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# Tissue-specific light-regulated expression directed by the promoter of a $C_4$ gene, maize pyruvate,orthophosphate dikinase, in a $C_3$ plant, rice

($C_4$ photosynthesis/gene evolution/transgenic rice)

MAKOTO MATSUOKA*†, YUICHI TADA‡, TATSUHITO FUJIMURA‡, AND YURIKO KANO-MURAKAMI§

*National Institute of Agrobiological Resources, Tsukuba, Ibaraki 305, Japan; ‡Plant Biotechnology, Life Science Institute, Mitsui Toatsu Chemicals, Inc., Mobara 297, Japan; and §Fruit Tree Research Station, Tsukuba, Ibaraki 305 Japan

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**ABSTRACT** **Pyruvate,orthophosphate dikinase (PPDK; EC 2.7.9.1) activity is abundant in leaves of $C_4$ plants, while it is difficult to detect in leaves of $C_3$ plants. Recent studies have indicated that $C_3$ plants have a gene encoding PPDK, with a structure similar to that of PPDK in $C_4$ plants. However, low expression makes PPDK detection difficult in $C_3$ plants. This finding suggests that high PPDK expression in $C_4$ plants is due to regulatory mechanisms which are not operative in $C_3$ plants. We have introduced a chimeric gene consisting of the gene encoding β-glucuronidase (GUS; EC 3.2.1.31) controlled by the PPDK promoter from a $C_4$ plant, maize, into a $C_3$ cereal, rice. The chimeric gene was exclusively expressed in photosynthetic organs, leaf blades and sheaths, and not in roots or stems. Histochemical analysis of GUS activity demonstrated high expression of the chimeric gene in photosynthetic organs, localized in mesophyll cells, and no or very low activity in other cells. GUS expression was also regulated by light in that it was low in etiolated leaves and was enhanced by illumination. These observations indicate that the mechanisms responsible for cell-specific and light-inducible regulation of PPDK observed in $C_4$ plants are also present in $C_3$ plants. We directly tested whether rice has DNA-binding protein(s) which interact with a previously identified cis-acting element of the $C_4$-type gene. Gel retardation assays indicate the presence in rice of a protein which binds this element and is similar to a maize nuclear protein which binds PPDK in maize. Taken together, these results indicate that the regulatory system which controls PPDK expression in maize is not unique to $C_4$ plants.**

Pyruvate,orthophosphate dikinase (PPDK; EC 2.7.9.1) catalyzes the formation of phospho*enol*pyruvate, the primary acceptor of $CO_2$ in $C_4$ plants. This reaction is one of the rate-limiting steps in the $C_4$ photosynthetic pathway (reviewed in ref. 1). The mRNA encoding this enzyme, which is one of the major transcripts in green leaves of $C_4$ plants, is translated in the cytoplasm as a large precursor and transported into chloroplasts with concomitant processing of a transit peptide (2). The expression of PPDK occurs primarily in the mesophyll and not in the bundle sheath cells of $C_4$ plants. Previous studies have indicated that this cell-specific expression of the protein is regulated at the transcriptional level (3, 4). Transcription of the gene is also regulated by light, which stimulates steady-state expression of PPDK mRNA (3, 5).

In contrast with PPDK activity in $C_4$ plants, PPDK activity in green leaves of $C_3$ plants has been difficult to detect (1). The difficulty in detecting PPDK activity in $C_3$ plants does not directly demonstrate that genes encoding PPDK are absent from $C_3$ plants or that this activity not expressed in green leaves in $C_3$ plants. There are reports of low PPDK activity in the leaves of some $C_3$ species, where the PPDK protein has been detected with antibodies directed against the enzyme (6, 7). The PPDK protein and mRNA were also detected in green leaves of rice, a $C_3$ plant (8, 9). Our recent studies have revealed that rice contains a PPDK gene with primary structure very similar to that of the maize PPDK gene (10). These findings indicate that the difficulty in detecting PPDK activity in $C_3$ plants is due not to lack of the PPDK gene but to low expression of the gene. This presents the possibility that $C_4$ plants have a unique regulatory system which permits high PPDK expression and is not present in $C_3$ plants.

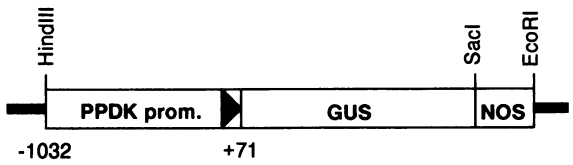


FIG. 1. Structure of the PPDK-GUS chimeric gene. The 5′-flanking region (from nt −1032 to nt +71) of the PPDK gene from maize (*Zea mays*) was fused to the *Escherichia coli* β-glucuronidase (GUS; EC 3.2.1.31) coding region followed by the polyadenylylation signal of the nopaline synthetase (NOS) gene in the plasmid vector pUC19.

We have addressed the differences in PPDK gene expression between $C_3$ and $C_4$ plants by introducing a reporter gene controlled by the promoter of the PPDK gene from maize into rice, at present the only mocotyledonous species which can be routinely transformed with foreign genes. Here, we describe expression of the chimeric gene, which occurs specifically in leaf mesophyll cells and is induced by light.

## MATERIALS AND METHODS

**Transformation and Selection of Transgenic Rice Lines.** The PPDK-GUS fusion gene (Fig. 1) was constructed as described previously (11). Transgenic control rice plants were transformed with pBI221 (Clontech), which contains the promoter of cauliflower mosaic virus 35S transcript. Protoplasts were isolated from suspension cells of rice (*Oryza sativa* cv. Nipponbare) according to a previously reported method (12). Rice protoplasts were cotransformed by electroporation with 10 μg of the 35S–hygromycin phosphotransferase plasmid and 20 μg of either PPDK-GUS or pBI221 (13). Clones of cells which developed from transformed protoplasts were cultured and regenerated as reported by Fujimura

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Abbreviations: GUS, β-glucuronidase; PPDK, pyruvate,orthophosphate dikinase.

†To whom reprint requests should be addressed at: Department of Plant Physiology, National Institute of Agrobiological Resources, Tsukuba, Ibaraki 305, Japan.

*et al.* (14). Transformed plants were selected from among the regenerants according to Tada *et al.* (13).

**GUS Assay.** Fluorometric assay and histochemical analysis of GUS activity were performed as previously described (11).

**Light Treatment of Etiolated Leaves.** Self-pollinated T2 transgenic rice seeds which had been shown to have GUS activity were germinated on vermiculite in the dark at 25°C for 2 weeks. The etiolated seedlings were transferred to continuous white light (about 100 millieinstein·$m^{-2}$·$sec^{-1}$) for the indicated time. The secondary leaves from 10 seedlings were used for the GUS assays and for RNA isolation.

**Hybridization Analysis.** Rice DNA was prepared as previously described (15). Genomic Southern hybridization and Northern hybridization were performed as described elsewhere (9).

**DNA Gel Retardation Assay.** Nuclear extracts used in studies of DNA-binding activity in rice and maize were isolated from the leaves of light-grown seedlings as previously described (16). The binding reaction was carried out as previously reported (16). Competition reactions were conducted with competitors added in the molar ratios described in the text. Protein–DNA complexes were separated from unbound DNA by electrophoresis on nondenaturing polyacrylamide gels.

## RESULTS

**Expression of GUS Activity in Transgenic Rice.** A chimeric gene was constructed by fusing the 5′-flanking region of the PPDK gene from maize to the coding region of the GUS gene (Fig. 1) and electroporated into rice protoplasts with a plasmid containing a gene encoding hygromycin phosphotransferase under the control of the cauliflower mosaic virus 35S transcript promoter (35S promoter). GUS activity was detected in the leaves of 14 plants, each of which contained one to several copies of the intact fusion gene per diploid genome (data not shown).

Further analysis of the 14 transformants revealed high levels of GUS activity in the leaf blades, leaf sheaths, and rachis tissues. GUS activity was highest in the leaf blade in all 14 transformants (Table 1). GUS activity was low in glumes and was extremely low or undetectable in the nonphotosynthetic tissues we analyzed, stems and roots. GUS activity under the control of the 35S promoter as a control for nonspecific expression was expressed in all of the tissues we tested, including leaf blades, leaf sheaths, rachis, glumes, stems, and roots. The GUS activity in leaves of PPDK-GUS transformants was almost half of that in leaves of transgenic rice with the GUS gene controlled by the promoter of a rice light-harvesting chlorophyll *a*/*b* binding protein, which is one of the most highly expressed genes in leaves (17), indicating that the promoter of the maize ($C_4$) PPDK gene retains high transcriptional activity in leaves of $C_3$ plants. Furthermore, the organ specificity of the maize PPDK promoter activity is the same in rice as in maize.

Table 1. GUS activity controlled under the promoter of the PPDK gene in various organs of transgenic rice

| Plant | GUS activity, pmol of 4-MU released per min per mg of protein | | | | | |
|---|---|---|---|---|---|---|
| | Leaf blade | Leaf sheath | Rachis | Glume | Stem | Root |
| PPDK-GUS 1 | 30,200 | 11,800 | 3540 | 245 | ND | ND |
| PPDK-GUS 2 | 19,700 | 2,970 | 3760 | 115 | ND | ND |
| PPDK-GUS 3 | 4,540 | 245 | 518 | 72 | ND | ND |
| PPDK-GUS 4 | 4,770 | 346 | 1010 | 29 | ND | ND |
| PPDK-GUS 5 | 2,033 | 601 | | | ND | ND |
| PPDK-GUS 6 | 3,133 | 777 | | | ND | ND |
| PPDK-GUS 7 | 12,400 | 9,220 | 9320 | | 91 | ND |
| 35S-GUS | 5,645 | 1,282 | | | 1265 | 634 |
| Nontransformant | ND | ND | | | ND | ND |

ND, not detected (<10 pmol·$min^{-1}$·$mg^{-1}$); 35S, promoter of cauliflower mosaic virus 35S transcript; 4-MU, 4 methylumbelliferone.

The cell specificity of PPDK-GUS expression in rice was determined by observing the *in situ* histochemical staining resulting from GUS activity. A similar pattern of GUS staining was observed in several independent transformants. In leaf blade cross sections, deep staining was observed only in mesophyll cells, while little or no staining was found in the upper or lower epidermis, bundle sheath cells, or vascular bundles (Fig. 2*A*). Similar staining patterns were observed in leaf sheath cross sections, indicating strong activity only in mesophyll cells and no activity in other cell types (Fig. 2*B*). Faint staining relative to that observed in leaf mesophyll cells was observed in rachis tissue, while no staining was found in the pistil (Fig. 2*C*), corroborating direct measurement of GUS activity in the tissues (Table 1). Faint staining was also observed in the palea and lemma of glumes (Fig. 2*D*), also corroborating direct assays of GUS activity (Table 1). The only other staining observed in the transgenic rice plants was very faint and occurred in the root meristem immediately adjacent to the root cap (Fig. 2*E*). Thus the PPDK promoter specifically directs GUS expression in cells which contain chloroplasts, leaf mesophyll cells, and does not do so in nongreen cells and tissues. This pattern of gene expression in rice, a $C_3$ plant, is similar to that in maize, a $C_4$ plant.

**Light Induction of PPDK-GUS in Transgenic Rice.** Light-regulated expression of PPDK in $C_4$ plants has been well characterized (3, 5). We analyzed light-regulated expression of the PPDK promoter in transgenic $C_3$ plants. Seeds from three self-pollinated transformants (PPDK-GUS 1, 2, and 7 in Table 1, which all exhibited GUS activity) were germinated in the dark for 2 weeks then exposed to light. Fig. 3 shows the results of GUS assays conducted in the secondary leaves of 10 seedlings from each transformed line. In all three transformed lines, GUS activity in the leaves of etiolated seedlings was less than 15% of the levels observed in greening leaves of seedlings which had been exposed to light for 24 h. The induction of activity initiated after a lag phase of about 6 h and increased almost linearly through 24 h of exposure to light. No induction was observed in the control transformant line containing the 35S-GUS construct.

We also analyzed transcription of the PPDK-GUS construct in the PPDK-GUS 1 line during the greening process. The accumulation of GUS mRNA was observed after 5 h of illumination and it continued beyond 10 h of illumination (data not shown), suggesting that there is a system responsible for induction of PPDK in rice (a $C_3$ plant) which is similar to that found in maize (a $C_4$ plant).

**Rice Nuclear Protein(s) Bind the PPDK Promoter.** Our studies of transgenic rice plants indicate that the regulation of PPDK-GUS expression in rice is very similar to the regulation of PPDK expression in maize. Therefore, there must be a system which regulates PPDK in rice which is similar to that in maize. Previous studies of PPDK gene expression have indicated that the region from −327 to −1 is sufficient for transient expression of the PPDK gene in maize leaves (16). We analyzed rice nuclei for the presence of proteins which specifically bind this region of the PPDK promoter. Similar patterns of retarded mobility were observed after this fragment was incubated with nuclear extracts of green rice and maize leaves (Fig. 4*A*). This result indicates that rice leaves contain nuclear proteins which have binding properties similar to those found in maize leaves with regard to the 5′-flanking region of the PPDK gene.

The region between −316 and −289 has been shown to be essential for the expression of PPDK in maize leaves, and a maize nuclear protein, PPD-1, which binds this sequence

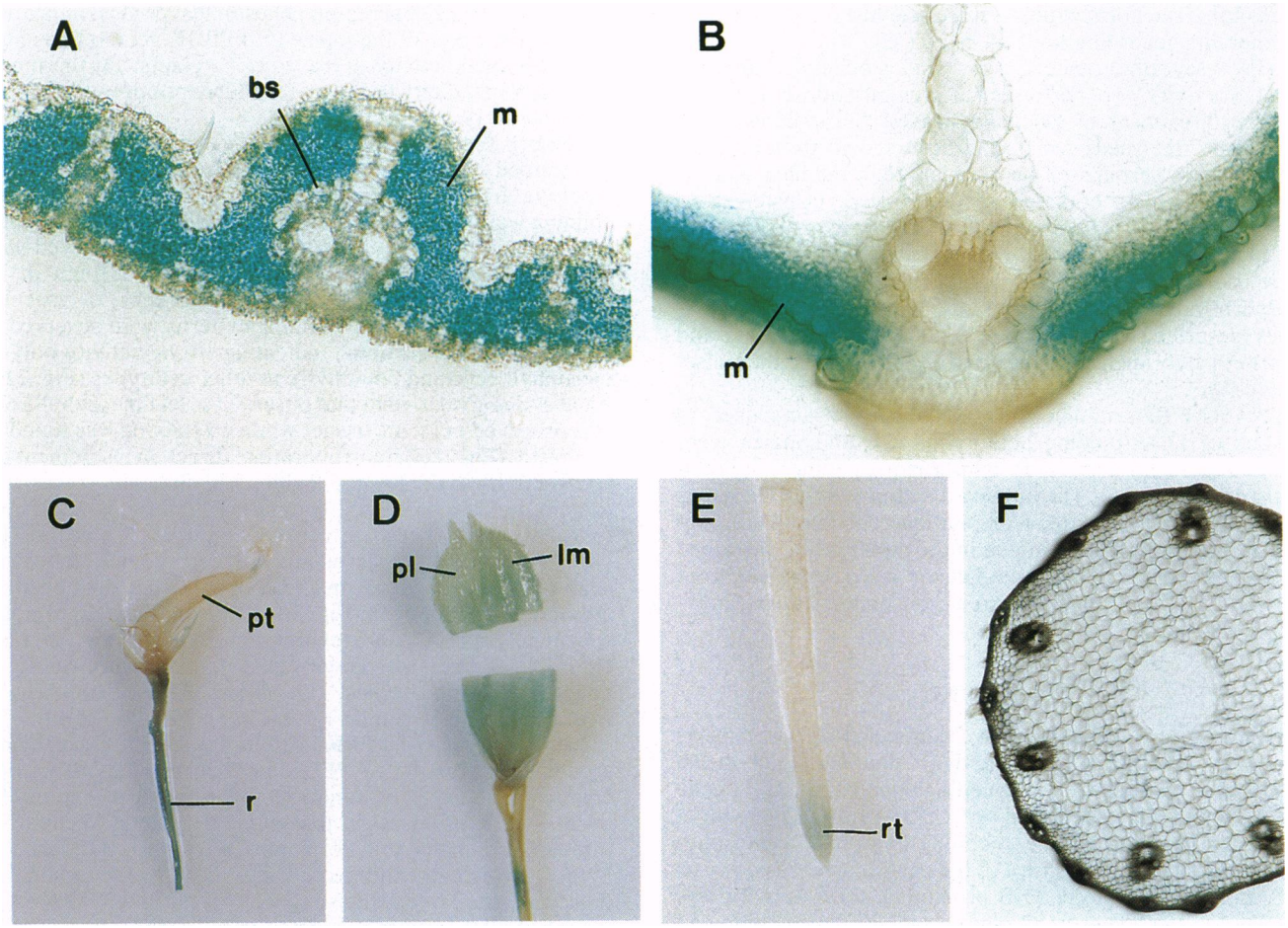


FIG. 2. *In situ* analysis of GUS activity (blue) in transgenic rice plants. (*A* and *B*) Cross sections of leaf blade (*A*) and leaf sheath (*B*) stained for GUS activity. m, Mesophyll cells; bs, bundle sheath cells. (×200.) (*C* and *D*) Floral tissues stained for GUS activity. pt, Pistil; r, rachis; pl, palea; and lm, lemma. (*E*) Root tissues stained for GUS activity. rt, Root tip. (*F*) Cross section of the stem. (×50.)

specifically has been identified (16). The mobility of this region was retarded after incubation with the rice extract in the same manner as that observed after incubation with maize nuclear extracts, and the binding specificity of the rice nuclear extract is identical to that of PPD-1 in maize (Fig. 4*B*), indicating that green rice leaves contain a similar or identical factor involved in light-regulated expression of the genes involved in $C_4$ photosynthesis.

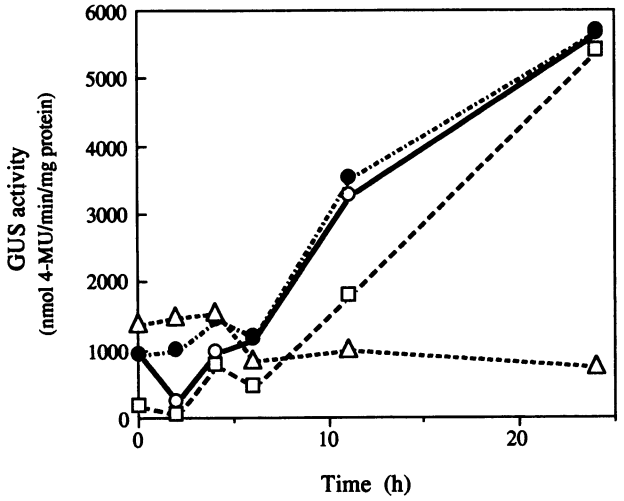


FIG. 3. Light induction of GUS activity during the greening of etiolated rice seedlings. Seedlings were grown in the dark for 2 weeks and subjected to light. GUS activities in greening leaves of the transformants (PPDK-GUS 1, ○; PPDK-GUS 2, ●; PPDK-GUS 7, □; and 35S-GUS, △) were measured at the indicated times after exposure to illumination began. 4-MU, 4-methylumbelliferone.

## DISCUSSION

We have demonstrated that the promoter region of a maize PPDK gene directs high-level expression of a chimeric gene (PPDK-GUS) in transgenic rice plants. Comparison with expression of a 35S-GUS construct indicates that GUS expression in transgenic rice is cell specific when directed by the maize PPDK promoter region. This expression is also light regulated in rice (a $C_3$ plant) in a manner similar to the light-regulated PPDK expression observed in maize (a $C_4$ plant). Thus, $C_3$ plants have a regulatory system which is able to direct cell-specific and light-regulated expression of a "$C_4$-type" gene. Furthermore, characterization of this regulatory system indicates that it is similar to that which directs PPDK expression in maize. This notion is confirmed by the identification of rice nuclear proteins which bind cis-acting elements of the PPDK gene and are similar in binding specificity to a nuclear factor which has been identified in maize, PPD-1.

High levels of PPDK activity are well documented in $C_4$ plants but have been difficult to observe in $C_3$ plants. A PPDK gene has been identified in the rice genome and has been shown to have a structure similar to that of the maize gene (9, 10). Extensive homology between these genes was observed not only in the coding regions but also in the exon–intron

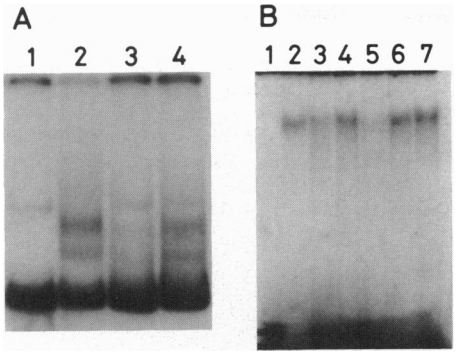


FIG. 4. Assay of binding between a rice nuclear extract and the promoter region of the maize PPDK gene. (*A*) $^{32}$P-labeled DNA fragment extending from −327 to −1 of the maize PPDK gene was incubated in the absence (lane 1) or presence (lanes 2–4) of nuclear extract prepared from green maize leaves (lane 2, 2.5 mg of protein) or from green rice leaves (lanes 3 and 4, 1 and 2.5 mg of protein, respectively). (*B*) An oligonucleotide extending from −316 to −298 of the 5′-flanking region of the maize PPDK gene was used as a probe. The oligonucleotide probe was incubated in the absence (lane 1) or presence (lanes 2–7) of nuclear extracts from green leaves of maize (lane 2, 3 mg of protein) or rice (lane 3, 1 mg of protein; lanes 4–7, 3 mg of protein). A 100-fold molar excess of unlabeled oligonucleotides was added to the reaction mixtures in lanes 5–7. These consisted of a fragment identical to the probe (lane 5), the sequence extending from −253 to −222 of the maize PPDK promoter region which correspond to a long direct repeat (lane 6) (18), and a fragment containing a cis-acting element of another $C_4$ gene, phospho*enol*pyruvate carboxylase (19).

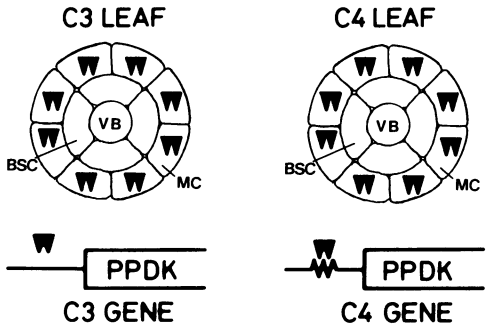


FIG. 5. Model accounting for the difference in the expression between the maize and rice PPDK genes. The $C_4$ plant, maize, has a gene which is essential to conduct $C_4$ photosynthesis. The $C_3$ plant, rice, has a gene homologous to the $C_4$-type PPDK gene which is expressed only at very low levels in rice leaves. The PPDK promoter of maize directs high expression of PPDK in leaf mesophyll cells of maize and of GUS in leaf mesophyll cells of rice. This suggests that leaf mesophyll cells of both species have a trans-acting factor(s) (W) which interacts with a cis-acting element in the maize PPDK gene (-W-). The interaction of such cis and trans factors is necessary for high expression of the gene (16). The simplest explanation for the low level of expression under these circumstances is that the rice gene does not contain the cis-acting element(s) necessary for high levels of PPDK expression. MC and BSC indicate mesophyll and bundle sheath cells, respectively; VB, vascular bundle.

relationships (10). Another study demonstrated that PPDK expression in green rice leaves occurs at very low levels (9). Thus, difficulty in detecting PPDK activity in $C_3$ plants was undoubtedly due to low expression of the gene.

Rice has a PPDK gene and a regulatory system which is able to direct high levels of cell-specific expression from the maize PPDK promoter. The question then arises as to what factor or factors determine the dramatic difference in PPDK expression between rice and maize. Evidence from genetic studies of gene regulation conducted long before the advent of modern molecular technology resulted in the concept of cis and trans regulators of gene expression, which have since been confirmed by molecular techniques. We have clearly demonstrated that trans elements required for cell-specific light-regulated PPDK expression are present in rice. Therefore, we must assume that although the structure of the coding regions of the rice and maize PPDK genes are very similar, there are probably different regulatory elements in the 5′-flanking regions of these genes which are responsible for the observed differences in expression (Fig. 5). The rice PPDK gene is expressed in leaf tissue at low levels and its expression is induced by light in a similar manner to the maize gene (9), providing further evidence that the regulatory elements involved in the light regulation and tissue specificity of PPDK gene expression are shared between $C_3$ and $C_4$ species. Thus, the difference between these genes may be only the presence or absence of a cis element(s) which determines the amount of expression rather than the tissue specificity or light responsiveness of the expression. Further analysis of the promoter activity of the rice gene will clarify this issue.

Differences in gene expression resulting from lack of or alterations in cis-acting elements have been observed in other organisms such as *Drosophila* (20), and alterations in cis-element structure are thought to play a role in the evolutionary variation of gene expression. All of the available evidence impinging upon the evolution of the $C_4$ plants indicates that they have arisen from $C_3$ plants and that this transition has occurred polyphyletically during the course of evolution (21–23). PPDK is one of the essential components of the $C_4$ pathway. As such, the development of the $C_4$-type PPDK gene is thought to be one of the primary events in establishing the ability to conduct $C_4$ photosynthesis. Our findings indicate that the transition from expressing PPDK at low levels to high levels may require only the acquisition of an enhancer element in the 5′-flanking region of the PPDK gene. Thus, the genetic transition required to establish an essential component of $C_4$ photosynthetic gene expression is much smaller than previously thought.

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