Significant Accumulation of $C_4$-Specific Pyruvate, Orthophosphate Dikinase in a $C_3$ Plant, Rice

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The $C_4$-$Pdk$ gene encoding the $C_4$ enzyme pyruvate, orthophosphate dikinase (PPDK) of maize ($Zea mays\text{cv Golden Cross Bantam}$) was introduced into the $C_3$ plant, rice ($Oryza sativa\text{cv Kitaake}$). When the intact maize $C_4$-$Pdk$ gene, containing its own promoter and terminator sequences and exon/intron structure, was introduced, the PPDK activity in the leaves of some transgenic lines was greatly increased, in one line reaching 40-fold over that of wild-type plants. In a homozygous 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 PPDK fused to the maize $C_4$-$Pdk$ promoter or the rice $Cab$ promoter only increased PPDK activity and protein level slightly. 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 maize and transgenic rice plants carrying the intact maize gene, the level of transcript in the leaves per copy of the maize $C_4$-$Pdk$ gene was comparable, and the maize gene was expressed in a similar organ-specific manner. These results suggest that the maize $C_4$-$Pdk$ gene behaves in a quantitatively and qualitatively similar way in maize and transgenic rice plants. The activity of the maize PPDK protein expressed in rice leaves was light/dark regulated as it is in maize. This is the first reported evidence for the presence of an endogenous PPDK regulatory protein in a $C_3$ plant.

Most terrestrial plants, including many important crops such as rice ($Oryza sativa$) and wheat ($Triticum aestivum$), assimilate $CO_2$ through the $C_3$ photosynthesis pathway and are classified as $C_3$ plants. However, some plants such as maize ($Zea mays$) and sugarcane ($Saccharum officinarum$) possess the $C_4$ photosynthesis pathway in addition to the $C_3$ pathway, and these are classified as $C_4$ plants. It is thought that $C_4$ plants evolved from $C_3$ plants in response to changes in atmospheric conditions, especially a drastic decline of $CO_2$ level (Ehleringer et al., 1991). 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). As a consequence, the transfer of $C_4$ traits to $C_3$ plants is one strategy being adopted for improving the photosynthetic performance of $C_3$ plants.

The $C_4$ pathway consists of three key steps: the initial fixation of $CO_2$ in the mesophyll cell cytosol by phosphoenolpyruvate carboxylase (PEPC) to form a $C_4$ acid, decarboxylation of a $C_4$ acid in the bundle sheath cells to release $CO_2$, and regeneration of the primary $CO_2$ acceptor PEP in the mesophyll cell chloroplasts by pyruvate, orthophosphate dikinase (PPDK; Hatch, 1987). The enzymes involved in the $C_4$ pathway are also present in $C_3$ plants, probably mostly in the photosynthetic mesophyll cells. However, the activities of these enzymes in $C_3$ plants are very low (Hatch, 1987). It is believed that genes for $C_4$ enzymes were derived from the corresponding ancestral genes of $C_3$ plants by acquiring mechanisms that gave high-level, cell-specific expression (Ku et al., 1996).

It is likely that any genes for $C_4$ 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). However, we recently reported that introduc-

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tion of the intact maize C_4-specific PEPC (C_4-Ppc) gene dramatically increased the activity of PEPC in transgenic rice leaves (Ku et al., 1999). To determine if this strategy could work for other C_4 enzymes, we turned our attention to another C_4 enzyme, PPDK.

PPDK (EC 2.7.9.1) catalyzes the following reaction:

\[
\text{Pyruvate} + \text{Pi} + \text{ATP} \Leftrightarrow \text{PEP} + \text{AMP} + \text{PPi}
\]

In the C_4 pathway, the reaction occurs in the forward direction to form PEP (Hatch, 1987). 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). Analysis of antisense-PPDK Flaveria bidentis plants indicated that this enzyme catalyzes one of the key rate-limiting steps in the C_4 pathway (Furbank et al., 1997). Genes for PPDK involved in the C_4 pathway (C_4-Pdk genes) have a dual promoter system to express two different transcripts for the chloroplastic and cytosolic forms of PPDK, with the former being specifically expressed at high levels in green leaves (Glackin and Grula, 1990; Sheen, 1991).

In this study, we compared the expression of the maize C_4-Pdk gene and two chimeric genes containing the PPDK cDNA in transgenic rice plants. We found that introduction of the intact maize gene leads to significant accumulation of PPDK protein in rice leaves. We also found that the maize PPDK protein in rice leaves can be controlled by the endogenous rice PPDK regulatory protein.

**RESULTS**

Expression of PPDK in Transgenic Rice Plants

Three different gene constructs were introduced into rice to express the maize PPDK in the chloroplasts of mesophyll cells. One of these constructs contained the intact maize C_4-Pdk gene, including its own promoter and terminator sequences and exon/intron structure (Fig. 1A). The other two contained the full-length cDNA encoding the maize chloroplastic PPDK, fused to the 5'-flanking region of the maize C_4-Pdk gene or the rice Cab promoter (Fig. 1, B and C).

It has been shown previously that the 1,032-bp 5'-flanking region of the maize C_4-Pdk gene can drive expression of a reporter gene in photosynthetic organs of transgenic rice at levels higher than that the cauliflower mosaic virus 35S promoter does (Matsuoka et al., 1993). The level of expression of these constructs in the transgenic rice plants was determined by assaying the activity of PPDK in leaf extracts of primary (T_1) transformants.

Transformants introduced with the intact maize C_4-Pdk gene exhibited a wide range of activities (Fig. 2A). About 80% of the transformants showed activities up to 5-fold that of wild-type plants, whereas the

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**Figure 1.** Constructs used for rice transformation. A, The construct with the maize C_4-Pdk gene. B, The Pdk promoter::cDNA construct with the maize chloroplastic PPDK cDNA fused to the 5'-flanking sequence of the maize C_4-Pdk gene. C, The Cab promoter::cDNA construct with the maize chloroplastic PPDK cDNA fused to the rice Cab promoter. The coding and the 5'- and 3'-non-coding regions are represented by shadowed and hatched boxes, respectively. Top diagram shows the restriction map of the maize C_4-Pdk gene. B, E, S, and X indicate BamHI, EcoRI, SphI, and XbaI sites, respectively. Short horizontal bars in A indicate the positions of the primers used for reverse transcriptase (RT)-PCR analysis, and MCS indicates a multicloning site that includes a BamHI site.
rest showed higher activities. The highest activity was 40-fold that of wild type, which corresponds to more than one-half of the PPDK activity of maize leaves. In contrast, the PPDK activity of transformants carrying the PPDK cDNA did not exceed 5-fold, irrespective of the promoter used (Fig. 2, B and C).

Figure 3 compares the levels of transcripts and translation products of the introduced gene in leaves of the three types of transformants. RNA gel blotting detected a single band of 3.5 kb in all the transformants, as well as in maize, but not wild-type rice plants. SDS-PAGE showed that a protein of 95 kD, which cross-reacted with anti-maize PPDK, was specifically increased in all transformants, although much less so in the transformants introduced with the cDNA constructs than in those introduced with the intact maize Pdk gene. In each type of transformant, the level of the PPDK protein increased with increasing level of PPDK transcript. The accumulation of PPDK transcript and protein in some of the rice plants introduced with the intact maize Pdk gene was quite remarkable. For example, in one plant (Fig. 3, lane 8) the transcript level was about 3-fold that in maize, and the PPDK protein accounted for about 20% of total soluble protein in the leaf.

Factors That Determine the Expression Level of the Introduced Gene

Our previous study has demonstrated that the level of transcript of the intact maize C₄-Ppc gene is determined by the copy number of the transgenes in transgenic rice (Ku et al., 1999). We examined if this was also the case for the intact maize C₄-Pdk gene using four independent homozygous lines, all of which have the transgenes in a single insertion site per haploid (Fig. 4). To estimate the copy number of the transgenes, genomic DNA was digested with BamHI, which excises a 3.4-kb fragment from the maize C₄-Pdk gene (see Fig. 1), and was probed with the same...
fragment excised from the plasmid for transformation. The levels of transcript and protein in the leaves correlated well with the copy number, giving straight lines in the plots against the copy number. The slope of the regression line of the plot of the transcript level was 1.11, an indication that the level of transcript per copy of the intact maize C₄-Pdk gene was comparable in maize and transgenic rice. In contrast, that of the plot of the protein level was 0.29, indicating that one copy of the maize gene in transgenic rice is capable of accumulating the PPDK protein only one-third as much as that in maize leaves on a total leaf-soluble protein basis.

The above finding, however, raises a question as to why the majority of the transformants introduced with the intact maize gene showed low PPDK activities (Fig. 2A). This result would be ascribable to the positional effects (Gelvin, 1998), silencing of transgenes (Gallie, 1998), and/or rearrangement of the introduced gene that occasionally occurs during the course of Agrobacterium tumefaciens-mediated gene transfer (Hiei et al., 1994). These possibilities were examined by DNA gel-blot analysis of low-expressing lines using two different probes specific to 5′- and 3′-terminal regions of the introduced gene (Fig. 5A). In the high-expressing homozygous line PD259 (lane 6), only the bands of the expected sizes were detected. The same results were obtained with the other three high-expressing homozygous lines (data not shown). All the low-expressing lines tested showed different band patterns. In a plant of lane 4, which did not accumulate the maize protein at all, a band corresponding to the 5′-side of the maize gene was totally absent. In plants of lanes 1 through 3 and 5, some to several bands were detected in addition to those expected for the intact gene, an indication of partial deletion and/or chimeric linking of the introduced gene. It is likely that such rearrangements could reduce transcriptional activity of the maize gene. In contrast, a plant of lane 1 seems to have multiple copies of the intact maize gene because a significant level of a set of the expected bands was detected, whereas the levels of unexpected bands were low. Nevertheless, this plant barely accumulated the maize protein. This result would be ascribable to the positional effects and/or silencing of transgenes.

Similar analysis was carried out with the transformants introduced with the Pdk promoter::cDNA construct (Fig. 5B). All plants tested accumulated small amounts of the maize PPDK protein, an indication that they have the entire coding region of the cDNA. Rearrangement of the introduced gene was observed in a plant of lane 7. The other three plants likely had
the introduced gene in an intact form, and two of them (lanes 9 and 10) contained several copies of the intact gene. These observations suggest that unlike the maize C₄-Pdk gene, the presence of multiple copies of the intact Pdk promoter and cDNA does not confer high-level expression of the PPDK protein, although the positional effects and gene silencing could also contribute to low expression levels in these transformants.

The Mode of Expression of the Maize C₄-Pdk Gene in Transgenic Rice Plants

The maize C₄-Pdk gene contains the dual promoter system, and two different transcripts of 3.5 and 3.0 kb for the chloroplastic and cytosolic forms of PPDK, respectively, are expressed in an organ-specific manner (Glackin and Grula, 1990; Sheen, 1991), with the former being expressed at high levels in green leaves (Glackin and Grula, 1990; Sheen, 1991) and the latter in reproductive organs (Aoyagi and Chua, 1988; Imaiizumi et al., 1997). Therefore, it is of importance to examine which transcript was expressed in leaves of the transformants with the intact maize Pdk gene. Because the two transcripts are difficult to distinguish from each other by RNA gel-blot analysis, RT-PCR analysis was carried out according to the method of Sheen (1991). The primer pairs used were PF-1 + PR-1 and PF-2 + PR-1 for transcripts of the chloroplastic and cytosolic forms, respectively (see Fig. 1). It is expected that RT-PCR products of 460 and 307 bp, respectively, are obtained with these primer pairs.

RT-PCR products were obtained from maize and the transgenic rice plants, but not from wild-type rice (Fig. 6). A single band of the expected size (460 bp) was detected with the primer pair used to detect transcript of the chloroplastic form (Fig. 6, a and b). With the other primer pair, two distinct bands of 307 bp were detected in leaves from transformants (Fig. 6, c and d).

Figure 5. DNA gel-blot analyses of low-expressing lines of transgenic rice plants of T₂ generation. A, Transformants introduced with the maize C₄-Pdk gene construct with PPDK activities in leaves less than 2-fold wild-type levels (lanes 1–5), together with PD259 (lane 6) for comparison. B, Transformants introduced with the Pdk promoter::cDNA construct (lanes 7–10). a, Location within the introduced gene of restriction sites and probes. E, H, and X indicate EcoRI, HindIII, and XbaI sites, respectively. Bidirectional arrows and numbers indicate fragments excised from the introduced gene and their sizes in kilobases, respectively. b, Polypeptide profiles after Coomassie Blue staining (left) and immunoblot profiles with anti-maize PPDK (right) of leaf-soluble protein. Arrowheads indicate the positions of the band of the maize PPDK protein. c, DNA gel-blot analysis. Restriction enzymes and probes used were indicated on the bottom side of panels. P1 and P2 in A and B represent the plasmid DNA used for transformation and the probes, respectively, of which amounts corresponded to 10 and one copy, respectively, per haploid genome of rice. M, Maize; R, wild-type rice.

Figure 6. RT-PCR analysis of the two different transcripts derived from the maize C₄-Pdk gene. A, Developing leaves. B, Hulled rice at a milky stage. Total RNA (5 μg from leaves and 2.5 μg from hulled rice) were used for cDNA synthesis, and 2 μL (a and c) and 0.02 μL (b) of cDNAs were used for the PCR reaction using PF-1 and PR-1 (a and b), and PF-2 and PR-1 (c) as primers (see Fig. 1). d, Electropherograms of total RNA used for the RT-PCR analysis after staining with ethidium bromide.
and 411 bp were detected (Fig. 6c). The 411-bp product is unlikely to be derived from contaminating genomic DNA because no bands were detected when the PCR reaction was performed without the cDNA synthesis step (data not shown), and probably resulted from incomplete splicing of the second intron (Sheen, 1991). Such incomplete splicing generates a new termination codon inside the third exon, and the resulting transcript could not contribute to synthesis of the functional protein.

In leaves, the ratio of the transcript levels for the chloroplast and cytosolic form were almost the same in maize and the two rice transformants with the intact maize Pdk gene (Fig. 6A). Taking the amounts of template used into account, transcript for the chloroplastic form was 100 times more abundant than that for the cytosolic form. In fact, this is likely to be an underestimate because the cDNA for the cytosolic form was amplified about three times more efficiently than that of the chloroplastic form (data not shown). Thus, transcripts from the maize C4-Pdk gene would code almost exclusively for the chloroplastic form of PPDK in transgenic rice leaves, suggesting that essentially all of the PPDK protein would accumulate in the chloroplasts. This hypothesis was confirmed by N-terminal amino acid sequencing. The N-terminal sequences of the PPDK protein in leaves of maize and PD278 were both AVVDAAPIQT, which matches perfectly with residues Ala-63 to Thr-72 of the sequence deduced from the nucleotide sequence of the gene.

In hulled rice at a milky stage of the transgenic rice, in contrast, the transcript levels for the chloroplastic and cytosolic forms were almost the same (Fig. 6B). Because the pericarp of hulled rice at this stage, several days after flowering, was green in color, it is likely that transcript for the chloroplastic form accumulated in the photosynthetically active pericarp. Transcript for the cytosolic form likely accumulated in the endosperm, as previously demonstrated in the mature kernel of maize (Aoyagi and Bassham, 1984; Aoyagi and Chua, 1988).

Figure 7 shows distribution of the PPDK protein in various organs in wild-type plant, PD259 and PD278. In wild-type rice, hulled rice at a milky stage contained a significant level of the PPDK protein, likely to be cytosolic, as reported previously in reproductive organs of wheat (Aoyagi and Bassham, 1984; Aoyagi and Chua, 1988) and rice (Imaizumi et al., 1997). Other organs of wild-type rice also contained the PPDK protein, albeit at barely detectable levels. Introduction of the maize C4-Pdk gene increased the levels of the PPDK protein in all organs except root. Albeit very slightly, the level of PPDK protein in hulled rice was further increased in PD278. Taking the results of RT-PCR analysis (Fig. 6b) into account, it is possible that the chloroplastic and cytosolic forms of the maize PPDK protein were expressed in hulled rice at this stage.

Characterization of Transgenic Rice Plants with High Levels of PPDK Protein

As described above, introduction of the intact maize C4-Pdk gene into rice led to significant accumulation of the PPDK protein in the chloroplasts of leaves. In homozygous lines of these transformants, extraordinary levels of the PPDK protein accumulated in leaves. The PPDK protein in PD259 and PD278 accounted for 6% and 35%, respectively, of total leaf-soluble protein, or 4% and 16%, respectively, of total leaf nitrogen (Table I). In Figure 4C, it can be seen that the staining intensity of the band of PPDK protein was comparable with that of the large subunit of Rubisco in PD278. However, the activities of PPDK in these transformants were lower than expected, given the levels of the PPDK protein present in the leaves (Table I).

The accumulation of PPDK protein affected the contents of other components in the leaves and total leaf nitrogen (Table I). In PD259, total leaf nitrogen was increased by about 7%. The Rubisco content on a leaf area basis was increased slightly, although on a total leaf nitrogen basis it remained unchanged, and the chlorophyll content was decreased slightly. In PD278, which accumulated much more PPDK protein than PD259, total leaf nitrogen did not increase further, and the Rubisco and chlorophyll contents were significantly decreased on leaf area and total leaf nitrogen bases. These observations suggest that the rice plants can accumulate the maize PPDK protein up to some threshold level without significant changes in levels of other components by increasing total leaf nitrogen, but that the PPDK protein can accumulate above the threshold level only at the expense of other components.

Despite such significant accumulation of the PPDK protein, the transformants did not show abnormalities in growth behavior or fertility. Only PD278, es-
especially when grown under limited light conditions, showed lighter leaf color and lower rates of growth and germination than wild-type plants. Photosynthetic activity was not appreciably altered in any of the transgenic plants except PD278, which showed a slightly lower activity.

The activity of the chloroplastic form of PPDK is strictly regulated by light in C₄ plants (Burnell and Hatch, 1985). The effects of illumination on PPDK activity in the transgenic rice plants were investigated to examine whether the maize PPDK protein expressed in these plants is also subject to such regulation. As shown in Figure 8, the activity of PPDK was much higher in the light than in the dark in all of the plants examined, though the level of the PPDK protein was unchanged. This observation suggests that the PPDK regulatory protein is present in rice leaves and that it can control the maize protein. However, the degree of light activation did differ somewhat between maize and rice plants. The ratios of activity in the light/dark were 20.7 in maize, 6.4 in wild-type rice, 9.4 in PD259, and 6.4 in PD278. If it is assumed that the PPDK in maize was fully activated in the light, only 28% and 13% of the PPDK was activated by light in PD259 and PD278, respectively. The apparent inability to activate all of the maize PPDK might be due to differences in the substrate specificity of the maize and rice PPDK regulatory proteins, the relative abundance of the regulatory protein to the substrate, or a combination of both.

**DISCUSSION**

We have shown previously that a C₄-specific PEPC can be expressed at high levels in rice leaves by introduction of the intact maize C₄-Ppc gene (Ku et al., 1999). The present study shows similar results for a C₄-specific PPDK (Figs. 2–4). From analysis of low-expressing lines (Fig. 5A), it was found that for high-level expression, the maize gene in its intact form has to be inserted in an appropriate position of the rice genome. Once this has been done, the level of the maize protein increases with increasing copy number of transgenes (Fig. 4).

The present study provides information as to which part of the intact gene is responsible for high-level expression of C₄ enzymes. The transcriptional activity of the introduced gene cannot be the prime reason because expression of the maize PPDK cDNA under the control of the maize C₄-Pdk promoter or the rice Cab promoter did not significantly increase the activity and protein level of PPDK in rice leaves (Figs. 2 and 3). This observation agrees with previous studies in which strong promoters, such as Cab, rbcS, and 35S, were used for expression of the PPDK cDNA (Ishimaru et al., 1997, 1998; Sheriff et al., 1998) and of the PEPC cDNA (Hudspeth et al., 1992; Kogami et al., 1994; Gehlen et al., 1996). The 5' - and 3'-non-coding regions by themselves did not lead to high-level expression either because the cDNA constructs containing these regions were not effective (Figs. 2 and 3). Therefore, it is quite possible that the presence of introns or the terminator sequence, or a

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**Table 1. Distribution of leaf nitrogen in PPDK, Rubisco, and chlorophylls**

<table>
<thead>
<tr>
<th>Plant</th>
<th>PPDFK Activity</th>
<th>PPDFK per Soluble Proteina</th>
<th>Levels per Leaf Area²</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>fold %</td>
<td>mmol m⁻²</td>
<td>g m⁻²</td>
</tr>
<tr>
<td>Maize</td>
<td>54 9.60</td>
<td>–</td>
<td>Trace</td>
</tr>
<tr>
<td>Rice</td>
<td>1 0.15</td>
<td>141 ± 2.0 (100%)</td>
<td>3.59 ± 0.07 (29.1%)</td>
</tr>
<tr>
<td>PD259</td>
<td>12 6.10</td>
<td>151 ± 3.0 (100%)</td>
<td>3.78 ± 0.03 (28.6%)</td>
</tr>
<tr>
<td>PD278</td>
<td>22 34.60</td>
<td>152 ± 2.0 (100%)</td>
<td>3.08 ± 0.05 (23.1%)</td>
</tr>
</tbody>
</table>

- Determined from the densitogram of a Coomassie-stained gel.
- The levels of leaf constituents are presented as means ± SD obtained from leaves of two independent plants. Percentages in parentheses represent levels of nitrogen in the respective components per total leaf nitrogen.
- Determined by the method of Makino et al. (1986). The distribution of nitrogen in PPDFK was calculated assuming the nitrogen content of the PPDFK protein to be 16% by wt.
combination of both, is required for high-level expression.

Introns and the terminator sequences have been shown previously to enhance expression of some genes in plant cells. It has been demonstrated that introns can increase the level of functional mRNA by promoting correct splicing of transcripts (Tanaka et al., 1990; Luehrsen and Walbot, 1991) and that the terminator sequence increases the efficiency of 3’ processing and/or the stability of mRNA (Ingelbrecht et al., 1989). Moreover, it has been reported that an intron and a terminator, each of which enhances gene expression, can act in an additive manner (Mitsuhara et al., 1996). At present, we cannot specify which region(s) of the maize genes is responsible for high-level expression of C₄ enzymes in rice. However, it seems likely that the introns and terminator, and perhaps other enhancer sequences, act cooperatively to increase the level of functional and stable mRNA.

Previous studies using a reporter gene demonstrated that the promoters of maize C₄-specific genes encoding PEPC and the chloroplastic form of PPDK can function in rice in the same way as in maize. Both of these promoters drive organ-specific, mesophyll cell-specific, and light-dependent expression in rice leaves (Matsuoka et al., 1993, 1994). The present study showed that the dual promoter system of the maize C₄-Pdk gene also functions in rice as it does in maize; essentially only transcript for the chloroplastic form of PPDK accumulated in rice leaves and transcript for the cytosolic form was expressed in hulled rice (Fig. 6). Furthermore, the levels of mRNA transcribed from one copy of the intact maize C₄-Ppc (Ku et al., 1999) and C₄-Pdk (Fig. 4) genes were comparable in maize and transgenic rice. Taken together, these results indicate that the maize C₄-specific genes for PEPC and PPDK behave in a qualitatively and quantitatively similar way in maize and transgenic rice plants. The effectiveness of using intact C₄-specific genes for high-level expression of C₄ enzymes in C₃ plants has now been shown for PEPC and PPDK, both of which are located in the mesophyll cells of C₄ plants. We presume that the same strategy can be used to express other C₄ enzymes, at least those located in mesophyll cells of C₄ plants, in C₃ plants. It seems necessary, however, to use transgenes from phylogenetically closely related plants to achieve high-level expression (Ku et al., 1999).

A remarkable feature of high-expressing lines of the transgenic rice carrying the intact maize C₄-Pdk gene was the extraordinarily high levels of PPDK protein found in the leaves (Figs. 3B and 4C). In a homozygous line PD278, the PPDK protein amounted to 35% of total leaf-soluble protein (Table I). The PD278 plants showed lighter leaf color and lower growth rates. This phenotype closely resembles the characteristic symptoms of nitrogen starvation and suggests that the level of PPDK in these plants exceeded the limit that a foreign protein can be expressed without leading to nitrogen deficiency.

Such a significant accumulation of a foreign protein in transgenic plants has only been reported by McBride et al. (1994), who succeeded in expressing β-glucuronidase (GUS) inside the chloroplast at levels of 20% to 30% of total leaf-soluble protein in tobacco. They introduced a construct into the nuclear genome to express the T7 phage RNA polymerase inside the chloroplast, and the GUS gene fused to the T7 phage promoter into the plastid genome, for efficient expression of the GUS gene under the control of the T7 polymerase inside the chloroplast. It is likely that the high copy number of the plastid genome, up to 50,000 (Bendich, 1987), contributed to the high-level expression. In contrast, the copy number of the introduced genes for C₄ enzymes in our transgenic rice plants was around 10 (Ku et al., 1999) to 24 (Fig. 4) at most, indicating that these genes must have quite high expression activity.

Most of the transgenic rice plants did not show any deleterious phenotypes associated with the very high levels of the PPDK protein accumulated. The lower than expected activity of PPDK suggested that much of the enzyme was not activated (Fig. 8), reducing its potential impact on metabolism in the chloroplast. It should be noted that GUS, the other enzyme expressed to very high levels in leaves by McBride et al. (1994), would not be enzymatically active in the chloroplast due to the lack of substrate. It is also interesting to note that the highest reported levels of expression of a foreign protein in leaves of transgenic plants involved expression of the foreign protein in the chloroplasts (this study; McBride et al., 1994). In contrast, the highest reported level of expression of a foreign protein in the cytosol is about 12% of total leaf protein for PEPC (Ku et al., 1999), and significant accumulation of GUS in the cytosol has not yet been reported despite a great number of studies to develop efficient gene expression system using the GUS gene. This might simply reflect the relative volumes of the two compartments in mesophyll cells. For example, in barley (Hordeum vulgare) leaves, chloroplasts and the cytosol occupy 19% and 6.7%, respectively, of the total cell volume (Winter et al., 1993). Protein storage vacuoles of the endosperm, which can accommodate foreign proteins at high levels (Katsube et al., 1999), also occupy a significant volume of the aleurone cell. However, this does not exclude the possibility that the level of tolerance of foreign proteins could differ significantly between the various compartments in the cell.

We found that the activity of the maize PPDK protein expressed in transgenic rice leaves was light/dark regulated (Fig. 8) in a similar manner to that in maize. This finding is the first evidence for an endogenous PPDK regulatory protein in a C₃ plant. The transgenic rice plants expressing PPDK at high levels...
will be a valuable tool for studying the function of the endogenous PPDK in C₃ plants and its regulation by the PPDK regulatory protein, which are poorly understood. These plants also make an important contribution toward our goal of transferring C₄ traits to C₃ plants, and might have wider significance with respect to achieving high-level expression of foreign proteins in transgenic plants.

**MATERIALS AND METHODS**

**Constructs and Transformation of Rice (Oryza sativa)**

Three different constructs were used for transformation of rice cv Kitaake (Fig. 1). The first construct contained the 7.3-kb C₄-\textit{Pdk} gene of maize (\textit{Zea mays} cv Golden Cross Bantam; Matsuoka, 1990) with a partial deletion of 4 kb in the first intron to shorten the length of the introduced gene. The second construct was a chimeric gene containing a full-length cDNA for the maize chloroplastic PPDK (Matsuoka et al., 1988) fused to the 5'-flanking region (1,321 bp) of the maize C₄\textit{Pdk} gene. The third construct was a chimeric gene containing a full-length cDNA for the maize chloroplastic PPDK fused to the rice \textit{Cab} promoter (Sakamoto et al., 1991). These constructs were cloned into a binary vector pIG121Hm containing a hygromycin resistance gene (a generous gift from Prof. Kenzo Nakamura, Nagoya University, Nagoya, Japan). The resultant plasmids were introduced into calli derived from rice via \textit{Agrobacterium tumefaciens}-mediated transformation. Transgenic plants were regenerated from hygromycin-resistant calli and were planted in soil.

**Plant Growth Conditions**

Rice and maize were grown under natural light conditions in a temperature-controlled greenhouse (Tsuchida et al., 2001). When indicated, plants were grown in a growth chamber on a 25°C day/20°C night cycle with a day period of 14 h under illumination at a photon flux density of 500 \mu mol m⁻² s⁻¹.

**Extraction and Analyses of Soluble Protein**

Samples were ground in extraction buffer containing: 50 mM HEPES (N-2-hydroxyethylpiperazine-N’-2-ethanesulfonic acid)-KOH (pH 7.4), 5 mM pyruvate, 2 mM KH₂PO₄, 10 mM MgCl₂, 1 mM EDTA, 5 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 10 \muM leupeptin, 5% (w/v) insoluble polyvinylpyrrolidone, and 10% (w/v) glycerol, with a small amount of sea sand. The homogenate was centrifuged at 15,000 \textit{g} for 10 min at 4°C and the supernatant after centrifugation of the homogenate was incubated at 25°C for 1 h to activate PPDK. PPDK activity was assayed in the forward direction at 30°C, essentially by the method of Ashton et al. (1990). The assay mixture contained 50 mM HEPES-KOH (pH 8.0), 10 mM MgCl₂, 0.1 mM EDTA, 5 mM Glc 6-P, 10 mM NaHCO₃, 2 mM pyruvate, 1.25 mM ATP, 10 mM dithiothreitol, 2.5 mM KH₂PO₄, 5 mM NH₄Cl, 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 activities of maize and wild-type rice leaves, calculated on a protein basis, were 0.50 to 0.80 and 0.01 to 0.03 \mu mol mg⁻¹ protein min⁻¹, respectively.

**Determination of Leaf PPDK, Rubisco, Chlorophyll, and Total Nitrogen Contents**

A leaf blade was homogenized in extraction buffer and a part of the homogenate was used for chlorophyll and nitrogen determination (Makino and Osmond, 1991). For protein determination, the homogenate was supplemented with 0.1% (v/v) Triton X-100. After centrifugation, the supernatant was supplemented with 1% (w/v) lithium dodecyl sulfate and was subjected to SDS-PAGE. After staining with Coomassie Brilliant Blue R-250, the dye was extracted with formamide from a protein band of the gel and was quantitated spectrophotometrically (Makino et al., 1986). Calibration curves for PPDK and Rubisco were made with bovine serum albumin and purified rice Rubisco as standards, respectively.

**DNA Gel-Blot, RNA Gel-Blot, and RT-PCR Analyses**

DNA gel blotting was performed using a 32P-labeled probe and an image plate (Ku et al., 1999). Probes used were a 1.8-kb \texttt{HindIII}/XbaI fragment (probe 1) and a 3.4-kb \texttt{BamHI} fragment (probe 2) of the maize C₄\textit{Pdk} gene excised from the leaf sample buffer and boiled for 5 min immediately after extraction. The level of the PPDK protein in the total leaf-soluble protein was determined from the peak area in a densitogram of the gel recorded with a thin-layer chromatography scanner (CS-9300PC, Shimadzu, Kyoto). N-terminal amino acid sequencing and immunoblotting were performed as described previously (Tsuchida et al., 2001).
from the maize C₄-Pdk gene construct for transformation, and a 1.5-kb EcoRI fragments of the maize chloroplastic PPDK cDNA (probe 3) excised from pPPD1067 (Matsuoka et al., 1988; see Figs. 1 and 5).

Total RNA was isolated using guanidine thiocyanate, and RNA gel blotting was performed as described previously (Ku et al., 1999). A 2.0-kb PstI fragment of pPPD1067 was used as a probe.

RT-PCR analysis was performed essentially as described by Sheen (1991). The cDNA was synthesized from total RNA with oligo(dT)₁₂-₁₈ as the primer, and the resultant cDNA was diluted to a final volume of 20 μL with PCR Gold buffer (Applied Biosystems, Foster City, CA) containing 7.5% (v/v) dimethyl sulfoxide, 1.5 μM of each primer, and 300 μM deoxynucleotide triphosphates. After heating at 100°C for 10 min, the mixture was supplemented with AmpliTaq Gold DNA polymerase (Applied Biosystems) and the PCR buffer, to a final volume of 30 μL, and the polymerase was activated by heating at 95°C for 10 min. The PCR was carried out for 25 cycles of 30 s at 95°C, 100°C for 10 min, the mixture was supplemented with AmpliTaq Gold DNA polymerase (Applied Biosystems), and the PCR buffer, to a final volume of 30 μL, and the polymerase was activated by heating at 95°C for 10 min. The PCR was carried out for 25 cycles of 30 s at 95°C, 120 s at 60°C, and 180 s at 72°C. The primers used were PF-1, PF-2, and PR-1 (Fig. 1; Sheen, 1991).

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LITERATURE CITED


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