REVIEW ARTICLE

Molecular evolution and genetic engineering of C₄ photosynthetic enzymes

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Abstract

The majority of terrestrial plants, including many important crops such as rice, wheat, soybean, and potato, are classified as C₃ plants that assimilate atmospheric CO₂ directly through the C₃ photosynthetic pathway. C₄ plants, such as maize and sugarcane, evolved from C₃ plants, acquiring the C₄ photosynthetic pathway in addition to the C₃ pathway to achieve high photosynthetic performance and high water- and nitrogen-use efficiencies. Consequently, the transfer of C₄ traits to C₃ plants is one strategy being adopted for improving the photosynthetic performance of C₃ plants. The recent application of recombinant DNA technology has made considerable progress in the molecular engineering of photosynthetic genes in the past ten years. It has deepened understanding of the evolutionary scenario of the C₄ photosynthetic genes. The strategy, based on the evolutionary scenario, has enabled enzymes involved in the C₄ pathway to be expressed at high levels and in desired locations in the leaves of C₃ plants. Although overproduction of a single C₄ enzyme can alter the carbon metabolism of C₃ plants, it does not show any positive effects on photosynthesis. Transgenic C₃ plants overproducing multiple enzymes are now being produced for improving the photosynthetic performance of C₃ plants.

Key words: C₄ photosynthesis, gene evolution, phosphoenolpyruvate carboxylase, transgenic plants.

Introduction

Terrestrial plants are classified into three major photosynthetic types, namely, C₃, C₄ and Crassulacean acid metabolism (CAM) plants, according to the mechanism of their photosynthetic carbon assimilation. About 90% of terrestrial plant species, which include major crops such as rice (Oryza sativa), wheat (Triticum aestivum), soybean (Glycine max), and potato (Solanum tuberosum), are classified as C₃ plants, and they assimilate CO₂ directly through the C₃ photosynthetic pathway, also called the Calvin cycle or the photosynthetic carbon reduction (PCR) cycle. C₄ and CAM plants possess a unique photosynthetic pathway, in addition to the C₃ pathway, which allows them to adapt to specific environments. While C₃ plants grow well in temperate climates, CAM plants such as stonecrops and cactus adapt to extreme arid conditions, but their photosynthetic capacity is very low (Black, 1973). By contrast, C₄ plants such as maize (Zea mays) and sugarcane (Saccharum officinarum) adapt to high light, arid and warm environments and achieve higher photosynthetic capacity and higher water- and nitrogen-use efficiencies compared with C₃ plants (Black, 1973). Both C₄ and CAM plants evolved from ancestral C₃ species in response to changes in environmental conditions that caused a decrease in CO₂ availability. C₄ plants evolved in response to the low atmospheric CO₂ concentrations, while the CAM plants evolved either in response to the selection of increased water-use efficiency or for increased carbon gain (Ehleringer and Monson, 1993).
In leaves of C₃ plants, all of the photosynthetic reactions from the capture of solar light energy to assimilation of carbon into carbohydrates (triosephosphates) proceed in the chloroplasts of the mesophyll cells (Fig. 1A). The primary CO₂ fixation step in the C₃ pathway is catalysed by ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco). However, Rubisco also reacts with O₂ at its catalytic site (oxygenase reaction), leading to photorespiration. Photorespiration plays a role in protecting catalytic site (oxygenase reaction), leading to photore-
rate-limiting enzymes are strictly regulated in the leaves of C₄ plants. The activity of Rubisco is controlled by the Rubisco activase as it is in C₃ plants (Salvucci et al., 1987). Those of PEPC and PPDK are regulated through reversible protein phosphorylation by their specific regulatory proteins, being up-regulated in the light (Burnell and Hatch, 1985; Vidal and Chollet, 1997).

Since the discovery of the C₄ pathway, it has been postulated that the transfer of C₄ traits to C₃ plants should improve the photosynthetic performance of C₃ plants. Initially, conventional hybridization between C₃ and C₄ plants was carried out. This approach was available only in several plant genera and most C₃-C₄ hybrids were infertile (Brown and Bouton, 1993). Another approach that has been adopted in the last ten years involves the use of recombinant DNA technology. With this technology, understanding of the evolution of C₄ photosynthetic genes has been expanded and it is now possible to express C₄ enzymes at high levels and in desired locations in the leaves of C₃ plants. The evolution of C₄ genes together with techniques with which to overproduce C₄ enzymes in the leaves of C₃ plants is summarized here. The regulation and physiological impacts of overproduced C₄ enzymes in transgenic rice plants are also presented. The physiological impacts of the overproduction in potato, tobacco (Nicotiana tabacum) and Arabidopsis thaliana as well as rice have previously been reviewed in detail by Häusler et al. (2002).

Evolution of C₄ photosynthetic genes

C₄ photosynthetic genes had previously been considered to be specific for C₄ plants, since the activities of the corresponding enzymes are low in C₃ plants (Hatch, 1987) and their kinetic properties are usually different from those of C₄ enzymes (e.g. for PEPC, see Svensson et al., 1997; Dong et al., 1998). However, recent comparative studies have revealed that C₃ plants have at least two different types of genes, one encoding enzymes of ‘housekeeping’ function and the other very similar to the C₄ genes of C₄ plants, though expression of the latter is very low or even undetectable in C₃ plants. Based on this finding, it is postulated that the C₄ genes evolved from a set of pre-existing counterpart genes in ancestral C₃ plants, with modifications in the expression level in the leaves and kinetic properties of enzymes (Ku et al., 1996). Hereafter, the C₄ genes in C₄ plants and their homologues in C₃ plants are designated C₄-specific and C₄-like genes, respectively. In addition to C₄-specific or C₄-like genes, both C₃ and C₄ plants have other homologous genes for the housekeeping function. These are designated as C₃-specific genes. The number of homologous genes and the evolutionary origins of C₄-specific genes are different among C₄ enzymes and plant species (Monson, 1999). By contrast, modifications of C₄-like genes required for functioning in the C₄ pathway probably share common features in all the C₄-specific genes examined so far. In the following, the evolutionary origin of the maize C₄-specific PPDK gene is considered as an example.

Evolution of the maize C₄-specific PPDK gene

Maize has three different isoforms of PPDK, namely, the chloroplastic isoform involved in the C₄ pathway and two cytosolic isoforms (Sheen, 1991). The chloroplastic and one cytosolic isoforms are encoded by a single gene that has a dual promoter system (Glackin and Grula, 1990; Sheen, 1991; Fig. 2). This gene (designated Pdk1 hereafter) has two transcription initiation sites and transcription from these sites is regulated by different promoters located at their respective 5¢-flanking regions. Transcription at the

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**Fig. 2.** Comparison of the rice and maize Pdk1 genes that encode the chloroplastic and cytosolic isoforms of PPDK. Both genes have a dual promoter system and transcription starts at two different sites indicated by bent arrows, giving rise to transcripts different in size. The larger transcript encodes the chloroplastic isoform and the smaller one encodes the cytosolic isoform. The coding regions common to the two transcripts are represented by filled boxes, and the 5¢- and 3¢-non-coding regions by open boxes. Hatched boxes in maize exon 1 and rice exon 1 represent regions that encode the transit peptide, and those in maize exon 2 and rice exon 2 represent the coding regions unique to the small transcript. ATG and TGA indicate the initiation and termination codons, respectively. The gene structures reported previously (Imaizumi et al., 1997) are modified.
first initiation site produces large transcripts for the chloroplastic isoform, while that from the second site produces small transcripts for the cytosolic isoform. The large transcripts are expressed highly specifically in the mesophyll cells of green leaves at a high level and expression is induced by light, while the small transcripts are expressed at a high level in roots but at a low level in the mesophyll cells (Sheen, 1991). By definition, the genes encoding the chloroplastic and cytosolic isoforms in the maize \( Pdk_1 \) are \( C_4 \)-specific and \( C_3 \)-specific genes, respectively: they have previously been designated \( C4ppdkZm1 \) and \( cyppdkZm1 \), respectively, by Sheen (1991). The second cytosolic isoform is encoded by a gene with a single promoter (previously designated \( cyppdkZm2 \) by Sheen (1991) but \( Pdk2 \) hereafter), which shows high homology to the \( C_3 \)-specific gene in \( Pdk_1 \). This gene is expressed at a very low level in the mesophyll cells (Sheen, 1991) and thus \( C_3 \)-specific. The cytosolic isoform of PPDK accumulates significantly in kernels (Aoyagi and Bassham, 1984; Aoyagi and Chua, 1988), though it is uncertain which of the two genes is expressed in this organ.

Rice also has three different isoforms of PPDK, and two different genes have been identified. One has a dual promoter system and encodes the chloroplastic and cytosolic isoforms (Imaizumi et al., 1997) and the other, with a single promoter, encodes the cytosolic isoform (Moons et al., 1998). The former gene is highly homologous to the maize \( Pdk_1 \) (Imaizumi et al., 1997). It has 21 exons and the positions of introns are essentially the same as those in the maize gene, except that the exon 1 and 3 of the maize gene are split into two exons in the rice gene (Fig. 2). The deduced amino acid sequences are 88% homologous in the mature protein portion and 56% homologous in the transit peptide portion. In addition, this gene is expressed in rice plants essentially in the same way as the maize \( Pdk_1 \) does in maize, except for the expression level of the large transcripts in green leaves: the large transcripts are expressed specifically in photosynthetic organs but at low levels, while the smaller ones in reproductive organs at high levels and roots at a low level (Imaizumi et al., 1997). Thus, this gene is a counterpart of the maize \( Pdk_1 \). The other gene encoding the cytosolic isoform has been identified as a cDNA clone from rice roots (\( osppdka \); Moons et al., 1998), but its genomic clone has not yet been isolated. Since the 3’ region of the cDNA is highly homologous to the exons in the 3’ region of the rice \( Pdk_1 \) (Moons et al., 1998), it seems likely that this gene is a counterpart of the maize \( Pdk2 \).

From the comparison between the maize and rice genes, the postulated evolutionary origin of PPDK genes is depicted in Fig. 3. From a single ancestral gene, two genes encoding a cytosolic isoform were derived. One was an ancestral \( Pdk2 \) gene, which would evolve to become the \( Pdk_2 \) genes of \( C_3 \) and \( C_4 \) plants. The other was an ancestral \( Pdk_1 \) gene with a single promoter. This gene subsequently evolved to become the \( Pdk_1 \) gene with a dual promoter system in an ancestral \( C_3 \) plant, acquiring a sequence for the transit peptide, and finally evolved to become the \( Pdk_1 \) gene of a \( C_4 \) plant by acquiring a mechanism(s) for high-level expression. The evolutionary scenario for the \( C_4 \)-
specific PPDK gene might differ from this in the C₄ species of Flaveria. Until now, only Pdk1 with a dual promoter system, but not Pdk2, has been identified in both C₃ and C₄ species of Flaveria (Rosche et al., 1994; Rosche and Westhoff, 1995). If Pdk2 were to be missing, the evolution in this species probably proceeded without gene duplication.

Modifications required for the evolution of the maize C₄-specific PPDK gene have been investigated by comparing expression patterns of C₄-like and C₄-specific genes in rice and maize plants. When a reporter gene (GUS gene) was expressed in rice plants under the control of the promoter of the C₄-specific gene in the maize Pdk1 (~1032 to +71, relative to the transcription initiation site), GUS was expressed highly specifically in the mesophyll cells of green leaves at a high level and in a light-responsive manner (Matsuoka et al., 1993). Its expression level was even higher than that under the control of the cauliflower mosaic virus 35S promoter. On the other hand, when the GUS gene was expressed in maize plants under the control of the promoter of the C₄-like gene in the rice Pdk1 (~1419 to +512, a region upstream from the initiation codon), GUS was expressed in both the mesophyll and bundle sheath cells at low levels, although its expression was light responsive (Nomura et al., 2000a). These results clearly show that a cis-acting element(s) for light-responsive expression is present in the rice promoter, but that those for cell-specific and high-level expression are missing and had to be acquired during the course of evolution from a C₄-like to a C₄-specific gene (Fig. 3). Some of these cis-acting elements in the promoter of the maize C₄-specific gene have been identified (Sheen, 1991; Matsuoka and Numazawa, 1991; Imaizumi et al., 1997; Nomura et al., 2000a). Another important implication of the results is that trans-acting elements (e.g. transcription regulators) required for the expression of the C₄-specific gene are present in the leaves of the C₃ plant, rice.

The expression pattern of the promoter of the C₃-specific gene in Pdk1, on the other hand, does not differ much between the maize and rice genes. The C₃-specific promoters from the maize and rice Pdk1 both directed expression of the GUS gene in non-photosynthetic organs such as grains and roots in transgenic rice (Nomura et al., 2000b).

Thus, modifications of Pdk1 required for the evolution from a C₄-like to a C₄-specific gene are relatively simple; namely, gain of the cis-acting elements for cell-specific and high-level expression in the promoter region. As described later, however, cis-acting elements for high-level expression are not restricted to the promoter region.

Evolution of other C₄-specific genes

The same evolutionary scenario can be applied to the C₄-specific PEPC gene. The promoter of the maize C₄-specific gene (~1212 to +78) directed high-level, mesophyll-cell-specific and light-inducible expression of the GUS gene in transgenic rice (Matsuoka et al., 1994). The C₄-specific PPDK and PEPC are both located in the mesophyll cells of C₄ plants. Quite recently, it has been found that C₄-specific genes for the enzymes located in the bundle sheath cells might evolve in similar ways. The promoter of the C₄-specific PEP-CK gene of a turf grass Zoysia japonica (PEP-CK type) was fused to the 5′ side of the GUS gene and introduced into rice plants by Agrobacterium-mediated gene transfer. Histochemical localization of GUS activity is shown. Cross-sections of leaf blade (a), leaf sheath (b) and stem (c). Scale bars 0.1 mm. M Nomura, M Matsuoka, unpublished results.
gene modifications for the evolution of C₄-specific genes followed similar mechanisms.

How to overproduce C₄ enzymes in the mesophyll cells of C₃ plants

In leaves of C₃ plants, photosynthesis and subsequent carbon and nitrogen metabolism proceed mainly in the mesophyll cells. To alter carbon metabolism in leaves of C₃ plants, C₄ enzymes have to be overproduced in these cells. From the evolutionary origins of the C₄-specific genes and the expression patterns of their promoters in the leaves of C₃ plants, it was anticipated that the introduction of the intact C₄-specific genes into C₃ plants would lead to high-level and cell-specific expression of C₄ enzymes. In fact, this strategy was effective in overproducing C₄ enzymes specific to the mesophyll cells of C₃ plants. By contrast, to overproduce enzymes specific to the bundle sheath cells, conventional techniques with gene constructs containing a strong promoter fused to a cDNA were effective (Table 1).

<table>
<thead>
<tr>
<th>C₄ enzyme (location in C₃ plants)</th>
<th>Introduced construct</th>
<th>Highest enzyme activity(a) (increase in fold)</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>PEPC (MC)</td>
<td>Intact maize gene</td>
<td>110 3–4</td>
<td>Ku et al., 1999</td>
</tr>
<tr>
<td>PPDK (MC)</td>
<td>Rice Cab prom::maize FL C₄ cDNA</td>
<td>5 &lt;0.1</td>
<td>Fukayama et al., 2001</td>
</tr>
<tr>
<td></td>
<td>Maize Pdk1 C₄ prom::maize FL C₄ cDNA</td>
<td>5 &lt;0.1</td>
<td>Fukayama et al., 2001</td>
</tr>
<tr>
<td></td>
<td>Intact maize gene</td>
<td>40 0.5</td>
<td>Fukayama et al., 2001</td>
</tr>
<tr>
<td>NADP-ME (BSC)</td>
<td>Rice Cab prom::rice FL C₃ cDNA</td>
<td>&lt;5 &lt;0.1</td>
<td>Tsuchida et al., 2001</td>
</tr>
<tr>
<td></td>
<td>Rice Cab prom::maize FL C₄ cDNA</td>
<td>30 0.6</td>
<td>Tsuchida et al., 2001</td>
</tr>
<tr>
<td></td>
<td></td>
<td>70 –</td>
<td>Takeuchi et al., 2000</td>
</tr>
<tr>
<td>PEP-CK (BSC)</td>
<td>Rice Cab prom::Zoysia FL C₄ cDNA</td>
<td>– 0.1(b)</td>
<td>Miyao M. et al., unpublished results</td>
</tr>
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<td></td>
<td>Maize C₄ PEPC prom::Urochloa C₄ cDNA(c)</td>
<td>– 0.5(d)</td>
<td>Suzuki et al., 2000</td>
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<tr>
<td></td>
<td>Maize Pdk1 C₄ prom::Urochloa C₄ cDNA(c)</td>
<td>– 0.5(d)</td>
<td>Suzuki et al., 2000</td>
</tr>
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</table>

(a) Highest enzyme activities among the primary transgenic plants are listed.
(b) Highest level of the enzyme protein relative to the level in Zoysia leaves is presented.
(c) The Urochloa C₄-specific PEP-CK cDNA was fused to a sequence of the transit peptide for targeting to chloroplasts.
(d) Highest activities of the secondary transgenic plants relative to the activity of Urochloa leaves are presented.

MC, mesophyll cells; BSC, bundle sheath cells; prom, promoter; FL, full-length.

Enzymes located in the mesophyll cells of C₄ plants

The first trial of this kind was conducted with the intact maize PEPC gene expressed in transgenic rice plants (Ku et al., 1999). The maize gene of 8.8 kb that contained all exons and introns and its own promoter and terminator sequences was introduced into rice plants. As expected, the activity of PEPC in the leaf protein extract was greatly increased up to 110-fold that of non-transformants or 3-fold the maize activity. The level of the PEPC protein accounted for 12% of total leaf soluble protein at most. The introduction of the intact maize gene was also effective in overproducing PPDK in rice leaves (Fukayama et al., 2001). The introduction of the intact maize Pdk1 of 7.3 kb increased the PPDK activity in rice leaves up to 40-fold that of non-transformants or about half of the maize activity. In a homozygous transgenic line, the PPDK protein accounted for 35% of total leaf soluble protein or 16% of total leaf nitrogen, much above the levels of foreign protein in transgenic plants reported previously. The C₄-specific gene of the maize Pdk1 was exclusively expressed in the leaves of these transgenic rice plants while the C₄-specific gene was expressed in grains, an indication that the maize Pdk1 is expressed in rice plants with an organ specificity similar to that in maize plants.

To examine whether or not the promoter sequence of the C₄-specific gene is sufficient for the high-level expression, the full-length cDNA encoding the maize C₄-specific PPDK was expressed under the control of the promoter of the C₄-specific gene of the maize Pdk1 or the rice Cab promoter (Fukayama et al., 2001). Cab encodes the light-harvesting chlorophyll-binding protein and is expressed at high levels in photosynthetically active organs (Sakamoto et al., 1991). The introduction of these gene constructs, however, increased the activity of PPDK in rice leaves only up to several fold that of non-transformants (Fukayama et al., 2001). Thus, the transcriptional activity of the C₄-specific promoter cannot be the prime reason for high-level expression. The 5' and 3'-noncoding regions by themselves cannot lead to high-level expression either, since the constructs containing the full-length cDNA with these regions were not effective. Therefore, it is quite possible that, in addition to the promoter region, the presence of introns or the terminator sequence, or a combination of both, is required for high-level expression.

In transgenic rice plants containing the C₄-specific PEPC or PPDK gene, the levels of transcripts and protein and the activity of C₄ enzyme in the leaves all correlated well with the copy number of the introduced gene (Ku et al.,
of the C4 enzyme in C3 plants. The intact maize C4-specific plants have to be used to achieve high-level expression that transgenes from phylogenetically closely related specific gene, however, seems to have some limitation in genic rice plants (Ku et al., 1999; Fukayama et al., 2001). As described above, the promoters of C4-specific PPDK and PEPC genes have the cis-elements for organ- and cell-specific expression. It is likely that the maize C4-specific genes behave in a qualitatively and also quantitatively similar way in both maize and transgenic rice plants.

Overproduction by the introduction of the intact C4-specific gene, however, seems to have some limitation in that transgenes from phylogenetically closely related plants have to be used to achieve high-level expression of the C4 enzyme in C3 plants. The intact maize C4-specific PEPC gene was not expressed at high levels in tobacco leaves, because of incorrect transcription initiation (Hudspheth et al., 1992). Not only incorrect initiation and termination of transcription, but also incorrect splicing could occur when genes from monocots are introduced into dicots (Goodall and Filipowicz, 1991). Thus, phylogenetic distance may hamper the expression of genes from C4 plants in the leaves of C3 plants.

Conventional techniques for overproduction of transgenes, namely, the introduction of a chimeric gene containing cDNA for a C4 enzyme, fused to a strong promoter alone or together with enhancer sequences, can also increase the activity of C4 enzymes in the leaves of C3 plants, although the increase does not exceed several fold that of non-transformants (for a review see Matsuoka et al., 2001).

Enzymes located in the bundle sheath cells of C4 plants

Since the intact C4-specific genes for these enzymes would be expressed specifically in the bundle sheath cells of C3 plants, they cannot be used for overproduction in photosynthetically active mesophyll cells. More conventional techniques were applied and have proven successful. The expression of the maize C4-specific NADP-ME cDNA under the control of the rice Cab promoter increased the activity of NADP-ME in rice leaves to 30- or 70-fold that of non-transformants (Takeuchi et al., 2000; Tsuchida et al., 2001). The level of the NADP-ME protein was also increased up to several per cent of total leaf soluble protein. Such high-level expression was unique to the cDNA for the C4-specific NADP-ME, and the expression of the cDNA for the rice C3-specific isoform under the control of the same promoter increased the activity only some fold (Tsuchida et al., 2001). This observation suggests that expression of the rice C3-specific NADP-ME is suppressed at co- and/or post-transcriptional levels by some regulation mechanisms intrinsic to rice, while that of the foreign C4-specific isoform can escape from such suppression. The Zoysia C4-specific PEP-CK was also overproduced by introduction of a cDNA construct (M Miyao et al., unpublished results).

Overproduction of C4 enzymes in a different intracellular compartment

The C4 enzymes described above were all overproduced in the same intracellular compartment in C3 plants as in C4 plants, namely, PEPC and PEP-CK in the cytosol and PPDK and NADP-ME in the chloroplasts. The intracellular location of foreign enzyme can be altered by use of targeting signals. To overproduce PEP-CK in the chloroplasts of the mesophyll cells of rice leaves, the cDNA of the C4-specific PEP-CK from Urochloa panicoides was fused to a sequence of the transit peptide for targeting to chloroplasts, and expressed under the control of the maize C4-specific PEPC or PPDK promoter (Suzuki et al., 2000). The PEP-CK activity of transgenic rice leaves reached about half of that in the Urochloa leaves. Similarly, bacterial enzymes were overproduced in the chloroplasts of transgenic C3 plants (Häusler et al., 2001; Panstruga et al., 1997).

Factors affecting the expression levels of transgenes

In general, expression of transgenes is hampered by many mechanisms including the positional effects (Gelvin, 1998), silencing (Gallie, 1998; Chandler and Vaucheret, 2001) and rearrangement (Hiei et al., 1994) of transgenes. During the course of the study of overproducing C4 enzymes, it was found that the rearrangement occurs frequently during the gene transfer mediated by Agrobacterium tumefaciens. A significant fraction of transgenic rice plants introduced with the intact maize C4-specific gene showed activities of C4 enzymes comparable to or even lower than that of non-transformants (Ku et al., 1999; Fukayama et al., 2001). DNA gel-blot analysis of these low-expressing lines showed that transgenes in all lines tested sustained partial deletion and/or chimeric linking (Fukayama et al., 2001). Such rearrangement is not peculiar to long transgenes with complex exon-intron structures, and it did occur in five out of nine transgenic rice plants introduced with a cDNA construct of 4.4 kb (Miyao et al., 2001). It is possible that cis-acting elements and/or the transit sequence are selectively deleted from an introduced gene, altering the level and/or location of a C4 enzyme in transgenic C3 plants.

As described above, overproduction of C4 enzymes in C3 plants can be achieved by introducing appropriate gene constructs. It is also necessary to screen a number of transgenic plants to obtain a desired expression level of a C4 enzyme and to confirm the enzyme location in the leaves of C3 plants.

Regulation and physiological impacts of C4 enzymes overproduced in C3 plants

PEPC

The activity of PEPC in higher plants is regulated by two different mechanisms; one the reversible protein phos-
phosphorylation of a conserved serine residue near the N-terminus, and the other through various metabolite effectors such as glucose-6-phosphate (Glc6P), malate, aspartate, and glutamate (Vidal and Chollet, 1997). Upon phosphorylation, PEPC becomes more sensitive to the activator Glc6P and less sensitive to the feedback inhibitor malate, being more active in vivo (Vidal and Chollet, 1997). The phosphorylation itself is also inhibited by malate through the conformational change of PEPC (Bakrim et al., 1998). In leaves of C_4 plants, PEPC is phosphorylated in the light and dephosphorylated in darkness and its activity is modulated in response to changes in light intensity (Vidal and Chollet, 1997). The maize PEPC expressed in transgenic rice leaves also underwent activity regulation via phosphorylation, but in an opposite manner (Fukayama et al., 2002). It remained dephosphorylated and less active during the daytime and became phosphorylated and more active in the night in transgenic rice leaves. Since the activity of the endogenous rice PEPC was also down-regulated during the daytime, it is likely that both the maize and rice PEPC undergo activity regulation by the same mechanisms in rice leaves. Bacterial PEPC lacks the phosphorylation site (Vidal and Chollet, 1997) and can escape from down-regulation via phosphorylation.

Another issue affecting potential activity of foreign PEPC in the leaves C_3 plants is the cytosolic concentrations of potential inhibitors and activators. The concentrations of inhibitors of higher plant PEPC are high in the cytosol of the mesophyll cells of C_3 plants, about 1 mM for malate and around 40 mM for aspartate and glutamate (Heineke et al., 1991). Bacterial PEPC is also inhibited by aspartate, and in addition, it requires acetyl-CoA as an activator (Chen et al., 2002). Taken together, the actual in vivo activity of foreign PEPC, either from higher plants or bacteria, in the leaves of transgenic C_3 plants is lower than maximum extractable activities, especially when measured in the presence of activators.

Until now, several transgenic C_3 plants overproducing PEPC have been produced and analysed precisely (Matsuoka et al., 2001). All the transformants analysed so far show a higher level of malate (Hudspeth et al., 1992; Kogami et al., 1994; Häusler et al., 1999) or OAA (Fukayama et al., 2002) in the leaves compared to that of non-transformants, an indication that foreign PEPC is partly active in these transformants. Physiological impacts of the elevated PEPC activity reported to date have varied with no clear consensus. Among these, stimulation of respiration in the light and destabilization of stomatal opening have been observed in different plant species overproducing PEPC of different origin, namely, transgenic potato overproducing PEPC from Corynebacterium glutamicum (Gehlen et al., 1996) and transgenic rice overproducing the maize C_4-specific PEPC (Fukayama et al., 2002). The stimulated respiration is consistent with an anaplerotic function of the C_4-specific PEPC, which replenishes the tricarboxylic acid cycle with organic acids to meet the demand of carbon skeletons for amino acid synthesis (Champigny and Foyer, 1992). The effects on stomatal movement were observed only under non-steady-state conditions. Transient closure of stomata at the onset of gas-exchange measurements and after a step increase in light intensity were reported in the transgenic potato and rice, respectively. Accelerated stomatal opening, by contrast, was observed in the transgenic potato, but not in the transgenic rice. PEPC has long been implicated in the synthesis of malate as an osmotically active solute in stomata (Assmann, 1993). At present, it remains obscure if foreign PEPC is expressed in guard cells of these transformants.

In view of the critical function of PEPC in photosynthesis of C_4 and CAM plants, some researchers had expected that overproduction of PEPC alone would improve the photosynthetic performance of C_3 plants. A research group claims that the photosynthetic rate under saturating light can be greatly increased by overproduction of the maize PEPC in transgenic rice plants (Jiao et al., 2001). Their results, however, require reconsideration, since the correlation between the photosynthetic rate and the level of the PEPC protein in transgenic rice leaves has not yet been confirmed. Other groups all reported negative effects on photosynthesis, and the photosynthetic rate was slightly lowered by the overproduction. It has previously been reported that the O_2 inhibition of photosynthesis was mitigated by overproduction of the maize PEPC, and suggested that the maize PEPC may participate in photosynthetic CO_2 fixation in transgenic rice leaves (Ku et al., 1999). Later experiments, however, indicated that the initial CO_2 fixation product, determined by 14CO_2 labelling experiments with transgenic rice plants showing a 50-fold elevation in extractable PEPC activity, was exclusively the C_3 compound 3-phosphoglycerate (Fukayama et al., 2002). The apparent reduction of O_2 inhibition can be explained by more marked suppression of photosynthesis by overproduction of PEPC at 2% O_2 than at 21% O_2 (Matsuoka et al., 2000). In a recent paper (Agarie et al., 2002), it has been proposed that the lower photosynthetic rate resulted from the reduced capacity of regeneration of inorganic phosphate. However, a significant reduction of the photosynthetic rate was observed at very low intercellular CO_2 concentrations and a low O_2 concentration where the Rubisco activity but not the phosphate regeneration limits photosynthesis (Fukayama et al., 2002). It is more likely that the suppression of photosynthesis results from the enhanced respiration by elevated PEPC.

Transgenic Arabidopsis overproducing PEPC from a cyanobacterium Synechococcus vulcanus has recently been reported (Chen and Izui, 2002). The Synechococcus PEPC has some advantages for raising the in vivo activity
of PEPC in the leaves of C₃ plants. It does not undergo activity regulation via phosphorylation or, in contrast to other bacterial PEPCs, is not inactivated by malate, and moreover, it does not require acetyl-CoA for activation (Chen et al., 2002). Expression of this PEPC in Arabidopsis led to stunting and bleaching of leaf colour (Chen and Izui, 2002). Similar phenomena were observed in transgenic potato overproducing the potato enzyme that had been modified for a higher affinity for PEP and lowered sensitivity toward malate (Rademacher et al., 2002). It has been demonstrated that carbon flow in these transgenic potato plants was redirected from soluble sugars and starch to organic acids and amino acids (Rademacher et al., 2002).

**PPDK**

In leaves of C₄ plants, 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). Unlike phosphorylation of PEPC, PPDK is dephosphorylated in the light and dephosphorylated in darkness, and upon phosphorylation it is completely inactivated. Such a strict activity regulation is a prerequisite for proper operation of the C₄ pathway since the synthesis of PEP upon phosphorylation is completely inactivated. Such a serious damage occurs. 

There are four reports on transgenic plants, which overproduce PPDK derived from higher plants; transgenic Arabidopsis (Ishimaru et al., 1997), potato (Ishimaru et al., 1998), rice (Fukayama et al., 2001) overproducing the maize C₄-specific PPDK, and transgenic tobacco overproducing PPDK from a CAM plant Mesembryanthemum crystallinum (Sheriff et al., 1998). Physiological impacts were minimal and no changes in the photosynthetic characteristics were observed in these transfectants, even in the transgenic rice with a 40-fold increase in activity (Fukayama et al., 2001). In general, the reaction of PPDK is freely reversible, depending on concentrations of substrates, activators, and inactivators (Burnell and Hatch, 1985). This could be the reason why the overexpression of PPDK does not result in significant effects on carbon metabolism in the leaves.

**NADP-ME**

In contrast to PEPC and PPDK, there is no specific regulatory protein for higher plant chloroplastic NADP-ME. The activity of the C₄-specific NADP-ME is increased under illumination through increases in pH and concentration of Mg²⁺ in the chloroplast stroma (Edwards and Andreo, 1992). Two sets of transgenic rice plants overproducing the maize C₄-specific isoform (Takeuchi et al., 2000; Tsuchida et al., 2001) and the rice C₃-specific isoform of NADP-ME (Tsuchida et al., 2001) have been reported. The transformants overproducing the rice enzyme with some fold increase in activity did not show any detectable differences in their growth, while those overproducing the maize enzyme at the same activity level showed serious stunting and bleaching of leaf colour, due to enhanced photoinhibition of photosynthesis under natural light conditions. It is proposed that the action of the maize NADP-ME in the chloroplasts increases the NADPH/NADP ratio and suppresses photorespiration, rendering photosynthesis more susceptible to photoinhibition (Takeuchi et al., 2000; Tsuchida et al., 2001). The C₄-specific NADP-ME has a higher Vₘ value, lower Kₘ values for substrates, and higher optimum pH, as compared with the C₃-specific isoform (Casati et al., 1997). Such features are suitable for strict regulation of the enzyme activity in the bundle sheath cell chloroplasts of C₄ plants, but they allow the enzyme to continue operating in the leaves of C₃ plants even when serious damage occurs.

**Future applications of overproduction of C₄ enzymes**

A major objective of overproduction of C₄ enzymes in C₃ plants is to improve the photosynthetic performance. As described above and reviewed recently (Häusler et al., 2002), none of the positive effects on photosynthesis have been observed in transgenic C₃ plants overproducing a single C₄ enzyme. Transgenic C₃ plants overproducing multiple enzymes are being produced and analysed in some research groups (Häusler et al., 2002). Although the introduction of the ‘C₄-like’ pathway into the mesophyll cells of C₃ plants is one strategy being adopted (Mann, 1999; Surridge, 2002), whether or not this pathway can operate with desirable effects on C₃ photosynthesis is a matter of controversy (Edwards, 1999; Leegood, 2002; Häusler et al., 2002). Considering the C₄ pathway operating in a single cell found in some aquatic organisms (for a review see Leegood, 2002), it might be possible that the C₄-like pathway could support C₃ photosynthesis under some stress conditions such as drought, in which the CO₂ availability is limited.

Apart from photosynthesis, overproduction of a single C₄ enzyme seems to have some positive effects on physiology of C₃ plants. It has been reported that overproduction of the chloroplastic, but not cytosolic, PPDK increased the number of seeds per seed capsule and the weight of each seed capsule in transgenic tobacco (Sheriff et al., 1998), and that overproduction of the maize C₄-specific PEPC improved resistance to aluminium of root elongation in transgenic rice (Miyao et al., 2001). Of course, it is of prime importance to elucidate mechanisms for these effects and to confirm whether or not similar phenomena can be generally observed in different plant
species. Taking account of a variety of housekeeping functions of the C₃-specific enzymes, it is not unlikely that overproduction of C₄ enzymes could improve various features of C₃ plants.

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