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cDNA cloning and characterization of a C4 PEPC kinase from *Flaveria trinervia*

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1. Introduction

In C_4 plants, phospho*enol*pyruvate carboxylase (EC 4.1.1.31; PEPC) plays a key role in photosynthetic CO_2 fixation. The enzyme activity is controlled by reversible phosphorylation at its conserved Ser residue near the *N*-terminus in response to light (Vidal *et al*., 1997) and other environmental stimuli (Ueno *et al*., 2000). The phosphorylation state of PEPC is considered to be largely modulated by an activity of protein kinase specific to PEPC (PEPC-PK; McNaughton *et al*., 1991).

The regulatory phosphorylation of PEPC is also observed with CAM plants and C_3 plants. Recently, cDNA clones for PEPC-PK were obtained from these plants and characterized (Hartwell *et al*., 1999; Taybi *et al*., 2000). However, the cDNA has not yet been cloned from C_4 plants.

Flaveria is a genus composed of C_3 and C_3-C_4 intermediate species, as well as C_4 species (Ku *et al*., 1991), and hence becoming valuable model plants for the study of evolution and development of C_4 photosynthesis. Here, we report the first isolation and characterization of a cDNA for PEPC-PK from a dicot C_4 plant, *Flaveria trinervia* (FtPEPC-PK). We show that this molecular species of PEPC-PK is mainly involved in the regulatory phosphorylation of the PEPC for C_4 photosynthesis (C_4-form PEPC). We also discuss the possibility that the gene for PEPC-PK has co-evolved with the gene for PEPC in the course of evolution of C_4 photosynthesis in *Flaveria*.

2. Materials and Methods

2.1 cDNA cloning

For construction of cDNA library, total RNA was extracted from leaves of *F. trinervia* at noon. Using purified poly(A)$^+$-rich RNA, the cDNA library was prepared by the cDNA synthesis kit (Amersham Pharmacia). By screening cDNA library with a *F. trinervia* PEPC-PK fragment prepared by PCR with degenerate primers, thirty-four positive clones were obtained from 3.0×10^5 plaques. Partial sequence analysis indicated that 26 clones in 34 were derived from the same single gene encoding PEPC-PK.

2.2 Assay of recombinant FtPEPC-PK activity

An expression plasmid for FtPEPC-PK was constructed using pET-43b(+) as a vector. A FtPEPC-PK protein fused with NusA protein was expressed in *E. coli* BL21, and purified by a Hitrap Chelating HP column as described in (Ueno *et al*., 1997). Phosphorylation reactions were conducted at 30˚C for 15 min in a reaction mixture (Ueno *et al*., 1997) with 1 μg protein kinase substrate (PEPC, α̃casein, histone IIIS from Sigma) and 70 ng enzyme.

2.3 Genomic-Southern blot analysis

Five microgram each of genomic DNA from *F. trinervia* (C_4) or *F. pringlei* (C_3) was digested with *Eco*RI, *Bam*HI, *Sac*I or *Xba*I and separated by electrophoresis. The DNA-transferred membranes were hybridized with a PCR-generated probe in the coding region of *F. trinervia PEPC-PK* from cDNA, and washed with 0.1 ×SSPE, 0.1% SDS at 65˚C.

2.4 Northern-blot analysis

Ten microgram each of total RNA from each tissue of *F. trinervia* (C_4) or *F. pringlei* (C_3) was separated on 1.2% agarose gel, and then transferred to nylon membranes. The membranes were hybridized with the same probe as used in genomic-Southern analysis or *Rbcs* probe prepared from the coding region of *F. trinervia Rbcs*, and then washed with 0.1 ×SSPE, 0.1% SDS at 60˚C.

3. Results and Discussion

3.1 Sequence analysis

Isolated FtPEPC-PK clone contained an open reading frame for a protein of 281 amino acid residues with a molecular mass of 31.8 kDa. As previously noted for other PEPC-PKs from C_3 and CAM plants (Hartwell *et al*., 1999; Taybi *et al*., 2000), FtPEPC-PK also showed the highest consensus to the kinase domains of CDPKs and calmodulin-dependent protein kinases (CaMKs), though regulatory regions inherent in these enzymes are lacking. Notably, phylogenetic analysis showed that amino acid sequence of FtPEPC-PK is most similar to that from a C_3 plant, tomato, rather than those from CAM plants, ice plant and *Kalanchoe*.

3.2 Property of FtPEPC-PK protein

The recombinant FtPEPC-PK efficiently phosphorylated C_4-form PEPC from maize, but not phosphorylated a mutant PEPC whose phosphorylatable Ser residue had been substituted for Asp (S15D) as observed previously with native PEPC-PK from maize (Ueno *et al*., 1997) (Fig. 1A). FtPEPC-PK was Ca^{2+}-independent as observed by its insensitivity to Ca^{2+} or EGTA (Fig. 1B). FtPEPC-PK did not phosphorylate conventional substrate of protein kinases such as casein and histone IIIS, while a recombinant CDPK from rice (OsCDPK7; Saijo *et al*., 2000) phosphorylated them quite efficiently (Fig. 1C). The wild-type PEPC was a poor substrate for OsCDPK7. Thus FtPEPC-PK has quite high substrate specificity for PEPC as already noted with maize (Wang and Chollet, 1997), and substrate recognition mechanism of PEPC-PK and CDPK seems quite different from each other. Finally, the interesting effect of oxidant and reductant on the FtPEPC-PK activity was found as shown in Fig. 1D. An inclusion of DTT in the reaction mixture caused pronounced activation of FtPEPC-PK, in contrast, oxidized-form glutathione (GSSG) inhibited the enzyme as observed with PEPC-PK purified from maize (Saze et al., in preparation).

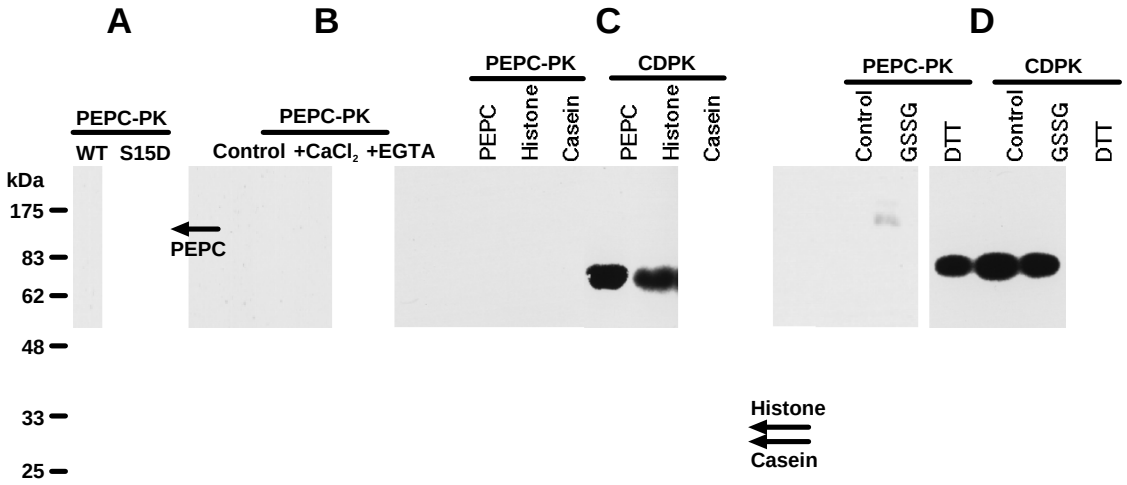

Fig. 1 . Phosphorylation assay by FtPEPC-PK. (A) Phosphorylation of recombinant wild-type and S15D mutant PEPCs from maize by FtPEPC-PK. (B) The effect of 0.2 mM $CaCl_2$ or 1 mM EGTA on the phosphorylation of PEPC byFtPEPC-PK to PEPC. (C) Comparison of substrate specificity between FtPEPC-PK and OsCDPK. PEPC, histone IIIS and casein were subjected to the phosphorylation reaction. (D) Effect of 5 mM GSSG or 1 mM DTT on the activity of FtPEPC-PK and OsCDPK.

3.3 Searching for FtPEPC-PK relatives in F. trinervia *(C_4) and* F. pringlei *(C_3)*

Genomic-Southern blot analysis of *F. trinervia* and *F. pringlei*, using a probe in the coding region of *F. trinervia PEPC-PK* from cDNA, indicated that *PEPC-PK* exists as a small multiple-copy gene family and this was also the case with *F. pringlei* (Fig. 2). In other words, the *FtPEPC-PK* gene and several close relatives have not changed largely after divergence of C_3 and C_4 species.

3.4 Tissue specific expression pattern of FtPEPC-PK

The transcript, approximately 1.5 kb in size, was found to be abundant in leaves, whereas it was very weakly expressed in stems and roots (Fig. 3A). The transcript level in leaves was high at noon but negligibly low at midnight, suggesting that the expression is under strict diurnal control. These expression patterns are very similar to those of the C_4-form PEPC (*ppcA*; Ernst *et al*., 1997), thus we concluded that this FtPEPC-PK is mainly involved in the phosphorylation of the C_4-form PEPC.

3.5 Comparison of the expression level of PEPC-PK *in* F. trinervia *(C_4) with that in* F. pringlei *(C_3)*

As shown in Fig. 3B, the transcript level in leaves of *F. trinervia* was quite high, whereas that of *F. pringlei* was below detection. The result clearly establishes that there is at least one gene of *PEPC-PK* specified for the C_4 photosynthesis in *F. trinervia* as judged from the expression level. Since similar results had already been obtained for the *PEPC* gene (Ernst *et al*., 1997), we suggest that this gene for *PEPC-PK* had seemingly co-evolved with the gene for *PEPC* during the course of evolution to acquire C_4 photosynthesis in *Flaveria*.

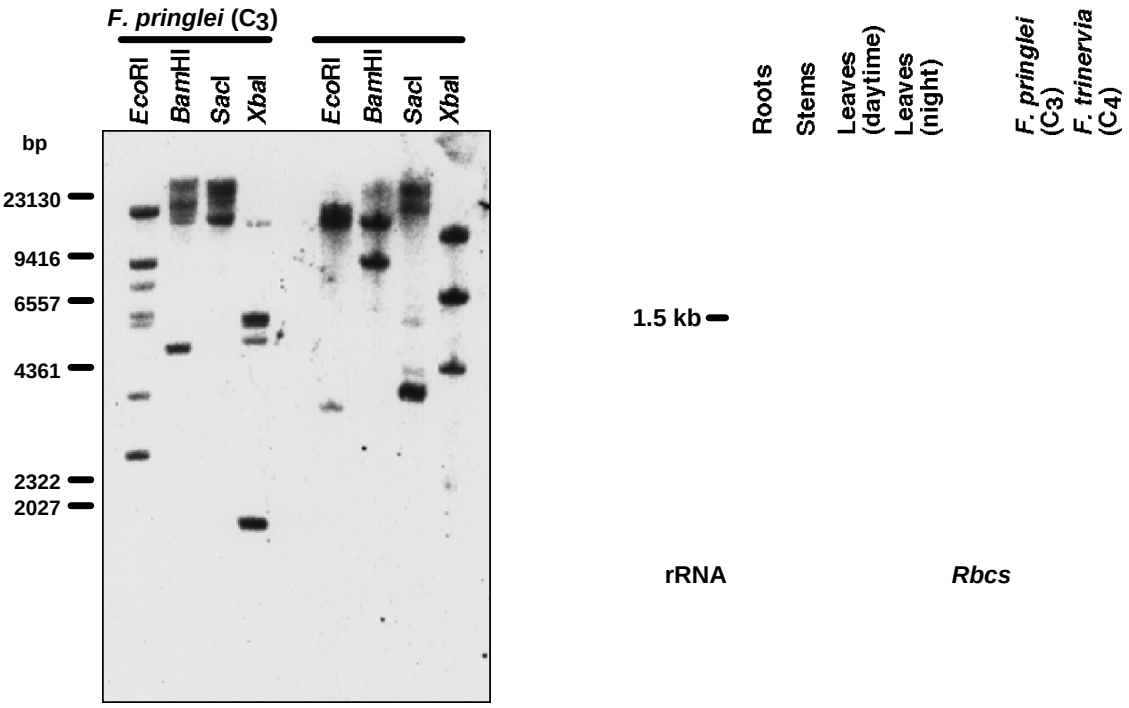

Fig. 2. Genomic-Southern blot analysis of the *PEPC-PK* gene in the *F. trinervia* (C_4) and *F. prenglei* (C_3). The molecular weight markers are shown on the left.

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