Maize C₄-form phosphoenolpyruvate carboxylase engineered to be functional in C₃ plants: mutations for diminished sensitivity to feedback inhibitors and for increased substrate affinity

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Abstract

Introducing a C₄-like pathway into C₃ plants is one of the proposed strategies for the enhancement of photosynthetic productivity. For this purpose it is necessary to provide each component enzyme that exerts strong activity in the targeted C₃ plants. Here, a maize C₄-form phosphoenolpyruvate carboxylase (PEPC, EC 4.1.1.31) was engineered for its regulatory and catalytic properties so as to be functional in the cells of C₃ plants. Firstly, amino acid residues Lys-835 and Arg-894 of maize PEPC, which correspond to Lys-773 and Arg-832 of Escherichia coli PEPC, respectively, were replaced by Gly, since they had been shown to be involved in the binding of allosteric inhibitors, malate or aspartate, by our X-ray crystallographic analysis of E. coli PEPC.

The resulting mutant enzymes were active but their sensitivities to the inhibitors were greatly diminished. Secondly, a Ser residue (S780) characteristically conserved in all C₄-form PEPC was replaced by Ala conserved in C₃- and root-form PEPCs to decrease the half-maximal concentration (S₀.₅) of PEP. The double mutant enzyme (S780A/K835G) showed diminished sensitivity to malate and decreased S₀.₅ (PEP) with equal maximal catalytic activity (Vₘₐₓ) to the wild-type PEPC, which will be quite useful as a component of the C₄-like pathway to be introduced into C₃ plants.

Key words: C₄ photosynthesis, genetic engineering, PEP carboxylase, site-directed mutagenesis, Zea mays.
Introduction

Phosphoenolpyruvate carboxylase (PEPC, EC 4.1.1.31) catalyses an irreversible β-carboxylation of phosphoenolpyruvate (PEP) in the presence of HCO$_3^-$ and Mg$^{2+}$ to yield oxaloacetate and orthophosphate (O’Leary, 1982). In higher plants, the enzyme has many faceted physiological roles, which are shared by specific isoforms. Particularly in C$_4$- and crassulacean acid metabolism (CAM) plants, PEPC plays a key role in photosynthesis by performing the initial fixation of atmospheric CO$_2$. Most PEPCs are subject to allosteric regulation. Their effectors show a wide variety depending on the species of organisms (Izui et al., 2004), and they often affect kinetic properties differently, even among isoforms in the same organism (Dong et al., 1998; Svensson et al., 2003). However, the effectors of higher plant PEPCs are confined to a set of metabolites; namely, PEPCs of dicot plants are activated by glucose 6-phosphate (G6P) and inhibited by malate or aspartate, and those of monocot plants are further activated by glycine. Furthermore, higher plant PEPCs are regulated by reversible phosphorylation by a specific protein kinase called PEPC kinase (PEPC-k), and the phosphorylated PEPC shows lower sensitivity to malate and aspartate (Vidal and Chollet, 1997; Tsuchida et al., 2001; Nimmo, 2003). Recently, PEPC-k knockdown Flaveria bidentis transgenic plants were produced using reverse genetics, and no obvious differences were observed in several photosynthetic parameters between transgenic plants and non-transformants, at least under normal greenhouse conditions (Furumoto et al., 2007).

For the coming global shortage of food in the near future, a second Green Revolution is required, which may bring about a tremendous improvement in the productivity of crop plants. The photosynthetic productivity of C$_4$ plants is usually 1.5–2-fold higher than that of C$_3$ plants under arid, hot, and light-intensive conditions owing to their CO$_2$-concentrating metabolic cycle, the C$_4$ pathway. For this reason, several groups of investigators have been trying to use genetic engineering to introduce a ‘C$_4$-like pathway’ into C$_3$ plants that have no Kranz anatomy (Surridge, 2002). The ‘C$_4$-like pathway’ is supposed to function in a single mesophyll cell and ultimately pumps p CO$_2$ from the cytoplasm to the chloroplasts where the net CO$_2$-fixation is performed. The discovery of this pathway in Hydrilla verticillata by Bowes et al. (2002) reinforced the feasibility of this strategy. Theoretical analysis by von Caemmerer (2003) also supported some advantages of this approach under water-limited conditions.

Previously, tobacco transformants expressing maize C$_4$-form PEPC were first produced, but no enhancement of photosynthetic activity could be seen (Kogami et al., 1994). Although several genes for the enzymes involved in the C$_4$ pathway had been successfully introduced and expressed in C$_3$ plants individually or in combination, thereafter, no substantial improvement in photosynthesis due to an operational ‘C$_4$-like pathway’ has been obtained (reviewed by Matsuoka et al., 2001; Häusler et al., 2002; Miyao and Fukayama, 2003; Raines, 2006). During the course of these experiments, several problems to be solved became apparent. One is that the overexpressed foreign enzymes do not necessarily exert their expected catalytic activities in the targeted C$_3$ cells, since they were not well matched with cellular levels of ligands. Thus it is necessary to construct engineered enzymes of the ‘C$_4$-like pathway’, whose catalytic and regulatory properties are modified to be able to function in any given intracellular environment of C$_3$ plants (Häusler et al., 2002). In the case of PEPC, there was a very small effect of overexpression of maize C$_4$-form PEPC on the cellular metabolism of rice and this was presumed to be because the intracellular levels of PEP and malate were too low and too high, respectively, for the functioning of the maize C$_4$-form PEPC (Fukayama et al., 2003). In fact, the $K_m$ value of this enzyme for PEP is 1.5 mM, being about 30-fold larger than that of a non-photosynthetic form (root-form) PEPC of maize, and the $K_i$ value for malate is 0.8 mM, being about 4-fold larger than the root-form PEPC (Dong et al., 1998). The same tendency was also observed with several isoform PEPCs of Flaveria trinervia, a C$_4$ dicot plant (Bläsing et al., 2000).

In addition, all of the transformants analysed to date show high levels of malate, which is enough to diminish PEPC activity in vitro (Kogami et al., 1994). In the intracellular environment, the foreign C$_4$-form PEPC appears not to be fully active. Therefore, it is worthwhile to prepare an engineered enzyme, which fits to the ‘C$_3$ environment’ and especially the low PEP- and high malate concentrations, for further metabolic engineering.

For molecular engineering on the enzyme, it is important to determine the key residues that influence these catalytic and regulatory properties. In recent years, a Ser residue involved in affinity for PEP was identified in the F. trinervia C$_4$-form PEPC (at position 774, corresponding to 780 in the maize C$_4$-form PEPC) (Bläsing et al., 2000). This Ser residue is conserved characteristi- cally in C$_4$-isoform enzymes among various plant species, while in other isoforms this site is an Ala residue. Replacement of this Ser of C$_4$-isoform PEPC to Ala successfully decreased its $K_m$ value for PEP (Bläsing et al., 2000).

Aspartate and malate are common allosteric inhibitors in both maize and Escherichia coli enzymes. The aspartate-binding site was first revealed from the analysis of the crystal structure of the E. coli enzyme (Kai et al., 2003). Both the crystal structures of E. coli PEPC (Kai et al., 1999) and the maize C$_4$-form PEPC (Matsumura et al., 2002) were determined. The former was resolved as an inactive state complexed with aspartate, while the latter was resolved as an active state not complexed with
aspartate. In the *E. coli* PEPC structure, aspartate was ligated with four residues, Arg-587, Lys-773, Arg-832, and Asn-881. These residues are widely conserved among PEPCs. Two of them, Arg-587 and Asn-881 are indicated to be essential for catalytic activity as well. The replacement Arg-587 to Ser diminished its catalytic activity (Yano et al., 1995). In the case of a sorghum *C₄*-isofrom enzyme, deletion of the four C-terminal residues, which included an Asn corresponding to Asn-881 of maize *C₄*-form PEPC, destroyed its catalytic activity (Dong et al., 1999). As for the residues, Lys-773 and Arg-832, there has been no experimental evidence to show their involvement in the aspartate binding, except for our structural analysis.

In this report, mutant *E. coli* PEPCs were first produced with either Lys-773 or Arg-832 replaced by Gly. Then mutant maize PEPCs were produced with corresponding residues, Lys-835 and Arg-894, replaced in the same way. The maize PEPC showed greatly diminished inhibitor sensitivity as a result of replacement of these residues. Based on these findings, the intention was to produce an engineered maize *C₄*-form PEPC with altered affinity to ligands, which would exert its activity sufficiently in the *C₃* environment of host plants. To this end, two residues of the maize *C₄*-form PEPC were substituted independently or simultaneously. Lys-835 which is involved in Asp binding was substituted with Gly; and Ser-780 which is involved in PEP affinity was substituted with Ala. The resulting recombinant enzymes were investigated for their kinetic properties. The double mutant enzyme showed the higher affinity for PEP and lower sensitivity to allosteric inhibitors than the wild-type enzyme (WT), demonstrating for the first time that these properties could be conferred to the enzyme almost additively. The possible use of this mutant enzyme in metabolic engineering is discussed.

**Materials and methods**

**Construction of plasmids**

The enzymes, chemicals, and bacterial strains in this study were used according to the previous report (Takahashi-Terada et al., 2005). The following pT3, pTM94 and pEM94 (Dong et al., 1997) derivatives were constructed by site-directed mutagenesis in this work; pT3-K773G, pT3-R832G, pEM-K835G, pEM-R894G, pEM-K835G/R894G, pEM-S780A, and pEM-S780A/K835G. The original pT3 plasmid contains a major part of the gene (*ppc*) for *E. coli* PEPC, consisting of the coding region and a part of the promoter region in pUC18 (Takara) (Terada et al., 1995). The original pEM94 plasmid contains the complete coding region of the maize *C₄*-form PEPC in pET32a (Novagen) (Dong et al., 1997). The site-directed mutagenesis was carried out by the overlap extension method using PCR (Ho et al., 1989). The mutagenized primer sets used in this work were as follows: 5’-GCTGGTGTGGGCAGACCCCTTA-CATCAC-3’ and 5’-GGCTGGTGTGGGCAGACCCCTTA-CATCAC-3’ for pEM-K835G, 5’-GGCTGGTGTGGGCAGACCCCTTA-CATCAC-3’ and 5’-GGCTGGTGTGGGCAGACCCCTTA-CATCAC-3’ for pEM-R894G, 5’-GGCTGGTGTGGGCAGACCCCTTA-CATCAC-3’ and 5’-GGCTGGTGTGGGCAGACCCCTTA-CATCAC-3’ for pEM-R894G, 5’-GGCTGGTGTGGGCAGACCCCTTA-CATCAC-3’ and 5’-GGCTGGTGTGGGCAGACCCCTTA-CATCAC-3’ for pEM-R894G, 5’-GGCTGGTGTGGGCAGACCCCTTA-CATCAC-3’ and 5’-GGCTGGTGTGGGCAGACCCCTTA-CATCAC-3’ for pEM-R894G, 5’-GGCTGGTGTGGGCAGACCCCTTA-CATCAC-3’ and 5’-GGCTGGTGTGGGCAGACCCCTTA-CATCAC-3’ for pEM-R894G, 5’-GGCTGGTGTGGGCAGACCCCTTA-CATCAC-3’ and 5’-GGCTGGTGTGGGCAGACCCCTTA-CATCAC-3’ for pEM-R894G, 5’-GGCTGGTGTGGGCAGACCCCTTA-CATCAC-3’ and 5’-GGCTGGTGTGGGCAGACCCCTTA-CATCAC-3’ for pEM-R894G.

**Complementation test of mutant PEPCs for growth of E. coli F15 (Δppc), expression and purification of recombinant PEPCs**

Procedures for the complementation test of mutant PEPCs, their expression and purification were the same as described previously by Takahashi-Terada (1995). The genes for WT and mutant PEPCs of *E. coli* and maize were cloned into the pT3 and pTM plasmids, respectively, and expressed in *E. coli* F15. Since the *E. coli* PEPC protein comprises about 30% of the total soluble protein, the crude cell extracts were used for assays without further purification. On the other hand, WT and mutant maize enzymes were expressed in *E. coli* (BL21DE3) with the pET system (Novagen). These enzymes were purified with a Ni²⁺-chelating affinity column (GE Healthcare, UK) as a fusion protein with tags of 159 amino acid residues at the N-terminus. Our previous studies had shown that the kinetic properties are not significantly affected by the tag peptide (Dong et al., 1997). Therefore, the purified enzymes were used for kinetic analysis without truncation of the tag peptide. The purity of each recombinant enzyme was estimated to be more than 90% by SDS–PAGE (data not shown).

**Measurement of PEPC activity**

PEPC activity was measured spectrophotometrically as described by Terada and Izu (1991) and Takahashi-Terada et al. (2005). Briefly, the standard assay mixture for *E. coli* PEPC contained, in a total volume of 1.0 ml, 100 mM TRIS–HCl (pH 8.5), 2 mM PEP, 1.0 mM acetyl-CoA (an allosteric activator), 10 mM KHCO₃, 10 mM MgSO₄, 0.1 mM NADH, 1.5 IU of malate dehydrogenase, and the
enzyme. The assay mixture for maize PEPC contained, in a total volume of 1.0 ml, 100 mM HEPES-NaOH (pH 7.3), 2 mM PEP, 10 mM KHCO₃, 10 mM MgSO₄, 0.1 mM NADH, 1.5 IU of malate dehydrogenase, and the enzyme. The reaction was initiated by the addition of 2 mM or 0.5 mM PEP. The protein concentration was routinely determined using a protein assay kit (Bio-Rad) based on dye binding (Bradford, 1976) with crystalline bovine serum albumin as a standard. When necessary, the molar concentration of the maize C₄-form PEPC subunit was estimated from its molar extinction coefficient at 280 nm (7.5 ± 10⁴ M⁻¹ cm⁻¹). The value had been determined previously by amino acid analysis of the acid hydrolysate of PEPC in a solution of known absorbance (Y Ueno, K Izui, unpublished data). More simply, the PEPC concentration of 1.0 mg ml⁻¹ estimated by the dye-binding method above corresponds to an absolute concentration of 0.72 mg ml⁻¹, and this solution gives an absorbance of 0.5 at 280 nm.

Results

Complementation test of mutant enzymes

Mutant PEPCs of E. coli, K773G and R832G, as well as wild-type PEPC (WT) could complement the glutamate-requiring phenotype of E. coli F15, a deletion mutant of the gene for PEPC (Fig. 1A). This indicates that the mutant enzymes were active, at least to the extent to support cell growth. Among the maize mutant PEPCs, K835G complemented well and R894G complemented slightly, whereas the double mutant K835G/R894G did not (Fig. 1B). Crude protein extract was prepared from each of the cell lines grown on the agar plate containing glutamate (Fig. 1B), and subjected to the western blot analysis using the anti-maize PEPC antibody (Fig. 1C). The signals for PEPCs, WT, K835G, and R894G, were detected with an expected protein size, while that of the double mutant was not. Thus R894G was accumulated in the E. coli cells, but the enzyme activity was insufficient to support good growth of the mutant F15. On the other hand, no accumulation of the double mutant K835G/R894G was observed, the reason for which remains unknown.

Kinetic properties of enzymes with mutations at the inhibitor binding site

The crude extracts from the transformed F15 cells which expressed E. coli mutant PEPCs, either K773G or R832G, were assayed for PEPC activity, since each of the mutant PEPCs was able to complement the F15 phenotype. However, only a very low activity was observed for K773G probably due to its instability in the cell extracts. For R832G, a high activity was observed. The concentrations required for 50% inhibition ($I_{0.5}$) for aspartate and malate in the presence of 1 mM acetyl-CoA were 1.7 mM and 1.8 mM, respectively, for the WT PEPC, while those values increased to 15.3 mM and 3.6 mM for R832G. In the absence of acetyl-CoA, the extent of the desensitization became more profound for both inhibitors (data not shown).

Maize mutant PEPCs, whose putative residues involved in the binding with allosteric inhibitor (Lys-835 and Arg-894) had been replaced with Gly, were purified to homogeneity and their sensitivity to malate or aspartate was investigated. As shown in Fig. 2 and Table 1, remarkable desensitization to these inhibitors was observed. Further kinetic measurements showed that their major catalytic properties were retained even after these mutations.

Effect of replacement of Ser-780 to Ala on kinetic properties

For the C₄-form PEPC from a dicot plant F. trinervia, replacement of the conserved Ser, which corresponds to Ser-780 of the maize PEPC, to Ala had been shown to decrease the concentration of PEP required for half-maximum velocity ($S_{0.5}$) (Bläsing et al., 2000). To test
whether this also holds true for PEPC from a monocot plant, maize, a mutant enzyme S780A was produced and its kinetic properties were investigated. As shown in Fig. 3A and Table 2, the $S_{0.5}$ value of S780A was decreased to about one-quarter of WT. The saturation curve of S780A for Mg$^{2+}$ was weakly sigmoidal, similarly to WT but the $S_{0.5}$ value was not changed (Fig. 3B). The apparent increase of $V_{\text{max}}$ for S780A relative to WT was due to the difference in the degree of PEP saturation, since the PEP concentration employed was sufficiently low so that the extents of its saturation were different from each other. The $I_{0.5}$ values for S780A were also apparently slightly larger than WT for the same reason. It is known that the $I_{0.5}$ values increase with increasing concentrations of PEP through heterotrophic interaction between these ligands.

Kinetic properties of double mutant S780A/K835G
In order to obtain an enzyme with high affinity to PEP and low sensitivity to allosteric inhibitors, a double mutant PEPC S780A/K835G was produced. The $S_{0.5}$ value increased about 2-fold as compared with S780A, but it was still 40% of WT (Fig. 3A; Table 2). Sensitivity to malate and aspartate was almost completely lost, although the extent of slight inhibition was larger for malate than aspartate (Fig. 2). Measurement of activity at higher concentrations of the inhibitors up to 40 mM did not give $I_{0.5}$ values for both inhibitors (Table 1). Although stronger desensitization was observed for S780A/K835G than K835G, this appears to be due to the difference in their $S_{0.5}$ values. In fact, when the concentration of PEP was lowered to 0.5 mM for S780A/K835G so as to bring the extent of PEP saturation equal to K835G at 2 mM, the inhibition curve of S780A/K835G was almost superimposable on the curve of K835G (Fig. 2A, B). The sensitivities to allosteric activators, glucose 6-phosphate and glycine, were not affected by the introduced mutations (Tables 1, 2).

### Discussion
In combination with our previous X-ray crystallographic analysis (Kai et al., 1999), the present study using site-directed mutagenesis strongly suggests that the two conserved basic residues, Lys-835 and Arg-894 of maize C$_4$-form PEPC, are involved in the binding with the allosteric inhibitors. The mutant enzymes, which had been desensitized to the negative feedback inhibitors but retained their catalytic activity, may be useful for a variety of metabolic engineering approaches. Although simultaneous desensitization to aspartate and malate was always observed by the replacement of a single amino acid residue, the degree of inhibition by malate was always larger than that of aspartate (Fig. 3). This tendency is presumably due to the interaction of malate at the catalytic site as a product analogue at higher concentrations.

Blasing et al. (2000) showed that the $S_{0.5}$ value for PEP decreased by about one-third when the C$_4$-characteristic...
Ser-774 of a C_4-form PEPC in _F. trinervia_ was replaced with Ala which is conserved in non-C_4-type PEPCs. It was also observed here with the maize C_4-form PEPC. It was also observed that the same replacement of the corresponding Ser-780 diminished the S_{0.5} value for PEP to one-quarter. Thus the role of this Ser was established for both dicot and monocot C_4-form PEPCs.

By the introduction of a double mutation S780A/K835G, we succeeded in producing an enzyme with low S_{0.5} and high I_{0.5}, demonstrating that these properties could be conferred simultaneously to PEPC. This mutant enzyme may be of use to make a C_4-like cycle in C_3 plants. To date, two different enzyme forms that would be less down-regulated and would show strong activity in plant cells have been used: one study exploited an engineered potato PEPC (Rademacher _et al._, 2002) and the other a PEPC from the cyanobacterium _Synechococcus vulcanus_ (SvPEPC) (Chen _et al._, 2004). In the former case, the N-terminal truncated enzyme or the enzyme mutated at the regulatory phosphorylation site (Ser to Asp) was used. Both modifications resulted in enzymes with lowered sensitivity to malate inhibition. In the latter case, recombinant SvPEPC was malate/aspartate-insensitive in the normal cytosolic pH range (Chen _et al._, 2004). Although these modifications and protein sources were also available for metabolic engineering, maize PEPC was considered to be necessary for this technology, especially in applying it to rice. Since an extremely high level of expression was achieved in rice by the introduction of partial maize genomic DNA containing the gene for the C_4-form PEPC (Miyao and Fukayama, 2003), this system will be quite useful for the expression of the engineered PEPC developed here. To construct the plasmid, a fragment (approximately 4 kb in size) of genomic DNA of the Ppc gene containing exons 8 and 9 where the mutation sites reside is subcloned and mutagenized by a conventional method, and then the mutagenized fragment is inserted back to the original plasmid.

Recently it was shown that, in rice, in which the maize PEPC was highly expressed, a nocturnal phosphorylation of PEPC was observed as an unexpected result, while the diurnal phosphorylation as in C_4 plants was not (Fukayama _et al._, 2006). Because the malate-sensitivity of PEPC is altered by phosphorylation on its conserved Ser residue, the non-phosphorylated daytime form PEPC was thought to be less active in the transgenic rice. To avoid this problem, the phosphorylation-independent malate-insensitive feature is necessary for ideal PEPC functioning in rice C_3 cell environments.

Figure 4 shows our present strategy for the enhancement of photosynthetic CO_2 fixation in a C_3 plant, tobacco. In scheme (A), the engineered maize C_4-form PEPC and maize PEP carboxykinase (PCK) (Furumoto _et al._, 1999) are both expressed in the chloroplast, so as to convert HCO_3^- to CO_2 at the expense of energy. Since Rubisco utilizes CO_2 and not HCO_3^- as a substrate, an increase in CO_2 supply to the microdomain around Rubisco would facilitate its CO_2-fixation reaction. Scheme (B) shows a minimal C_4-like pathway to be installed. Bicarbonate (HCO_3^-) in the cytoplasm is fixed onto PEP to produce oxaloacetate (OAA), and then OAA is transported into chloroplasts by a translocator, for example, a malate/Pi exchange translocator (Eastmond _et al._, 1997).

<table>
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<th>PEP (+5 mM G6P)</th>
<th>PEP (+ 5 mM Gly)</th>
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Table 2. Kinetic parameters for PEP and Mg^{2+} of maize mutant PEPCs in the presence and absence of allosteric activators

Assay conditions are the same as described in the Materials and methods. V_{max} denotes maximal activity (unit mg^{-1} protein) attainable at infinite concentrations of ligands. S_{0.5} denotes ligand concentration (mM) required for 50% of V_{max}. nd, not determined.
or a dicarboxylate transporter (Jeong et al., 2004). In the chloroplasts, OAA is decarboxylated to supply CO₂ to Rubisco by PCK. PEP formed in the chloroplasts is exported to the cytoplasm as the substrate for PEPC, by the action of a PEP/Pi translocator (Knappe et al., 2003). We are planning to introduce not only the enzymes but also the translocators into a C₃ plant to complete the cycle. Although several systems of C₄ photosynthesis in single cells have been envisaged and no successful improvement of photosynthetic productivity has been achieved yet, there still remains many trials to be done before this idea is abandoned (Mitchell and Sheehy, 2006). It should be emphasized that no work has yet been performed that introduced metabolite translocators to complete the C₄-like cycle.

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References


