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Phylogenic and phosphorylation regulation difference of phosphoenolpyruvate carboxykinase of C3 and C4 plants

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ABSTRACT

In C4 plants, phosphoenolpyruvate carboxykinase (PEPCK) plays a key role in the C4 cycle. PEPCK is also involved in gluconeogenesis and is conserved in both lower and higher organisms, including in animals and plants. A phylogenic tree constructed from PEPCK sequences from bacteria to higher plants indicates that the C4 Poaceae PEPCKs are conserved and have diverged from the PEPCKs of C3 plants. The maximum enzymatic activities of wild-type and phosphorylation mimic PEPCK proteins indicate that there is a significant difference between C3 and C4 plant PEPCKs. The conserved PEPCK phosphorylation sites are regulated differently in C3 and C4 plants. These results suggest that the functions of PEPCK have been conserved, but that sequences have diverged and regulation of PEPCK is important in C4 plants, but not in herbaceous and, in particular, woody C3 plants.

1. Introduction

Phosphoenolpyruvate carboxykinase (PEPCK) is an enzyme that catalyzes the reversible reactions, decarboxylation of oxaloacetate (OAA) and carboxylation of phosphoenolpyruvate (PEP) (Hatch and Mau, 1973). The decarboxylation of OAA, catalyzed by PEPCK, plays a crucial role in a process fundamental to C4 plants- the C4 cycle of photosynthesis, in which the concentration of CO_2 is increased in bundle sheath (BS) cells, minimizing photosynthesis and increasing the efficiency of photosynthesis. Based on the type of enzyme utilized in the decarboxylation phase of the C4 cycle, C4 plants are classically divided into three subgroups: NADP-malic enzyme (ME)-type, NAD-ME-type, and PEPCK-type (Hatch et al., 1975). In PEPCK-type plants, PEPCK is present in the bundle sheath (BS) cells, where it catalyzes the decarboxylation of OAA and supplies CO_2 to the Calvin cycle (Hatch and Osmond, 1976; Walker et al., 1997). Decarboxylase activity of PEPCK has also been detected in more and more NADP-ME-type and NAD-ME-type species, such as maize, *Flaveria* species, *Cleome gynandra*, and Guinea grass (Christin et al., 2011; Koteyeva et al., 2015; Walker et al., 1997; Wang et al., 2014). As PEPCK activity has been detected in the BS cells in several C4 plants, the PEPCK pathway is thought to be an accessory pathway for the NADP-ME and NAD-ME pathways. It has been hypothesized that combining PEPCK with either NADP-ME or NAD-ME could robustly maintain high photosynthetic efficiency under a broad range of light regimes because of the decreasing need to maintain high concentrations and concentration gradients of transport metabolites (Wang et al., 2014).

The functions of PEPCK are not limited to the C4 cycle. The PEPCK-catalyzed conversion of OAA to PEP is an early and rate-limiting step in the gluconeogenesis pathway in all plants, and PEPCK plays an important role in different stages of plant growth and development. PEPCK may be involved in the conversion of fats to sugars during seed germination (Leegood and ap Rees, 1978; Martín et al., 2007), the accumulation of soluble sugars and dissimilation of organic acids during fruit ripening (Famiani et al., 2016; Huang et al., 2015a,b), and the metabolism of nitrogenous assimilates and the increase in seed protein content (Beihaghi et al., 2015; Delgado-Alvarado et al., 2007; Leegood and Walker, 2003). It has been proven that PEPCK plays an important role in malate metabolism, which is significant during dark-induced stomatal closure (Penfield et al., 2012). In rice leaves, PEPCK is present in the stomata, hydathodes, and parenchyma cells close to the xylem and phloem, where it contributes to nitrogen recycling (Bailey and Leegood, 2016).

PEPCK also plays important roles in species other than plants. In fact, PEPCK is an ancient enzyme present in all known groups of living organisms (Aich and Delbaere, 2007). PEPCKs are traditionally divided into two groups based on nucleotide substrate specificity: ATP-dependent PEPCKs, which are mainly present in

Abbreviations: PEPCK, phosphoenolpyruvate carboxykinase; OAA, decarboxylation of oxaloacetate; PEP, carboxylation of phosphoenolpyruvate; ME, malic enzyme; BS, bundle sheath.

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bacteria, yeast, and plants, and GTP-specific PEPCKs, which are mostly present in higher eukaryotes, major archaea, and also in some bacteria (Aich and Delbaere, 2007; Fukuda et al., 2004). Interestingly, significant sequence homology is found among the members of each group, whereas there is no statistically remarkable homology between the PEPCKs from the two different classes (Fukuda et al., 2004). Furthermore, based on structural evidence, the oxaloacetate-binding and metal-binding active site residues are highly conserved in both ATP- and GTP-dependent PCKs (Cotelesage et al., 2005; Dunten et al., 2002; Holyoak and Nowak, 2001; Leduc et al., 2005; Matte et al., 1996; Sudom et al., 2001; Trapani et al., 2001). In 2015, PPi-PEPCK was identified for the first time from *Entamoeba histolytica*, a eukaryotic human parasite (Chiba et al., 2015).

The activity of PEPCK is regulated by many kinds of external and internal factors, such as light, biotic and abiotic stress, and the pH value of the system. The phosphorylation of PEPCK has been observed in all CAM leaves and C3-tissues that have been studied to date and also in some C4 leaves (Walker et al., 1997; Walker and Leegood, 1996). In Guinea grass, PEPCK is dephosphorylated in illuminated leaves and phosphorylated in dark-adapted leaves (Walker and Leegood, 1996). PEPCK is also phosphorylated in the cotyledons and endosperm of many germinating seeds. For example, in cucumber (*Cucumis sativus* L.) cotyledons, dephosphorylation of PEPCK is stimulated by illumination (Walker and Leegood, 1995, 1996). The phosphorylation of PEPCK was also detected in the ripening fruit flesh of grapes, tomatoes, cherries and plums (Walker et al., 2016). Using mass spectrometry, four phosphorylated residues (Ser55, Thr58, Thr59, and Thr120) were identified in the maize PEPCK protein, ZmPCK1 (Chao et al., 2014), and the phosphorylation of these four residues was positively regulated by light. Because the decarboxylase activity of PEPCK in leaves is higher in the dark than in the light, it is possible that the depressing effect of light on PEPCK decarboxylation activity might be mediated by reversible phosphorylation (Chao et al., 2014).

The catalytic action of PEPCK is conserved across species but it has also undergone significant divergence, playing different roles in different species. In plants, PEPCK has evolved functions in the C4 cycle, but it is not clear how these functions have arisen. In this study, our goal was to answer two key questions regarding PEPCK evolution. What kinds of changes in PEPCK allowed it to become a key enzyme in C4 photosynthesis, and is the regulation of PEPCK evolutionarily conserved? To answer these questions, we examined the evolutionary relationships between PEPCKs from representative bacteria, fungi, and C3 and C4 plant species by constructing phylogenetic trees, identifying protein sequences that may be responsible for differential regulation of PEPCKs, and comparing the decarboxylase activity of PEPCKs from selected C4 and C3 plants *in vitro*. We found that the maximum enzymatic activities of PEPCKs from C4 plants and the woody C3 plant, *Populus trichocarpa*, were significantly higher than those in C3 herbs. Because we found divergence in the N-terminal region containing known PEPCK phosphorylation sites, we also assayed the decarboxylation activity of specific amino acid mutants of PEPCKs that mimic phosphorylation. Our findings suggest that although plant PEPCKs are ancient enzymes descended from bacteria, there has been considerable divergence in the N-terminal region that has likely led to divergence in the regulation of PEPCK activity by phosphorylation.

2. Materials and methods

2.1. Sequence retrieval and phylogenetic analysis

The PEPCK coding sequences (CDS) and protein sequences used in this study were retrieved from the Phytozome database (v11.0, http://phytozome.jgi.doe.gov) and the NCBI protein database (http://www.ncbi.nlm.nih.gov/protein). The ID and CDS of PEPCKs are listed in Supplementary Table S1.

CDS of PEPCKs were aligned using CLUSTAL-X program (version 1.83) with the default parameters. Only positions with column scores over 15 were included in the following analysis. Statistical confidence for the evolutionary trees was assessed by bootstrap (1000 replicates). The phylogenetic tree was drawn using the MEGA7 program.

2.2. PEPCK *cloning and site-directed mutagenesis*

The full-length *PEPCK* coding sequences were amplified from cDNA obtained from the leaves of wild-type *Z. mays*, *S. bicolor*, *M. maximus*, *O. sativa*, *P. trichocarpa*, and *A. thaliana*.

The constructs for expression of PEPCK in bacteria were generated by cloning the cDNA into the vector pET28a (Novagen). Site-directed mutagenesis was performed using a double-stranded plasmid mutagenesis kit according to the manufacturer's protocol (QuickChange Lightning Site-Directed Mutagenesis Kit; Agilent Technologies). Mutations were verified by two independent sequence analyses of the same DNA strand.

The primers used for cDNA cloning, generating expression constructs, and site-directed mutagenesis are listed in Supplementary Table S2.

2.3. *Protein expression and* in vitro *enzyme assays*

Recombinant PEPCK proteins were expressed in *Escherichia coli* strain BL21 (RIL) and purified using an immobilized metal affinity resin (Profinity IMAC Ni-Charged Resin; Bio-Rad) as previously described (Jiang et al., 2016).

PEPCK decarboxylase activities of the purified recombinant proteins were measured using a previously described method (Chao et al., 2014) with some modifications. The decarboxylase activity reaction mixture (800 μl) contained 40 mM HEPES-KOH (pH 8.0), 0.25 mM ATP, 0.5 mM $MnCl_2$, and 0.1 mM oxaloacetate (Sigma). After incubation for 10 min at 25 °C, reactions were stopped by heating at 100 °C for 2 min. To determine the amount of PEP generated, 200 μl of each reaction solution was mixed with 800 μl of 30 mM K_2HPO_4/20 mM NaH_2PO_4 (pH 7.0), 10 mM $MgCl_2$, 1 mM ADP, 1.5 mM NADH, and 6 U lactate dehydrogenase (Sigma). Each PEP assay was initiated by addition of 0.5 U pyruvate kinase (Sigma; Walker and Leegood, 1995).

Decarboxylase activity was determined spectrophotometrically at 25 °C by following NADH oxidation at 340 nm. One unit of PEPCK activity corresponds to the production of 1 μmol/min NAD^+ at 25 °C. Carboxylase activity was calculated as $Vt \times \Delta A/\Delta t \times \varepsilon \times L \times Vs \times Pc$, where ΔA is the change in absorbance at 340 nm, Δt is the change in time, Vt is the total volume of the reaction mixture, Vs is the volume of the protein extract, Pc is the concentration of total protein in the extract, and L is the cuvette optical path. The extinction coefficient (ε) of NADH is 6220 M^{-1} cm^{-1}. To ensure the accuracy of the results, the enzyme activity of each reaction was measured every 30 s, and denatured crude extracts were used as a control. For the decarboxylation assay, the change in absorbance for the pyruvate kinase assay represents only a quarter (200 μl/800 μl) of each protein extraction sample. Therefore, the total decarboxylase activity was corrected by multiplying the value of the formula given above by four.