

Phosphoenolpyruvate carboxylase kinase involved in C₄ photosynthesis in *Flaveria trinervia*: cDNA cloning and characterization¹

Yuhei Tsuchida^a, Tsuyoshi Furumoto^b, Atsushi Izumida^{a,2}, Shingo Hata^b, Katsura Izui^{b,*}

^aLaboratory of Plant Physiology, Graduate School of Agriculture, Kyoto University, Kyoto 606-8502, Japan

^bLaboratory of Plant Physiology, Graduate School of Biostudies, Kyoto University, Sakyo-ku, Kyoto 606-8502, Japan

Received 22 August 2001; revised 28 September 2001; accepted 28 September 2001

First published online 15 October 2001

Edited by Richard Cogdell

Abstract In C₄ plants, phosphoenolpyruvate carboxylase (PEPC; EC 4.1.1.31), a key enzyme in C₄ 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 C₄ plant, *Flaveria trinervia*, which encodes the specific protein kinase (FtPEPC-PK) involved in the phosphorylation of C₄-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 C₄ plant (*F. trinervia*) but very low expression in a C₃ 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 C₄ photosynthesis. © 2001 Published by Elsevier Science B.V. on behalf of the Federation of European Biochemical Societies.

Key words: C₄ photosynthesis; Phosphoenolpyruvate carboxylase; Phosphoenolpyruvate carboxylase kinase; Regulatory phosphorylation; *Flaveria trinervia*

1. Introduction

In C₄ plants, phosphoenolpyruvate carboxylase (EC 4.1.1.31; PEPC) plays a key role in photosynthetic CO₂ 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].

*Corresponding author. Fax: (81)-75-753 6140.
E-mail address: izui@kais.kyoto-u.ac.jp (K. Izui).

¹ The nucleotide sequence data reported in this paper have been submitted to the DDBJ/EMBL/GenBank databases under the accession number AB065100.

² Present address: Kakegawa Research Center, Sakata Seed Co., Ltd., Yoshiokamizoguchi, Kakegawa, Shizuoka 436-0115, Japan.

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

The regulatory phosphorylation of PEPC is also observed with Crassulacean acid metabolism (CAM) plants [7] and C₃ 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 C₄ plants.

Flaveria is a genus composed of C₃ and C₃–C₄ intermediate species, as well as C₄ species [12], and hence becoming valuable model plants for the study of evolution and development of C₄ photosynthesis. Here, we report the first cloning and characterization of a cDNA for PEPC-PK from a dicot C₄ plant, *Flaveria trinervia*. The involvement of this PEPC-PK in the regulatory phosphorylation of PEPC for C₄ photosynthesis (C₄-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 C₄-form PEPC during the course of acquiring C₄ 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 × 10⁵ 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 C₃ 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'-GC-GGATCC(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-³²P]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 × 10⁵ 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,

5'-CAGAGCTCATGAAGGAACTCTGAAC-3', and 5'-TTGA-ATTCTTAGGTCAGATCCGCCATTG-3', the entire protein-coding sequence was amplified. The resulting PCR product was cloned between *SacI* and *EcoRI* 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 μ Ci), 50 mM Tris-HCl (pH 8.0), 5 mM MgCl₂, 1 μ g purified recombinant maize PEPC [14] or 1 μ g each of conventional protein kinase substrate (α -casein or histone H1S 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 μ g each of genomic DNA from *F. trinervia* (C₄) or *Flaveria pringlei* (C₃) was digested with *EcoRI*, *BamHI*, *SacI* or *XbaI* 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 \times SSPE, 0.1% SDS at 65°C.

2.5. Northern blot analysis

10 μ g each of total RNA from tissues of *F. trinervia* (C₄) or *F. pringlei* (C₃) 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 \times 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-

		I	II	III	
Ft PEPC-PK	1	..MKETLNND.....YHITEEIGRGRFGTVYRCYSTVSGDS...FACKTIDKRLND.HTDRECLQK	56		
Le PEPC-PK	1	..MFQTLKND.....FOICEEIGRGRFGTVYRCFSPATGES...YACKSIDKNLLID.STDRECLDK	56		
Mc PEPC-PK	1	..MCESFKRE.....YQLCEEIGRGRFGTVYRCYNPSSSTEDSDTPLAVKSIDKRLFLDDDET.DRECLDK	61		
Kf PEPC-PK	1	..MSEALSRV.....YEVCEEIGRGRFGTVYRCYNPSSSTEDSDTPLAVKSIDKRLFLDDDET.DRECLDK	56		
At PEPC-PK1	1	MTCSQTLGNNT...NKYQICEEIGRGRFGTVSRVYAPATGDF...FACKTIDKASLSD.DLDRACLDN	62		
At PEPC-PK2	1	MTREFELENN.....YQLCEEIGRGRFGTVYRCFSPATGES...YACKTIDKRVLLID.ALDRECIET	58		
Os CDPK7	71	ADLGSVLGHPTPNLRDLYAMGRKLCGGQGTITLCTELSTGVD...YACKSIDSKRLIT.KEDIEDVRR	135		
Rn CaMKII	1	..MASTTTCTR...FTDEYQLFEELKGAISVVRRCMKIPTGQE...YAKIINTKKLSA...RDHQKLER	60		
		IV	V	VIA	
Ft PEPC-PK	57	EPKILHILGGNPNIVQIYRLYEDDNYVHMIIDLCDSD.PDLFDRISNRNGVFSFTEAATVFAPLMSAISYC	125		
Le PEPC-PK	57	EPKILQLLGGNPNILHLYKVYEDDFFLHMVTDPCFN.SDLYERVS...SGSLSESAAAAILTLQVSAISYC	123		
Mc PEPC-PK	62	EPKILHLLSPHPNIIQIHNLFDSHTLLIVTDLCQE.ETLYERIIS.NGPFSEPDAAAIQCLAEALAH	129		
Kf PEPC-PK	57	EPKTMCLSSHPNIVSTHEAYEDDSSLHLIMDLCEPGLDLYQLLSG...GAVPEQEAASVIAASLMEGIAHC	124		
At PEPC-PK1	63	EPKLMALLSYHPNIVQIHDLDIDTSLSTIFMELVHPSVSIYDRLVSSG.TFFEPQTASFVAKILQALSHC	131		
At PEPC-PK2	59	EERLMAMLPHPNIIIRIFDLYETEDSLAIVMELVDPMTIYDRLISAGGRLSSESASVAKIILQALSHC	128		
Os CDPK7	136	EIQIMHHLGSHKNVVAIKGAYEDQLYVHVMELCAGG..ELFDRIIQ.RGHYSERKAAELTRIVGVVEAC	202		
Rn CaMKII	61	EARICRLK.HPNIVRLHKSISEEGFHYHFDLVGTG.GELFEDIVA.REYSEADASHCIIQILSVNHC	127		
		VIB	VII	VIII	
Ft PEPC-PK	126	HRLGLIAHRDLKPDNVLFDSE...GGLKLADFGSAEWFAMNDRRTMSGIVCTPYVVAPEVLVSGMEVNEKVD	192		
Le PEPC-PK	124	HMMGVAHRDLKPDNVLFDSE...NRLKLADFGSAEWFAGCEGRMMSGVVGCTPYVVAPEVLVLMGKEYNEKVD	190		
Mc PEPC-PK	130	HRNVVAHRDLKPDNVLFDSE...NRLKLADFGSAEWFAGAGD.REMRGVVGCTPYVVAPEVLVSGKDYNEKAD	195		
Kf PEPC-PK	125	HRRGVCHRDVKNPNVLFDSV...GRLKLADFGSAEWF...GDGRGMSGVVGCTPYVVAPEVLVQGREYNEKVD	189		
At PEPC-PK1	132	HRVGVVHRDLKPDNVLFDLRN...DTVKICDFGSGIWLGEGE...TTEGVVGCTPYVVAPEVLVMGYSYGEKVD	197		
At PEPC-PK2	129	HRCDVVRDLKPDNVLFDLVS...GGVKLCDFGSAVNLG.GE...TAEGVVGCTPYVVAPEVLVVMGRKYDEKVD	193		
Os CDPK7	203	HSLGVVHRDLKPDNVLFDLVS...GGVKLCDFGSAVNLG.GE...TFTDVVGSPYVVAPEVLL.KHYGPEAD	269		
Rn CaMKII	128	HLNGIVHRDLKPDNVLFDLVS...GGVKLCDFGSAVNLG.GE...QAWFGFACTPGVLSFPEVLRLKDFYGPVVD	196		
		IX	X	XI	
Ft PEPC-PK	193	VWSAGVILYIMLAGVPPPHGDSPADTFFAVLRGNLRFETRLFRSVSPEAKDLLRKMCKDVSRRRFSADQV	262		
Le PEPC-PK	191	VWSAGVILYIMLSGVPPPHYGETPTTETFAVLRGNLRFETRNFRSVSPEAKDLLRKMICKDVSRRRFSAEQV	260		
Mc PEPC-PK	196	VWSAGVILYIMLAGVPPPHYGETVEETFEAVLRGNLRFETARIFRNVSQARDLLRKMCKDVSRRRFSAEQV	265		
Kf PEPC-PK	190	VWSAGVILYIMLAGVPPPHYGETAQDIFEAVMRGNLRFETFRAPRNVSAGAKDLLRMLCRDVSRRRFSAEQV	259		
At PEPC-PK1	198	LWSAGVVLVYIMLAGTTPPHYGETAEEDIFEAVLRGNLRFETKIFRGVSSMAKDLRLKLIKCDASRRRFSAEQA	267		
At PEPC-PK2	194	IWSAGVILYIMLAGVPPPHYGETAEEDIFEAVLRGNLRFETPKKFGSVSSMAKDLRLKLIKCDVSRRRFSAEQA	263		
Os CDPK7	270	VWTAGVILYIMLSGVPPPHWAEQGGIFDAVLKGFIDSDPWPVISESAKDLITKMLNPRPKERLTAHEV	339		
Rn CaMKII	197	MWACGVILYIMLVGYPVPPWDEQHRLYQQIKAGAYDEFSPEWDTVTPEAKDLINKMLTINPAKRITASEA	266		
Ft PEPC-PK	263	LRHPVVVNGGKTRSMADLT	281		
Le PEPC-PK	261	LRHPVVINGGETRSMADLN	279		
Mc PEPC-PK	266	LRHPVVTSGG...LANM	279		
Kf PEPC-PK	260	LRHSWILSAG...DDTNF	274		
At PEPC-PK1	268	LRHPWIQRAGETEERFI	284		
At PEPC-PK2	264	LRK...SFFSRFFFF	277		
Os CDPK7	340	LCHPWIRDHGVAPDRPLDPA	359		
Rn CaMKII	267	LKHPVICQRSTVASMHRQE	286		

Fig. 1. Alignment of the amino acid sequences of PEPC-PKs from *Flaveria trinervia* (Ft, AB065100), 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.

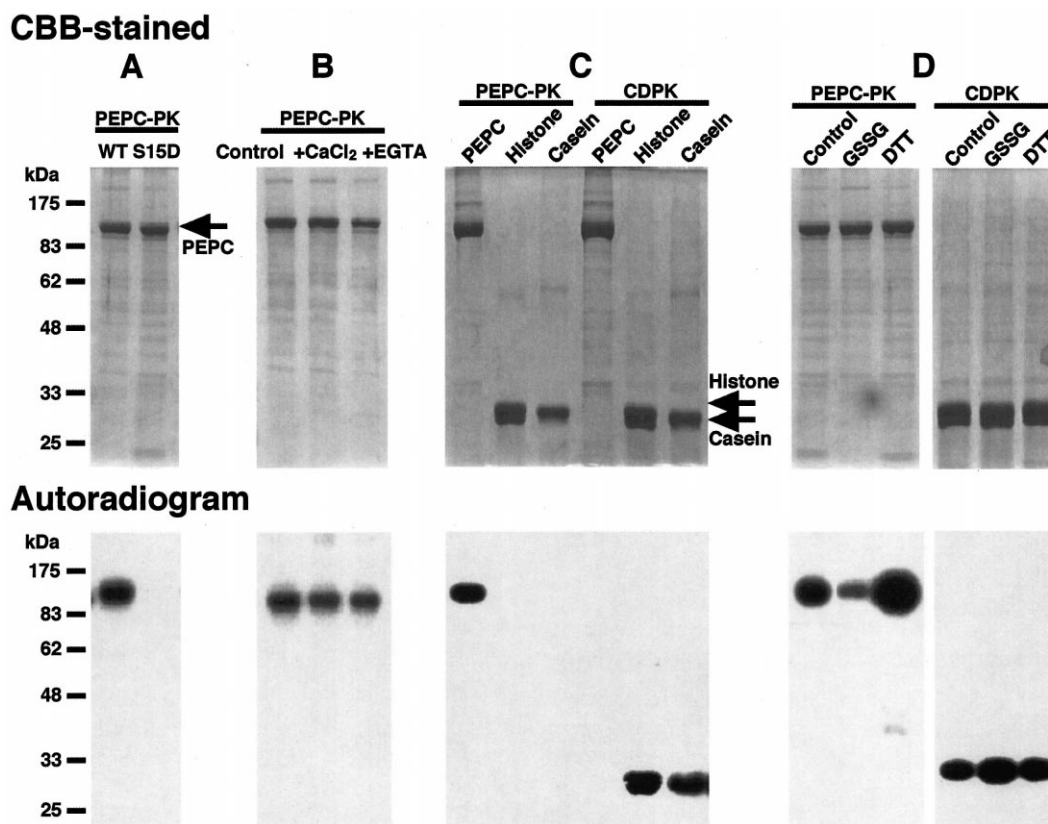


Fig. 2. Enzymatic property of FtPEPC-PK. A: Phosphorylation of recombinant wild-type and S15D mutant PEPCs from maize by FtPEPC-PK. B: The effect of 0.2 mM CaCl₂ or 1 mM EGTA on the phosphorylation of PEPC by FtPEPC-PK. C: Comparison of substrate specificity between FtPEPC-PK and OsCDPK. PEPC, histone 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.

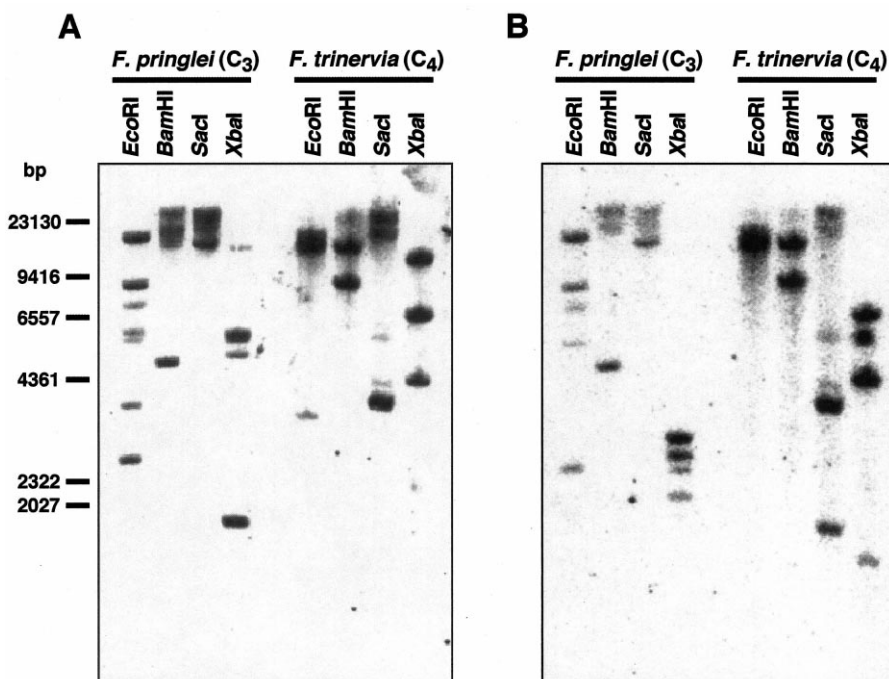


Fig. 3. Genomic Southern blot analysis of the *PEPC-PK* gene. 5 µg each of genomic DNA from *F. trinervia* or *F. pringlei* was digested with *EcoRI*, *BamHI*, *SacI* or *XbaI* and separated by electrophoresis. The DNA-transferred membranes were hybridized with a PCR-generated probe derived from the 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* (*Arabidopsis1* and *Arabidopsis2*) were 72, 60, 59, 52 and 51%, respectively. It is noteworthy that the C₄ plant PEPC-PK is most similar to that from a C₃ plant, tomato, rather than those from CAM plants, ice plant and *Kalanchoe*, in which PEPC plays a key role in CO₂ assimilation as well as in C₄ plants.

As shown in Fig. 2A, the FtPEPC-PK fused with NusA efficiently phosphorylated C₄-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 Ca²⁺ (Fig. 2B). FtPEPC-PK did not phosphorylate conventional substrate of protein kinases such as casein and histone H1s, 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, Ca²⁺-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* (C₃) and *F. trinervia* (C₄) 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* (C₃) and *F. trinervia* (C₄) (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* (C₃) than *F. trinervia* (C₄). These results indicated that several genes, being close to *PEPC-PK*, were present in both *F. pringlei* (C₃) and *F. trinervia* (C₄).

The expression of the gene for FtPEPC-PK was next inves-

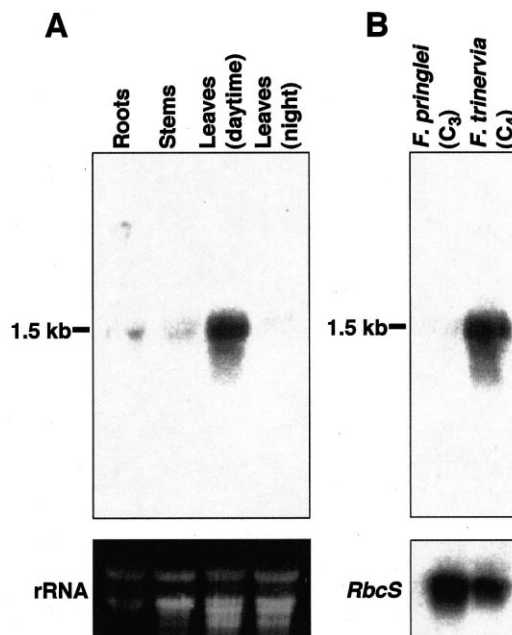


Fig. 4. Northern-blot analysis of *FtPEPC-PK*. A: Tissue-specificity and light-dependence of *FtPEPC-PK* expression in *F. trinervia*. B: Transcripts of *PEPC-PK* in leaves of *F. trinervia* and *F. pringlei* harvested during light period. 10 µg each of total RNA was separated on 1.2% agarose gel containing 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.

tigated 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 C₄-form PEPC (*ppcA*) [20], suggesting that this gene product be involved in the phosphorylation of the C₄-form PEPC. Furthermore, the transcript level in leaves was high at noon but negligible at midnight, suggesting that the expression is controlled diurnally.

The levels of transcripts of *FtPEPC-PK* were compared between *F. trinervia* (C₄) and *F. pringlei* (C₃). 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 C₄ photosynthesis in *Flaveria*.

In conclusion, we isolated the *FtPEPC-PK* gene, which is suggested to be involved in regulatory phosphorylation of C₄-form PEPC, the key enzyme in C₄ 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.

Acknowledgements: We wish to thank Dr. Hugh Nimmo (Glasgow University), Dr. Raymond Chollet (Nebraska University) and Dr. Peter Westhoff (Heinrich-Heine University) for their useful sugges-

tions, and Dr. Maurice S.B. Ku (Washington State University) for the seeds of *F. trinervia* and *F. pringlei*. We would like to thank Dr. Susanne von Caemmerer and Dr. Robert T. Furbank for their critical reading of this manuscript. The present work was supported in part by grants from a program of 'Research for the Future' from the Japan Society for the Promotion of Science (JSPS-RFTF 96 (0064)) and a Grant-in-Aid for Scientific Research on Priority Areas (No. 12025214).

References

- [1] Chollet, R., Vidal, R. and O'Leary, M.H. (1996) *Annu. Rev. Plant Physiol. Plant Mol. Biol.* 47, 273–298.
- [2] Vidal, J. and Chollet, R. (1997) *Trends Plant Sci.* 2, 230–237.
- [3] Foyer, C.H., Valadier, M.H., Migge, A. and Becker, T.W. (1998) *Plant Physiol.* 117, 283–292.
- [4] Ueno, Y., Imanari, E., Emura, J., Yoshizawa-Kumagaya, K., Nakajima, K., Inami, K., Shiba, T., Sakakibara, H., Sugiyama, T. and Izui, K. (2000) *Plant J.* 21, 17–26.
- [5] McNaughton, G.A.L., MacKintosh, C., Fewson, C.A., Wilkins, M.B. and Nimmo, H.G. (1991) *Biochim. Biophys. Acta* 1093, 189–195.
- [6] Dong, L., Ermolova, N.V. and Chollet, R. (2001) *Planta* 213, 379–389.
- [7] Nimmo, H.G. (2000) *Trends Plant Sci.* 5, 75–80.
- [8] Wang, Y.H. and Chollet, R. (1993) *FEBS Lett.* 328, 215–218.
- [9] Duff, S.M.G. and Chollet, R. (1995) *Plant Physiol.* 107, 775–782.
- [10] Hartwell, J., Gill, A., Nimmo, G.A., Wilkins, M.B., Jenkins, G.I. and Nimmo, H.G. (1999) *Plant J.* 20, 333–342.
- [11] Taybi, T., Patil, S., Chollet, R. and Cushman, J. (2000) *Plant Physiol.* 123, 1471–1481.
- [12] Ku, M.S.B., Wu, J., Dai, Z., Scott, R.A., Chu, C. and Edward, G.E. (1991) *Plant Physiol.* 96, 518–528.
- [13] Sambrook, J. and Russell, D.W. (1989) *Molecular Cloning: a Laboratory Manual*, 3rd edn., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
- [14] Ueno, Y., Hata, S. and Izui, K. (1997) *FEBS Lett.* 417, 57–60.
- [15] Gutierrez, R.A., MacIntosh, G.C. and Green, P.J. (1999) *Trends Plant Sci.* 4, 429–438.
- [16] Saijo, Y., Hata, S., Kyozuka, J., Shimamoto, K. and Izui, K. (2000) *Plant J.* 23, 319–327.
- [17] Wang, Y.H. and Chollet, R. (1993) *Arch. Biochem. Biophys.* 304, 496–502.
- [18] Li, B. and Chollet, R. (1994) *Arch. Biochem. Biophys.* 314, 247–254.
- [19] Zhang, X-Q. and Chollet, R. (1997) *Arch. Biochem. Biophys.* 34, 3260–3268.
- [20] Ernst, K. and Westhoff, P. (1997) *Plant Mol. Biol.* 34, 427–443.