowhead in (B) indicated the position of a band derived from incomplete digestion by *Xba*I.

Pdk promoter and cDNA cannot confer high level expression of the PPDK protein, although the positional effects and gene silencing could also hamper its expression.

Unlike the C_4 enzymes located in the mesophyll cells of C_4 plants, those located in the bundle sheath cells can be expressed at high levels in the mesophyll cells of rice by introduction of a chimeric gene containing the full-length cDNA for the C_4 enzyme fused to the *Cab* promoter, which direct mesophyll-specific expression in C_3 plants. The expression of

the maize C_4-specific NADP-ME cDNA under the control of the rice *Cab* promoter increased the activity of NADP-ME in rice leaves to 30-fold that of wild type rice or about 60% of the

Table 1 Increase in activities of C_4 enzymes in transgenic rice leaves

C_4 enzyme (Location in C_4 plants)	Introduced construct	Highest enzyme activity (Increase in fold) over rice activity	over maize activity
PEPC (MC)	Intact maize gene	110	3-4
PPDK (MC)	Rice *Cab* prom::maize FL cDNA	5	
	Maize *Pdk* prom::maize FL cDNA	5	
	Intact maize gene	40	0.5
NADP-ME	Rice *Cab* prom::rice FL cDNA	5	
(BSC)	Rice *Cab* prom::maize FL cDNA	30	0.6

Highest enzyme activities among the primary transgenic plants are listed. MC, mesophyll cells; BSC, bundle sheath cells; prom, promoter; FL, full-length.

NADP-ME activity of maize leaves (Tsuchida et al. 2001). Such a high level expression was unique to cDNA for the C_4-specific NADP-ME, and the expression of cDNA for the rice C_3-specific isoform increased the activity only several fold (Tsuchida et al. 2001). This observation suggests that expression of the rice C_3-specific NADP-ME is suppressed at co- and/or post-transcriptional levels by some regulation mechanisms intrinsic to rice, while that of the foreign C_4-specific isoform can escape from such suppression.

Table 1 summarizes the highest activities of C_4 enzymes in transgenic rice leaves introduced with six different constructs.

Physiological Impacts of Overproduction of C_4 Enzymes in Rice Plants

PEPC: Overproduction of PEPC did not affect the photosynthetic characteristics of rice plants. We previously reported that overproduction of PEPC mitigated the O_2 inhibition of net CO_2 assimilation (Ku et al. 1999). However, the initial CO_2 fixation product of the transformants was exclusively the C_3 compound 3-phosphoglycerate (not shown). The observed effect on O_2 inhibition likely resulted from stimulation of glycolysis and/or respiration by overproduction of PEPC. Significant changes were observed in features other than photosynthesis. Under nitrogen limiting conditions, transgenic rice overproducing PEPC grew better than wild type rice (not shown), an indication of increased nitrogen assimilation. Aluminum tolerance was also enhanced by overproduction of PEPC. As shown in Fig. 5, suppression of root elongation by aluminum was mitigated by overproduction of PEPC.

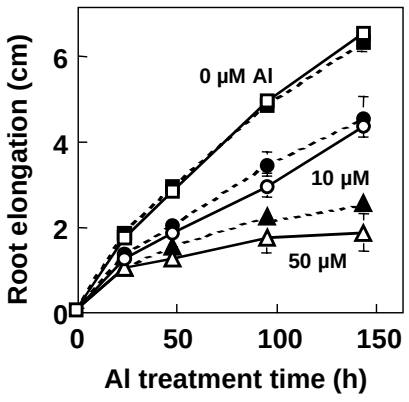

Fig. 5. Root elongation in the presence of aluminum. Rice plants two days after germination were floated on 0.3 mM $CaCl_2$ containing the designated concentrations of aluminum (pH 4.2) and grown in a growth chamber. (Open symbols) wild type rice; (closed symbols) transgenic rice with PEPC activity about 100-fold higher than wild type levels.

The mechanisms of these changes are obscure at present. It seems likely that overproduction of PEPC reinforced the anaplerotic function of the endogenous PEPC to stimulate synthesis of organic acids and nitrogen assimilation.

PPDK: Any significant changes in photosynthetic characteristics were not observed in transgenic rice overproducing PPDK. Sheriff et al. (1998) have previously demonstrated that the expression of chloroplast-targeted PPDK increased the number of seeds per seed capsule and the weight of each seed capsule in transgenic tobacco. We also observed similar phenomena in transgenic rice. The number of seeds per plant and the weight of each hulled rice of the transgenic rice were significantly larger than wild type rice (not shown). The mechanism of the increase in seed yield by PPDK is obscure at present. One possibility is that overproduction of PPDK in the chloroplast enhances photosynthesis in organs surrounding seeds, in which C_4-like photosynthesis is operative and contributes significantly to grain filling (see Imaizumi et al. 1997; King et al. 1998).

NADP-ME: Transgenic rice plants overproducing the maize NADP-ME showed serious stunting and leaf photobleaching, due to increased photoinhibition of photosynthesis under natural light conditions (Takeuchi et al. 2000; Tsuchida et al. 2001). It is likely that the maize NADP-ME in the chloroplasts acts to increase the NADPH/NADP ratio and suppress photorespiration, rendering photosynthesis more susceptible to photoinhibition. Such detrimental effects of the enzyme might imply significant flexibility of carbon metabolism in the mesophyll cells of C_3 plants, especially in terms of transport of metabolites between the cytosol and the chloroplasts.

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