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Phospho*enol*pyruvate carboxylase kinase involved in C_4 photosynthesis in *Flaveria trinervia*: cDNA cloning and characterization[1]

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Abstract In C_4 plants, phospho*enol*pyruvate carboxylase (PEPC; EC 4.1.1.31), a key enzyme in C_4 photosynthesis, is controlled by reversible phosphorylation of a conserved Ser residue near the N-terminus. We now report the first cloning of a cDNA from a C_4 plant, *Flaveria trinervia*, which encodes the specific protein kinase (FtPEPC-PK) involved in the phosphorylation of C_4-form PEPC. Several lines of supportive evidence are: strict substrate specificity of the recombinant enzyme, prominent light/dark response of the transcript level and abundant expression in leaves of C_4 plant (*F. trinervia*) but very low expression in a C_3 plant of the same genus (*Flaveria pringlei*). We also discuss the possibility that the *FtPEPC-PK* gene has co-evolved with the *PEPC* gene to participate in C_4 photosynthesis. © 2001 Published by Elsevier Science B.V. on behalf of the Federation of European Biochemical Societies.

Key words: C_4 photosynthesis; Phospho*enol*pyruvate carboxylase; Phospho*enol*pyruvate carboxylase kinase; Regulatory phosphorylation; *Flaveria trinervia*

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, being activated by glucose 6-phosphate and inhibited by L-malate [1,2]. This susceptibility is further modified by reversible phosphorylation at its conserved Ser residue near the N-terminus in response to light [2] and other environmental stimuli [3,4]. The phosphorylation state of PEPC is considered to be largely modulated by an activity of a protein kinase specific to PEPC (PEPC-PK), since the activity of protein phosphatase 2A involved in PEPC dephosphorylation seems to remain constant under various conditions [5,6].

The regulatory phosphorylation of PEPC is also observed with Crassulacean acid metabolism (CAM) plants [7] and C_3 plants [8,9]. Recently, cDNA clones for PEPC-PK were obtained from these plants and characterized [10,11]. 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 [12], and hence becoming valuable model plants for the study of evolution and development of C_4 photosynthesis. Here, we report the first cloning and characterization of a cDNA for PEPC-PK from a dicot C_4 plant, *Flaveria trinervia*. The involvement of this PEPC-PK in the regulatory phosphorylation of PEPC for C_4 photosynthesis (C_4-form PEPC) was clearly indicated. We also discuss the possibility that at least one of the genes for PEPC-PK has co-evolved with the gene for C_4-form PEPC during the course of acquiring C_4 photosynthesis ability in *Flaveria*.

2. Materials and methods

2.1. Cloning of PEPC-PK from Flaveria trinervia

F. trinervia plants were grown in a glasshouse for 2 months at 23°C day and night. A cDNA library was constructed by a cDNA synthesis kit (Amersham Pharmacia), using poly(A)$^+$-rich RNA extracted from leaves at noon. For screening this λgt10 library, approximately 3.0×10^5 phages were plated with *Escherichia coli* NM514, and blotted onto nylon membrane (Hybond-N$^+$, Amersham Pharmacia). The cDNA library was screened with a *PEPC-PK* fragment of *F. trinervia*, which had been amplified by polymerase chain reaction (PCR) with degenerate primers. Degenerate oligonucleotide primers with attached restriction sites were designed for two conserved regions among previously cloned PEPC-PKs of C_3 and CAM plants [10,11]. The forward primer, 5′-GGAATTCTG(T/C)GA(A/G)GA(A/G)AT(T/C/A)GG-3′ corresponded to CEEIG, and the reverse primer, 5′-GCGGATCC(T/C)TC(T/C/G/A)GG(T/C/G/A)GC(T/C/G/A)AC(A/G)TA(A/G)TA-3′, to YYVAPEV. The former motif in subdomain I is characteristically conserved among PEPC-PKs, while the latter motif in subdomain VIII is conserved in both PEPC-PKs and calcium-dependent protein kinases (CDPKs) (Fig. 1). The amplification reaction was carried out for 40 cycles (a 94°C denaturing cycle for 80 s, a 60°C annealing cycle for 1 min, and a 72°C extension cycle for 1 min). The amplified fragment (520 bp) was radiolabeled with [α-^{32}P]dCTP, and then hybridized on the membranes at 65°C for 1 day in a standard solution [13]. The membranes were washed in 2× saline–sodium phosphate–EDTA buffer (SSPE), 0.1% sodium dodecyl sulfate (SDS), and 0.1×SSPE, 0.1% SDS at 65°C. Thirty-four positive clones were obtained from 3.0×10^5 plaques. Plaque PCR with degenerate primers has revealed that 26 clones in 34 contained *PEPC-PK* cDNA. All clones had identical 3′-non-coding region (data not shown), indicating that 26 clones were derived from the same single gene encoding PEPC-PK. The longest (1459 bp) was fully sequenced, and designated *FtPEPC-PK*.

2.2. Expression and purification of the recombinant FtPEPC-PK

An expression plasmid for FtPEPC-PK was constructed using pET-43b(+) (pET system, Novagen) as a vector. With two oligo-primers,

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Abbreviations: CAM, Crassulacean acid metabolism; CaMK, calmodulin-dependent protein kinase; CDPK, calcium-dependent protein kinase; DTT, dithiothreitol; GSSG, oxidized-form glutathione; PEPC, phospho*enol*pyruvate carboxylase; PEPC-PK, phospho*enol*-pyruvate carboxylase kinase

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5′-CAGAGCTCATGAAGGAAACTCTGAAC-3′, and 5′-TTGAATTCTTAGGTCAGATCCGCCATTG-3′, the entire protein-coding sequence was amplified. The resulting PCR product was cloned between *Sac*I and *Eco*RI sites in the pET-43b(+). Sequencing of this plasmid confirmed that the *FtPEPC-PK* gene was correctly inserted into expected sites. An FtPEPC-PK protein fused at the N-terminal side with NusA protein was expressed in *E. coli* BL21 (DE3) cells with 1 mM isopropyl β-D-thiogalactopyranoside at 25°C for 3 h. The *E. coli* cells were harvested by centrifugation and were suspended with extraction buffer containing 20 mM sodium phosphate (pH 7.5), 0.1 M NaCl, 10 mM imidazole (pH 7.5), 15% ethylene glycol, 14 mM mercaptoethanol and 1 mM phenylmethylsulfonyl fluoride. After extraction of fusion protein from the cells disrupted with a French press, the enzyme was purified by a Hitrap Chelating HP column (Amersham Pharmacia) according to the manufacturer's protocol and the eluted protein (60–90 mM imidazole) were desalted using PD-10 column (Amersham Pharmacia).

2.3. Assay of FtPEPC-PK activity

Phosphorylation reactions were conducted at 30°C for 15 min in a reaction mixture consisting of 0.1 mM [γ-^{32}P]ATP (0.5 μCi), 50 mM Tris–HCl (pH 8.0), 5 mM $MgCl_2$, 1 μg purified recombinant maize PEPC [14] or 1 μg each of conventional protein kinase substrate (α-casein or histone IIIS from Sigma), and 70 ng purified enzyme. After the reaction, each sample was subjected to SDS–polyacrylamide gel electrophoresis (PAGE; 10% polyacrylamide), and then the radioactive bands on the gel were visualized by an imaging analyzer, BAS2000 (Fujifilm).

2.4. Southern blot analysis

5 μg each of genomic DNA from *F. trinervia* (C_4) or *Flaveria pringlei* (C_3) was digested with *Eco*RI, *Bam*HI, *Sac*I or *Xba*I and separated by electrophoresis on a 0.8% agarose gel. The DNA-transferred membranes were hybridized with a PCR-generated probe derived from the coding region (probe I, 507 bp), or a probe from the 3′-non-coding region (probe II, 357 bp) of *PEPC-PK* cDNA from *F. trinervia*. Probes I and II had sequences corresponding to the nucleotides 244–750 and 1048–1404 of the *FtPEPC-PK* (AB065100). Probe I covered the same region as used in the cDNA screening. Washes were performed with 0.1×SSPE, 0.1% SDS at 65°C.

2.5. Northern blot analysis

10 μg each of total RNA from tissues of *F. trinervia* (C_4) or *F. pringlei* (C_3) was separated on 1.2% agarose gel containing formaldehyde, and then transferred to nylon membranes. The membranes were hybridized with probe I or *RbcS* probe prepared from the coding region of *F. trinervia RbcS* (GenBank accession number X05037), and then washed with 0.1×SSPE, 0.1% SDS at 60°C.

3. Results and discussion

Isolated cDNA for FtPEPC-PK contained an open reading frame for a protein of 281 amino acid residues, with a molecular mass of 31.8 kDa, which contained minimum of the conventional protein kinase domain. In addition, rapid turn-

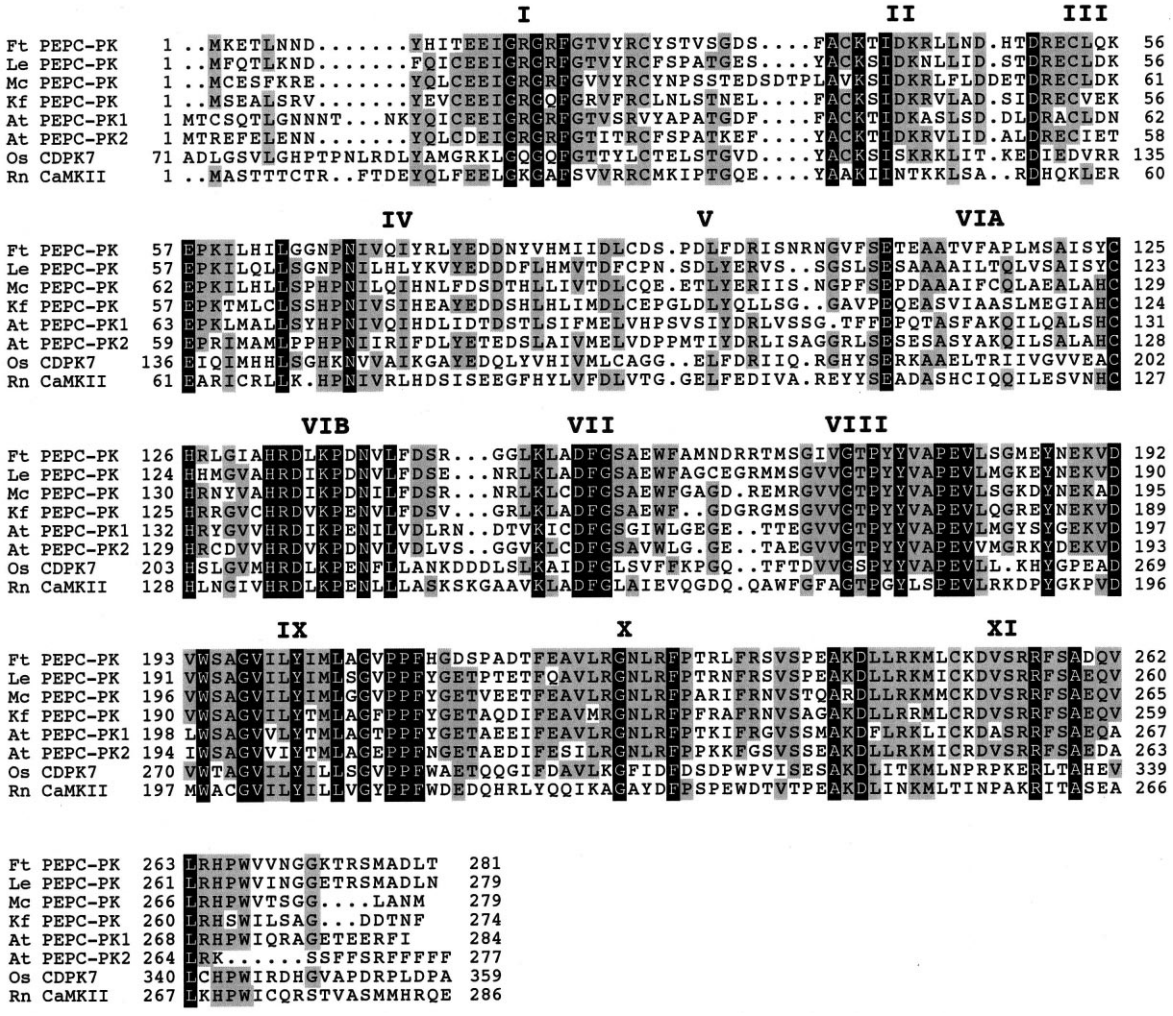

Fig. 1. Alignment of the amino acid sequences of PEPC-PKs from *Flaveria trinervia* (Ft, AB065100), tomato (Le, AF203481), ice plant (Mc, AF158091), *Kalanchoe fedtschenkoi* (Kf, AF162661) and *Arabidopsis thaliana* (At PEPC-PK1, AF162660 and At PEPC-PK2, AF358915), and the catalytic domains of CDPK from rice (Os, AB042550) and CaMKII from rat (Rn, J05072). Identical residues are dark shaded, and conserved residues are gray shaded. The twelve defined subdomains of the eukaryotic protein kinases are indicated by Roman numerals.

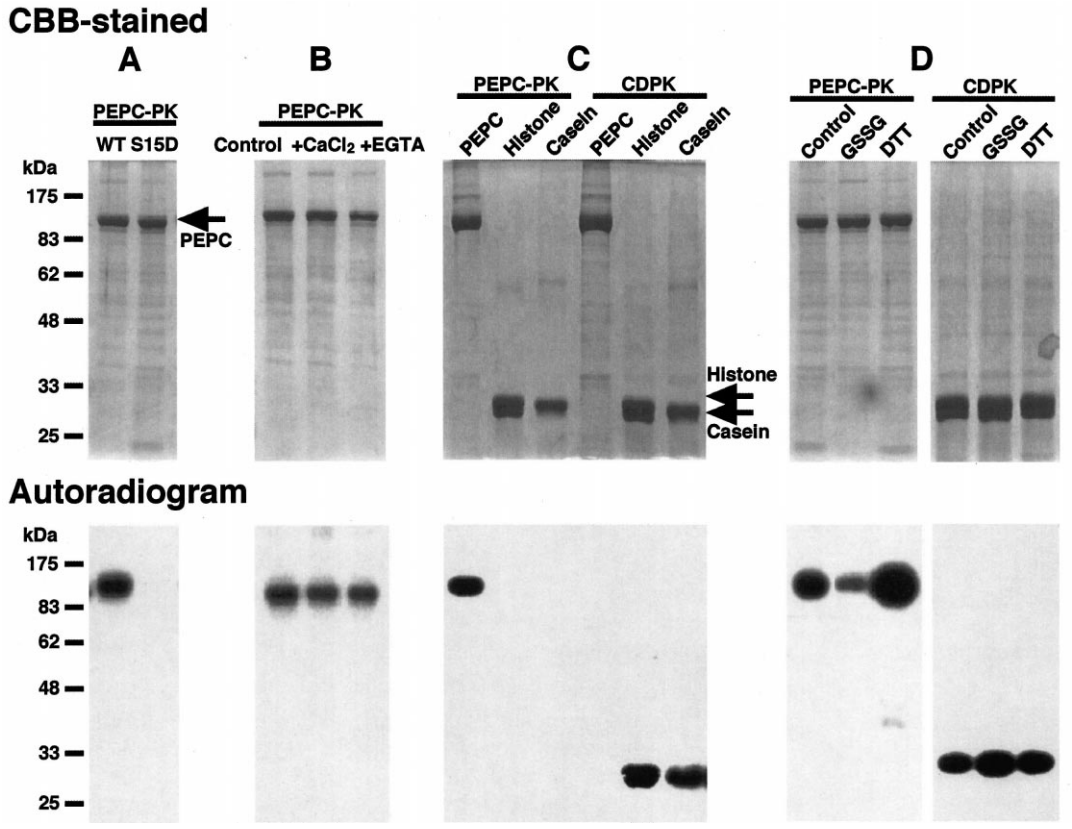

Fig. 2. Enzymatic property of FtPEPC-PK. A: Phosphorylation of recombinant wild-type and S15D mutant PEPCs from maize by FtPEPC-PK. B: The effect of 0.2 mM $CaCl_2$ or 1 mM EGTA on the phosphorylation of PEPC by FtPEPC-PK. C: Comparison of substrate specificity between FtPEPC-PK and OsCDPK. PEPC, histone IIIS and casein are subjected to the phosphorylation reaction. D:Effect of 5 mM GSSG or 1 mM DTT on the activity of FtPEPC-PK and OsCDPK. Each phosphorylation reaction was performed at 30°C for 15 min in a reaction mixture as described in the text. After reaction, the products were subjected to 10% SDS–PAGE. Upper panels show Coomassie-stained SDS–PAGE gels and lower panels show their autoradiogram.

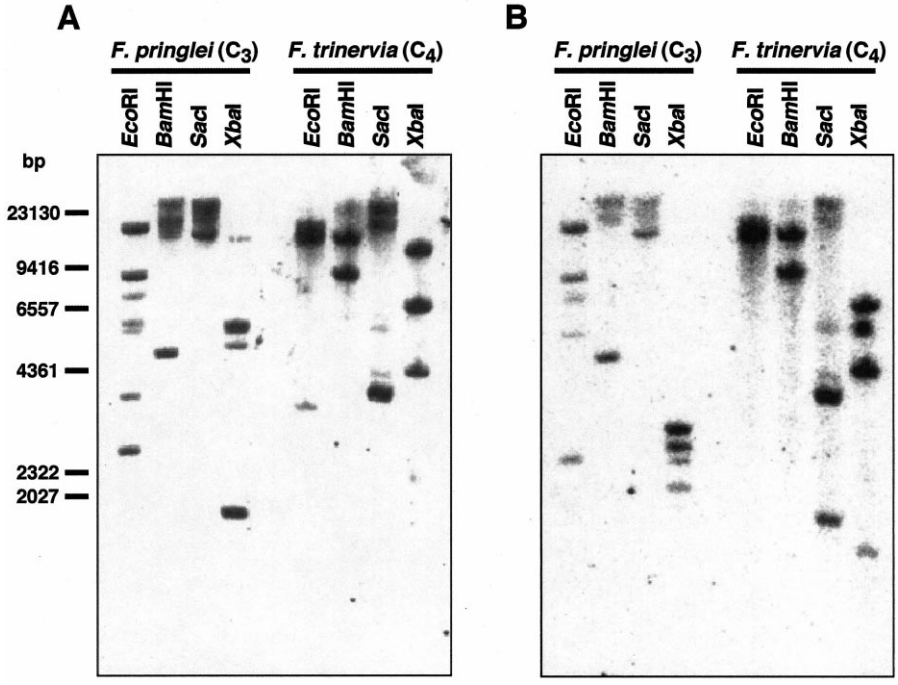

Fig. 3. Genomic Southern blot analysis of the *PEPC-PK* gene. 5 μg each of genomic DNA from *F. trinervia* or *F. pringlei* was digested with *Eco*RI, *Bam*HI, *Sac*I or *Xba*I and separated by electrophoresis. The DNA-transferred membranes were hybridized with a PCR-generated probe derived from the coding region (probe I, 507 bp) (A), or a probe from the 3′-non-coding region (probe II, 357 bp) (B) of *PEPC-PK* cDNA from *F. trinervia*. The molecular weight markers are shown on the left.

over of this mRNA was predicted as in the case of *PEPC-PK* from *Mesembryanthemum crystallinum* [11], since it contains a specific sequence motif (GAA, ATAGAT, and GTA elements) in the 3′-non-coding region which is known to be a determinant for short life of mRNAs [15]. Fig. 1 shows the alignment of the deduced amino acid sequence of FtPEPC-PK with five other PEPC-PKs and related protein kinases. As previously noted for other PEPC-PKs [10,11], the 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. The amino acid identities of FtPEPC-PK with PEPC-PKs from tomato, ice plant, *Kalanchoe* and *Arabidopsis* (*Arabidopsis*1 and *Arabidopsis*2) were 72, 60, 59, 52 and 51%, respectively. It is noteworthy that the C_4 plant PEPC-PK is most similar to that from a C_3 plant, tomato, rather than those from CAM plants, ice plant and *Kalanchoe*, in which PEPC plays a key role in CO_2 assimilation as well as in C_4 plants.

As shown in Fig. 2A, the FtPEPC-PK fused with NusA efficiently phosphorylated C_4-form PEPC from maize, but not phosphorylated a mutant PEPC whose phosphorylatable Ser residue had been substituted for Asp (S15D). FtPEPC-PK showed similar activities with or without Ca^{2+} (Fig. 2B). FtPEPC-PK did not phosphorylate conventional substrate of protein kinases such as casein and histone IIIS, while a recombinant CDPK from rice (OsCDPK7 [16]) phosphorylated them quite efficiently (Fig. 2C). The wild-type PEPC was a poor substrate for OsCDPK7. Thus FtPEPC-PK has quite high substrate specificity for PEPC as already noted with those from other plants [11,17–19], and substrate recognition mechanism of PEPC-PK and CDPK seems quite different from each other. Such site-specific activities, Ca^{2+}-insensitivity and substrate specificity of the recombinant enzyme were in accordance with previous observations using purified maize enzyme [14,17]. Finally, the interesting effect of oxidant and reductant on the FtPEPC-PK activity was found as shown in Fig. 2D. An addition of dithiothreitol (DTT) in the reaction mixture caused pronounced activation of FtPEPC-PK, in contrast, that of oxidized-form glutathione (GSSG) inhibited the enzyme as observed with PEPC-PK purified from maize (Saze et al., in preparation). To identify the cysteine residues involved in this redox-associated change of activity, we are presently in the process of carrying out site-directed mutagenesis of six cysteine residues from *F. trinervia*.

Southern blot analyses of genomic DNA isolated from *F. pringlei* (C_3) and *F. trinervia* (C_4) were performed using PCR-generated DNA fragments for the coding region (probe I, 507 bp) and for the 3′-non-coding region (probe II, 357 bp) of *FtPEPC-PK* cDNA, respectively. The PCR-amplified genomic DNA obtained by using primers for probe I was sequenced and found that it does not contain any intron and cleavage sites of *Eco*RI, *Bam*HI, *Sac*I and *Xba*I (data not shown). Even under highly stringent conditions, probe I gave more than two bands on each lane, suggesting that the *PEPC-PK* exists as a multi-copy gene in both *F. pringlei* (C_3) and *F. trinervia* (C_4) (Fig. 3A). A similar result was obtained in Southern blot analysis with probe II (Fig. 3B), though the overall intensity of the signals was weaker in *F. pringlei* (C_3) than *F. trinervia* (C_4). These results indicated that several genes, being close to *PEPC-PK*, were present in both *F. pringlei* (C_3) and *F. trinervia* (C_4).

The expression of the gene for FtPEPC-PK was next investigated by Northern blot analysis. Using probe I, the transcript, approximately 1.5 kb in size, was abundant in leaves, whereas it was very weakly expressed in stems and roots (Fig. 4A). Probe I recognizes not only this *FtPEPC-PK* but also a few closely related genes (Fig. 3A). However, this gene seems predominantly expressed in leaves, since all of the 26 clones isolated from cDNA library for leaves were from this gene. This tissue specific expression pattern is very similar to those of a gene for C_4-form PEPC (*ppcA*) [20], suggesting that this gene product be involved in the phosphorylation of the C_4-form PEPC. Furthermore, the transcript level in leaves was high at noon but negligible at midnight, suggesting that the expression is controlled diurnally.

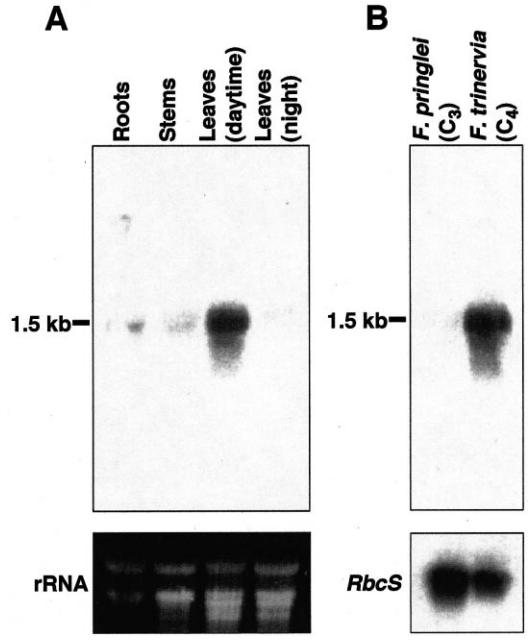

Fig. 4. Northern-blot analysis of *FtPEPC-PK*. A: Tissue-specificity and light-dependence of *FtPEPC-PK* expression in *F. trinervia*. B: Transcripts of *PEPC-PK* in leaves of *F. trinervia* and *F. pringlei* harvested during light period. 10 μg each of total RNA was separated on 1.2% agarose gel containing formaldehyde, and then transferred to nylon membranes. The membranes were hybridized with probe I. In the lower panels, rRNAs stained with ethidium bromide (A) and *RbcS* reprobed by *RbcS* cDNA from *F. trinervia* (B) are shown.

The levels of transcripts of *FtPEPC-PK* were compared between *F. trinervia* (C_4) and *F. pringlei* (C_3). As shown in Fig. 4B, the transcript level in leaves of *F. trinervia* was quite high, whereas that of *F. pringlei* was negligible. This observation is very similar to the case with the *ppcA* gene [20]. From these similarities of the transcript expression patterns, we hypothesized that this *FtPEPC-PK* may have acquired to be highly expressed in leaves as well as *ppcA* for functioning C_4 photosynthesis in *Flaveria*.

In conclusion, we isolated the *FtPEPC-PK* gene, which is suggested to be involved in regulatory phosphorylation of C_4-form PEPC, the key enzyme in C_4 photosynthesis. The present findings reinforce the physiological importance of regulatory phosphorylation of PEPC and evoke an interest in the molecular event underlying the co-evolution.

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