Differential Expression Pattern of C₄ Bundle Sheath Expression Genes in Rice, a C₃ Plant

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NADP-malic enzyme (NADP-ME) and phosphoenolpyruvate carboxykinase (PCK) are specifically expressed in bundle sheath cells (BSCs) in NADP-ME-type and PCK-type C₄ plants, respectively. Unlike the high activities of these enzymes in the green leaves of C₄ plants, their low activities have been detected in the leaves of C₃ plants. In order to elucidate the differences in the gene expression system between C₃ and C₄ plants, we have produced chimeric constructs with the β-glucuronidase (GUS) reporter gene under the control of the maize NADP-Me (ZmMe) or Zosia japonica Pck (ZjPck) promoter and introduced these constructs into rice. In leaves of transgenic rice, the ZmMe promoter directed GUS expression not only in mesophyll cells (MCs) but also in BSCs and vascular cells, whereas the ZjPck promoter directed GUS expression only in BSCs and vascular cells. Neither the ZjPck nor ZmMe promoters induced GUS expression due to light. In rice leaves, the endogenous NADP-Me (OsMe1) was expressed in MCs, BSCs and vascular cells, whereas the rice Pck (OsPck1) was expressed only in BSCs and vascular cells. Taken together, the results obtained from transgenic rice demonstrate that the expression pattern of ZmMe or ZjPck in transgenic rice was reflected by that of its counterpart gene in rice.

Keywords: C₄ plant — NADP-malic enzyme — Phosphoenolpyruvate carboxykinase — Promoter analysis — Rice.

Abbreviations: BSC, bundle sheath cell; MC, mesophyll cell; ME, malic enzyme; PCK, phosphoenolpyruvate carboxykinase; RT-PCR, reverse transcription–Polymerase chain reaction.

Introduction

C₄ plants can be classified into three groups, such as NADP-malic enzyme (NADP-ME), NAD-malic enzyme (NAD-ME) and phosphoenolpyruvate carboxykinase (PCK) types according to the major decarboxylase enzyme involved in the decarboxylation of C₄ acids in bundle sheath cells (BSCs) (Gutierrez et al. 1974, Hatch et al. 1975). The mesophyll cells (MCs) and BSCs, which are distinct anatomically and biochemically, of C₄ photosynthetic tissues act in tandem to concentrate atmospheric CO₂ into BSCs. In BSCs, NAD-ME, NADP-ME or PCK is needed to release CO₂ from C₄ acid for refixation. PCK in C₄ PCK-type plants is located in the cytosol of BSCs and decarboxylates the oxaloacetate via the following reaction:

\[
\text{Oxaloacetate} + \text{ATP} \rightarrow \text{PEP} + \text{ADP} + \text{CO}_2.
\]

Although this reaction is freely reversible in vitro, in higher plants it is likely that the carboxylation reaction is negligible under physiological conditions because of the low affinity of the enzyme for CO₂ (Ray and Black 1976, Urbina and Avilán 1989). On the other hand, NADP-Me acts in a wide range of metabolic pathways in plants and is not limited to C₄ metabolism; the enzyme catalyzes the oxidative decarboxylation of malate to pyruvate:

\[
\text{Malate} + \text{NADP}^+ + \text{pyruvate} + \text{CO}_2 + \text{NADPH} + \text{H}^+.
\]

Genes encoding C₄ enzymes are believed to have evolved by gene duplication from genes encoding non-photosynthetic isoformic forms (Sheen 1999). Thus, these C₄-type genes had gained some specific characteristics including high level expression in one cell type. Acquisition of new cis-regulatory elements for high level expression is therefore a key aspect of C₄ gene evolution (Ku et al. 1996). Furthermore, novel regulatory mechanism(s) had to be acquired for the cell- and organ-specific expression of the ancestral genes, along with the development of Kranz anatomy, which is imperative for the functioning of C₄ photosynthesis. In several studies, attempts have been made to elucidate the molecular basis of cell-specific expression of various genes required in C₄ photosynthesis. However, most of the previously studied genes are those encoding MC-located enzymes, phosphoenolpyruvate carboxylase (Ppc) or pyruvate, orthophosphate dikinase (Pdk) (Matsuoka et al. 1993, Matsuoka et al. 1994, Stockhaus et al. 1997). Only Flaveria bidentis NADP-Me, whose enzyme protein was located in BSCs, was studied in terms of its cis-regulatory elements for high level, BSC-specific expression (Marshall et al. 1996, Marshall et al. 1997). It was found that

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using a transgenic C₄ plant, *F. bidentis*, both 5′-upstream and 3′-downstream regions of the *Me* gene regulate to confer the expected expression pattern.

In this study, we have focused on the C₄ photosynthetic genes expressed specifically in BSCs, such as the *Pck* and *NADP-Me* genes, in order to examine the mechanisms for gene development during the evolution from C₃ to C₄ plants. To test whether the regulatory elements are present in the 5′ regions of C₄-type *Pck* and *NADP-Me* genes, we have directly compared the transcriptional activity of the promoter for *Pck* in *Zoysia japonica* (*ZjPck*) with that of the promoter for *NADP-Me* from maize (*ZmMe*) in transgenic rice, a C₃ plant.

**Results**

**Construction of chimeric *ZmMe*-GUS and *ZjPck*-GUS genes**

To examine the transcriptional activity of the promoters of *ZjPck* and *ZmMe* in a C₃ plant, rice, both of which are preferentially expressed in the BSCs of C₄ plants, we made two chimeric constructs with their promoters and a β-glucuronidase (*GUS*) reporter gene. For *PCK-GUS*, the sequence from –1,447 to +227 (relative to the transcription initiation site) of the *ZjPck* gene, consisting of the 5′-flanking region, exon 1 (containing only 5′-non-coding sequence), intron 1 and a partial sequence of exon 2, was used as a promoter (Fig. 1A). For *ME-GUS*, the sequence from –1,032 to +94 (relative to the transcription initiation site) of the *ZmMe* gene, consisting of the 5′-flanking region and 5′-non-coding sequence, was used as the promoter (Fig. 1B). The 3′-end site of each promoter sequence corresponded to the region just in front of the first ATG; consequently, the promoter sequences contained the entire 5′-non-coding sequences for the transcripts. Since the *ME-GUS* promoter did not induce *GUS* expression (see below), we also made another chimeric construct, *MEint-GUS*, under the control of the sequence from –1,032 to +463 of *ZmMe* (Fig. 1C). The promoter sequence for *MEint-GUS* contained the 5′-flanking region, exon 1, intron 1 and the partial coding sequence of exon 2 from *ZmMe*. We also introduced *ME-GUS* into maize to examine the expression pattern of this chimeric construct in a C₄ plant. We usually used T1 plants of transgenic rice to analyze *GUS* expression, with the exception of the light induction experiments, for which we used T3 seedlings. We always confirmed that *GUS* expression was inherited by the next generation with the co-segregation of the selection marker (data not shown).

**Expression of GUS activity under the control of the *ZjPck* promoter in transgenic rice**

The GUS activities controlled by the *ZjPck* promoter in various organs of mature transgenic rice plants were determined fluorometrically (Table 1). The GUS activity was observed in all organs we tested. The levels of GUS activity in leaves of *PCK-GUS* transformants were similar to that of the 35S-*GUS* transformant (Tada et al. 1991) and much lower than that of the maize *PPDK-GUS* transformant (Nomura et al. 2000). The cell specificity of GUS expression driven by the

<table>
<thead>
<tr>
<th>GUS activity [pmol of 4-MU min⁻¹ (mg protein)⁻¹]</th>
<th>Leaf blade</th>
<th>Leaf sheath</th>
<th>Stem</th>
<th>Root</th>
<th>Glume</th>
</tr>
</thead>
<tbody>
<tr>
<td>PCK-GUS31</td>
<td>1,154</td>
<td>766</td>
<td>236</td>
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<tr>
<td>35S-GUS</td>
<td>5,645</td>
<td>1,282</td>
<td>1,265</td>
<td>634</td>
<td>NT</td>
</tr>
<tr>
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<td>115,264</td>
<td>139,496</td>
<td>377</td>
<td>NT</td>
<td>378</td>
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<td>Non-transformant</td>
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<td>26</td>
<td>30</td>
<td>333</td>
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4-MU, 4-methylumbelliferone; NT, not tested; 35S, cauliflower mosaic virus 35S promoter; maize PPDK, maize *Pdk* promoter (Nomura et al. 2000).
ZjPck promoter was also determined by analyzing the in situ histochemical staining patterns in transgenic rice plants (Fig. 2A–C). In leaf cross-sections of transgenic rice plants, strong GUS expression was observed in BSCs and vascular cells, whereas no activity was seen in MCs (Fig. 2A, B). In the root, GUS staining was also limited to vascular cells (Fig. 2C). These results demonstrate that the 5'-flanking sequence, including the 5'-non-coding region, of ZjPck can drive the expression of the reporter gene in a cell-preferential manner, even in rice, a C₃ plant.

Expression of GUS under the control of the ZmMe promoter in transgenic rice and maize

We also examined GUS activity that was controlled by the 5'-flanking sequence of the ZmMe gene in a similar way to ZjPck. Surprisingly, when we used the ME-GUS construct, the chimeric gene was not expressed in transgenic rice at all and the GUS activity in all organs of transgenic plants we tested was almost the same as that in the non-transgenic rice plants (Tables 1, 2). This finding regarding GUS activity was not caused by failure to introduce the chimeric gene, since genomic Southern blot analysis revealed that almost all transgenic plants contained the entire region of the ME-GUS sequence (data not shown). We wondered if the 5'-flanking sequence we used perhaps did not contain the essential element(s) for expression in C₃ plants. Thus, we reconstructed a chimeric gene that contained the 5'-flanking region plus the entire sequences of exon 1 and intron 1 and a partial sequence of exon 2 (MEint-GUS, Fig. 1C). A high level of GUS activity was then observed in all organs that we tested (Table 2). Preferential expression was not seen in various organs and high GUS activity was not limited to photosynthetic organs but was also found in the root and glume. Such non-preferential expression of GUS was confirmed by in situ histochemical analysis (Fig. 2D–F). In the cross-sections of the leaf, strong blue staining was seen not only in MCs but also in the epidermis, BSCs and vascular cells.

Table 2  GUS activity under the Zmme promoter in various organs of transgenic rice plants

<table>
<thead>
<tr>
<th></th>
<th>Leaf blade</th>
<th>Leaf sheath</th>
<th>Stem</th>
<th>Root</th>
<th>Glume</th>
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<td>23,711</td>
<td>25,694</td>
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Pck and NADP-Me expression in transgenic rice

(Fig. 2D, E). Similarly, GUS activity was observed in the root (Fig. 2F). The pattern of MEint-GUS expression in transgenic rice did not mimic that of ZmMe in wild-type maize, which is expressed in a photosynthetic organ-specific and BSC-specific manner (Wedding 1989), even though the chimeric gene in rice was expressed at a high level, similar to ZmMe in maize.

We also introduced the ME-GUS construct into maize, the original plant of the ZmMe gene. In contrast to the non-expression of ME-GUS in transgenic rice, the same construct was specifically expressed in BSCs, identically to the endogenous ZmMe gene (Fig. 3A, B). This indicates that the promoter (−1,032 to +94) used for ME-GUS is sufficient for the BSC-specific expression in the original plant, maize (C₄ plant), but does not actively function at all in rice (C₃ plant).

Fig. 3  Histochemical localization of GUS activity in the transgenic maize plant. Cross-section (A) and surface (B) of the leaf blade from a maize plant transformed with the ME-GUS construct. Scale bars = 0.05 mm (A), and 0.02 mm (B).

Light dependence of the expression of PCK-GUS and MEint-GUS in transgenic rice

The expression of Pck and Me genes in C₄ plants is induced by light (Marshall et al. 1996, Finnegan et al. 1999, Tausta et al. 2002, Murmu et al. 2003). To determine whether the expression of these genes, ZjPck and ZmMe, is also regu-

Fig. 4  Light induction of GUS expression in etiolated transformed rice seedlings during the greening process. Seedlings were grown in the dark for 2 weeks and transferred to light. GUS activities in greening leaves of transformed rice plants carrying the PCK-GUS (A) and MEint-GUS (B) were measured by the formation of 4-methylumbelliferone (4-MU) at the indicated time after being transferred to light. The GUS activity controlled by the 35S promoter was stable at low levels during the light treatment. The results were obtained from three or four independent lines.
lated by light in rice, we analyzed the GUS activity driven by these promoters in transgenic rice plants under different light conditions (Fig. 4). T3 seeds, which were obtained by self-pollination of T2 plants carrying the transgenes in a homozygous manner, were germinated and grown in the dark for 2 weeks and then exposed to light. The second true leaves of 10 seedlings from each transformed line were assayed for GUS activity to normalize for individual differences. Although the levels of GUS activity were different in each line, the expression of PCK-GUS and MEint-GUS was not induced by light at all in any transformants tested and the GUS activity was almost at the same level during the greening process (Fig. 4A, B). Exposure to light did not induce GUS expression in seedlings carrying the 35S-GUS construct (Fig. 4B, Tada et al. 1991). These results indicated that neither the ZjPck promoter nor the ZmMe promoter retained the light-dependent expression in rice.

Expression of endogenous OsPck1 and OsMe1 genes in rice

We suspected that the reason why the ZjPck and ZmMe promoters did not retain their photosynthetic organ-specific and light-dependent expression in transgenic rice, in spite of retaining BSC-preferential expression of ZjPck, may be reflected by the difference in the expression pattern between endogenous rice Pck and Me genes, i.e. both endogenous genes are expressed nearly constitutively, but rice Pck is expressed preferentially in BSCs in the leaf and rice Me is not. To elucidate this possibility, we examined the expression pattern of their counterpart genes in rice. Based on the BLAST search using the whole genome sequence of rice (http://rgp.dna.affrc.go.jp), we found two kinds of endogenous rice Pck and rice Me homologous sequences in the rice genome. Phylogenetic comparison among these rice homologous genes and the ZjPck or ZmMe gene revealed that each rice homologous gene was most closely related to the ZjPck or ZmMe gene and therefore we predicted that these genes (endogenous rice Pck, accession no. AK102392, OsPck1; endogenous rice Me, accession no. D16499, OsMe1) are the counterparts of C4-type Pck and NADP- Me (Fushimi et al. 1994, Chi et al. 2004). The preferential expression patterns of both rice counterpart genes were not observed in the various organs of rice by reverse transcription-polymerase chain reaction (RT–PCR) analysis (Fig. 5). We also compared the expression patterns of these genes in rice leaves by in situ hybridization (Fig. 6). The expression of OsPck1 was observed mainly in BSCs and vascular cells, and also in epidermal cells (Fig. 6A), whereas that of OsMe1 was seen in almost

![Fig. 5](https://academic.oup.com/pcp/article-abstract/46/5/754/1841595)

Expression of the endogenous OsPck1 and OsMe1 genes in various rice organs. Total RNAs (5 µg) from various organs were reverse-transcribed with the oligo(dT) primer. The synthesized cDNAs were amplified with the specific primers described in Materials and Methods. LB, leaf blade; LS, leaf sheath; S, stem; R, root. Actin1 was used as a control.

![Fig. 6](https://academic.oup.com/pcp/article-abstract/46/5/754/1841595)

In situ hybridization analysis for the expression of OsPck1 and OsMe1 genes in rice leaves. Cross-sections of rice leaves were hybridized with antisense probes for OsPck1 (A) or OsMe1 (B), respectively. No hybridization signal was seen with the sense probes for OsPck1 (C) or OsMe1 (D), respectively. Scale bars = 0.1 mm.
Illumination time (h)

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<th>8</th>
<th>12</th>
<th>24</th>
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<tbody>
<tr>
<td>OsPck1</td>
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<td>Actin1</td>
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</table>

Fig. 7 Changes in the mRNA level of OsPck1 and OsMe1 genes in leaves of rice seedlings during illumination. Total RNAs (5 μg) from leaves were reverse-transcribed with oligo(dT) primer as described in Materials and Methods. The cDNAs were amplified by 20 cycles of PCR with specific primers, electrophoresed on an agarose gel and transferred to a nylon membrane. The amplified cDNAs on the membranes were hybridized with the fluorescein-labeled 3′-non-coding region of OsPck1, the 3′-non-coding region of OsMe1 and rice actin1 cDNA. Actin1 was used as a control.

all cells, i.e. MCs, BSCs and vascular cells (Fig. 6B). This indicates that the expression of the ZjPck and ZmMe genes in rice mimics that of the rice orthologous genes.

When we also examined the light induction of the expression for these endogenous genes in rice (Fig. 7), neither the expression of OsPck1 nor that of OsMe1 in leaves was induced by light. Taken together, the results of comparative analyses between ZjPck (or ZmMe) and its counterpart gene show that the expression pattern of the ZjPck (or ZmMe) gene is reflected by that of its counterpart gene in rice, and therefore some cis-acting elements for organ specificity and light induction may be conserved between ZjPck (or ZmMe) and OsPck1 (or OsMe1).

Discussion

Previously, we showed that the maize Pdk or Ppc promoter can drive GUS activity specifically in MCs in a light-dependent manner in transgenic rice plants (Matsuoka et al. 1993, Matsuoka et al. 1994, Nomura et al. 2000). Based on these results, we discussed the possibility that the genetic alterations required to give rise to the system for the cell-specific expression in C4 plants may be more limited than previously thought. We also inferred that C4-type Pck or NADP-Me might be preferentially expressed in BSCs in C3 plants. This study revealed that the previous hypothesis cannot extend to the BSC-specific genes tested here.

The GUS activity driven by the ZjPck promoter was preferentially localized to the vascular cells and BSCs where the rice counterpart OsPck1 expression was localized (Figs. 2A, B, 6A). When we used the ZmMe promoter containing the 5′-flanking sequence plus the entire sequences of exon 1 and intron 1 and a partial sequence of exon 2, a very high level of GUS activity was observed in all organs we tested, which was similar to the expression pattern of the rice counterpart OsMe1 (Figs. 2D, E, 6B). In many organisms, a significantly higher level of gene expression is observed from intron-containing transgenes than from otherwise identical intronless constructs (Callis et al. 1987, Buchman and Berg 1988, Choi et al. 1991, Duncker et al. 1997). While specific regulatory elements are found within some introns, a more general intron-mediated enhancement is thought to result from synergistic interactions between the factors involved in the various steps of gene expression from transcription to translation (Maniatis and Reed 2002, Le Hir et al. 2003).

Neither the ZjPck nor ZmMe promoter drove GUS expression in a light-dependent manner (Fig. 4). We thought the reason why the ZjPck promoter induced GUS expression in BSCs and the ZmMe promoter induced it in both MCs and BSCs in transgenic rice might be reflected by the difference in the expression pattern between endogenous rice Pck and Me genes. As we expected, the expression patterns of these transgenes were essentially the same as those of their counterpart genes in rice; the rice endogenous Pck gene was expressed in BSCs whereas the rice endogenous Me gene was expressed in MCs, BSCs and vascular bundle cells. Similar results have also been observed for the Panicum miliaceum aspartate aminotransferase gene (Aat) (Nomura et al. 2005). The expression pattern of the P. miliaceum mitochondrial Aat in transgenic rice, which is specifically expressed in BSCs of P. miliaceum, mimics that of its counterpart gene in rice. These data imply the evolution of BSC expression element(s) by modifying rudimentary progenitor elements that are already present in the C3 promoter.

In contrast to the presence of some consensus element, C4-type BSC-specific genes may contain specific cis-acting elements for BSC-specific expression that are only effective in the C4 plant. When we used the ZmMe promoter containing the 5′-flanking sequence lacking intron 1 and exon 2, no GUS activity was seen in any organs in transgenic rice (Table 2). Loss of the specific expression under the control of the maize promoter does not mean that the maize promoter does not contain transcriptional elements in its sequence. Indeed, the same promoter could specifically drive the GUS activity in BSCs in transgenic maize in the same manner as the maize endogenous gene (Fig. 3). These data suggest that cis-acting elements for BSC-specific expression in the maize NADP-Me gene might be added in the region from −1,032 to +94. This is consistent with the observation that BSC-specific expression of the NADP-Me gene in F. bidentis depends on a 5′-flanking region (Marshall et al. 1997). This consideration leads us to speculate that the gain of the cis-acting elements for BSC-specific expression had to
occur in the region from −1,032 to +94 bp. It might be a novel component for \( \text{NADP-Me} \) gene suppression in MCs. This model can explain why the rice promoter drives \( GUS \) expression in both MCs and BSCs, i.e. the pre-existing machinery works both in MCs and BSCs, while the rice promoter does not contain \( \text{cis} \)-elements responding to the novel components for its suppression.

In connection with this matter, both the \( \text{C}_4 \)-type promoter of the small subunit of the ribulose bisphosphate carboxylase gene (\( \text{RbcS} \)) from maize and the \( \text{C}_4 \)-type \( \text{RbcS} \) promoter from rice fused to the \( GUS \) reporter gene were expressed in MCs in transgenic rice (Kyozuka et al. 1993, Matsuoka et al. 1994). This implies that the expression pattern of the maize \( \text{RbcS} \) gene in transgenic rice also mimics that of its counterpart gene in rice. Schäffner and Sheen (1991) had suggested that the addition of the sequence of the maize \( \text{RbcS} \) promoter inhibited the expression of a reporter gene in maize mesophyll protoplast. They discussed that this upstream silencer of the maize \( \text{RbcS} \) gene might be important for MC-specific repression. Therefore, it might be possible that this upstream silencer is a \( \text{cis} \)-acting element essential for the establishment of the \( \text{C}_4 \)-type BSC-specific genes. Similar results have been found in \( \text{C}_4 \)-type MC-specific genes (Nomura et al. 2000). In the case of the \( \text{Pdk} \) gene, both maize \( \text{C}_4 \)-type \( \text{Pdk} \) and rice \( \text{C}_4 \)-like \( \text{Pdk} \) promoter induced \( GUS \) expression in MCs of rice. However, the promoter of the rice \( \text{C}_4 \)-like \( \text{Pdk} \) drove the expression of the reporter gene both in MCs and in BSCs of maize, while the maize \( \text{C}_4 \)-type \( \text{Pdk} \) promoter preferentially induced its expression in maize MCs. Therefore, the gain of \( \text{cis} \)-acting element(s) conferring the MC-specific expression was necessary for establishment of a \( \text{C}_4 \)-type \( \text{Pdk} \) gene.

Although we have not studied the expression profiles of rice counterpart genes of \( \text{Pck} \) and \( \text{NADP-Me} \), respectively, in transgenic \( \text{C}_4 \) plants, these data suggest that the \( \text{C}_4 \) promoter contains specific \( \text{cis} \)-acting element(s) for BSC-specific expression which might not be present in the rice promoter and might only be effective in \( \text{C}_4 \) plants. To date, it is not known which types of \( \text{cis} \)-and \( \text{trans} \)-regulatory elements constitute BSC expression modules at the molecular level and how regulatory networks for BSC-specific gene expression have evolved. Analysis of chimeric promoter–reporter genes in transgenic \( \text{F. bidentis} \) identified two sequences in the \( \text{S}\text{-flanking region of the Ppc} \) gene of the \( \text{C}_4 \) plant \( \text{F. trineria} \) that are necessary and sufficient for the MC-specific expression (Gowik et al. 2004).

We assume that the genetic transition required to establish a \( \text{C}_4 \)-type photosynthetic gene expression pattern was not complex (Matsuoka et al. 1993, Matsuoka et al. 1994). The comparatively small changes in the nucleotide sequence should be responsible for these changes that give rise to a novel model of expression. The identification of \( \text{C}_4 \)-specific components is important for a full understanding of how the \( \text{C}_4 \)-specific gene expression system has been established in the course of evolution from \( \text{C}_3 \) to \( \text{C}_4 \) plants.

**Materials and Methods**

**Construction of chimeric genes and transformations of rice and maize**

For the construction of PCK-GUS, the \( \text{S}\text{-flanking sequence containing the first intron} \), \( −1,447 \) to +227 of \( \text{ZjPck} \) inserted in pBlueScript II SK(−) (Stratagene, La Jolla, CA, USA) was digested with \( \text{Nae I} \) and blunted by T4 DNA polymerase (Takara Bio Inc, Otsu, Shiga, Japan), followed by digestion with \( \text{Xba I} \). The resulting fragment, \( −1,447 \) to +227 of the \( \text{ZjPck} \) promoter was inserted into pBI-Hm (kindly provided by Dr. Kenzo Nakamura at Nagoya University) digested with \( \text{Xba I} \) and \( \text{Sma I} \). The \( \text{ZmMe} \) gene, both maize \( \text{C}_4 \)-type \( \text{Pdk} \) and rice \( \text{C}_4 \)-like \( \text{Pdk} \) promoter induced \( GUS \) expression in MCs of rice. However, the promoter of the rice \( \text{C}_4 \)-like \( \text{Pdk} \) drove the expression of the reporter gene both in MCs and in BSCs of maize, while the maize \( \text{C}_4 \)-type \( \text{Pdk} \) promoter preferentially induced its expression in maize MCs. Therefore, the gain of \( \text{cis} \)-acting element(s) conferring the MC-specific expression was necessary for establishment of a \( \text{C}_4 \)-type \( \text{Pdk} \) gene.

Although we have not studied the expression profiles of rice counterpart genes of \( \text{Pck} \) and \( \text{NADP-Me} \), respectively, in transgenic \( \text{C}_4 \) plants, these data suggest that the \( \text{C}_4 \) promoter contains specific \( \text{cis} \)-acting element(s) for BSC-specific expression which might not be present in the rice promoter and might only be effective in \( \text{C}_4 \) plants. To date, it is not known which types of \( \text{cis} \)-and \( \text{trans} \)-regulatory elements constitute BSC expression modules at the molecular level and how regulatory networks for BSC-specific gene expression have evolved. Analysis of chimeric promoter–reporter genes in transgenic \( \text{F. bidentis} \) identified two sequences in the \( \text{S}\text{-flanking region of the Ppc} \) gene of the \( \text{C}_4 \) plant \( \text{F. trineria} \) that are necessary and sufficient for the MC-specific expression (Gowik et al. 2004).

We assume that the genetic transition required to establish a \( \text{C}_4 \)-type photosynthetic gene expression pattern was not complex (Matsuoka et al. 1993, Matsuoka et al. 1994). The comparatively small changes in the nucleotide sequence should be responsible for these changes that give rise to a novel model of expression. The identification of \( \text{C}_4 \)-specific components is important for a full understanding of how the \( \text{C}_4 \)-specific gene expression system has been established in the course of evolution from \( \text{C}_3 \) to \( \text{C}_4 \) plants.

**GUS assay**

Fluorometric assays for quantitative measurement and histochemical analysis of \( \text{GUS} \) activity were performed as previously described (Matsuoka and Sanada 1991). The histochemical \( \text{GUS} \) staining was performed for several hours for rice and maize transformants.

**Light treatment of etiolated transgenic rice plants**

T3 transgenic rice seeds from self-pollinated transformants were germinated and grown in vermiculite under darkness at 25°C for 2 weeks. The etiolated seedlings were transferred to continuous white light (approximately 100 µmol photons m\(^{-2}\) s\(^{-1}\)). Secondary leaves from 10 seedlings were used for \( \text{GUS} \) assays.

**In situ hybridization**

Plant materials were fixed with 4% (w/v) paraformaldehyde and 0.25% (v/v) glutaraldehyde in 0.1 M sodium phosphate buffer, pH 7.4, and embedded in Paraplast Plus (Oxford Labware, St Louis, MO, USA). Microtome sections (10 mm thick) were applied to glass slides treated with Vectabond (Vector Lab, Burlingame, CA, USA). In situ hybridization with digoxigenin-labeled sense or antisense RNA was conducted according to the method of Kouchi and Hata (1993). The
RNA probe was prepared from the 3'-untranslated region of OsPck1 or OsMe1 cDNA from rice.

RT-PCR analysis

Total RNAs (5 μg) from various organs were reverse-transcribed by Superscript II RNaseH- reverse transcriptase (Invitrogen, Carlsbad, CA, USA) with oligo(dT)20-28 primers. The synthesized cDNA was amplified in 20 cycles of PCR (94°C, 30 s; 55°C, 1 min; 72°C, 1 min). The sequences of oligonucleotide primers for PCR were as follows. OsPck1Fw, 5'-GTGACTGAGAAAACTTAT-3' and OsPck1Rv, 5'-GATCCTAACTAGTGCCTTC-3' for OsPck1 (accession no. AK102392); OsMe1Fw, 5'-CGCCGGGCGGTCATATTTT-3' and OsMe1Rv, 5'-ATGCTGCCCCATACATGTTG-3' for OsMe1 (accession no. D16499); and actin1Fw, 5'-TCTCCGTGGAGAA-GAGCTA-3' and actin1Rv, 5'-GCAATGGCGGGACATAGT-3' for actin1 (accession no. X16280; McElroy et al. 1990). The amplified cDNAs were electrophoresed on an agarose gel [1% (w/v)] and stained with ethidium bromide (Fig. 5). In Fig. 7, the amplified cDNAs after electrophoresis were transferred to nylon membranes and hybridized with the fluorescein-labeled 3'-non-coding region of OsPck1, OsMe1 or actin1 cDNA, respectively. Hybridization signals were detected by an anti-fluorescein–alkaline phosphatase conjugated antibody with CDP-Star as a substrate using a GeneImage Random-Prime Labeling and Detection System (Amersham Bioscience, Piscataway, NJ, USA).

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References


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