Regulatory phosphorylation of phospho*enol*pyruvate carboxylase in the leaves of *Kalanchoë pinnata, K. daigremontiana* and *Ananas comosus*

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Abstract

Phospho*enol*pyruvate carboxylase (EC 4.1.1.31, PEPC) in the three crassulacean acid metabolism (CAM) plants: *Kalanchoë pinnata*, *K. daigremontiana* and *Ananas comosus* (pineapple) undergoes regulatory phosphorylation during the dark period. We cloned PEPC kinase gene from two CAM *Kalanchoë* species using conventional RT-PCR approach. The PEPC kinase transcripts comprise only a protein kinase catalytic domain, encoding 272 - 276 amino acids with predicted M_r of 30.6 - 31.0 kDa. The expression of PEPC kinase gene in the *Kalanchoë* species was abundant at the beginning of dark phase, but that in pineapple cross-hybridized with *Kalanchoë* PEPC kinase probes was abundant at the end of dark phase. The PEPC kinase was encoded by a small gene family containing at least two members in each species. Treatment of detached leaves with the protein synthesis inhibitors cycloheximide and puromycin blocked the nocturnal appearance of PEPC kinase activity and maintained PEPC in the dephosphorylated state in the three CAM species. The calcium/calmodulin antagonist W7 blocked the apparent phosphorylation state of PEPC in pineapple, but not in *Kalanchoë* species. Furthermore, the transcript abundance of PEPC kinase matched the apparent *in vivo* phosphorylation state of PEPC in the *Kalanchoë* species, but unmatched that in the pineapple. These results implicated that the phosphorylation state of PEPC in *Kalanchoë* species is largely controlled by PEPC kinase transcript abundance, while that in pineapple may be controlled by both PEPC kinase transcript abundance and Ca^{2+}-dependent protein kinase (CDPK).

Additional key words: CAM species, PEPC kinase, protein phosphorylation, transcript abundance.

Introduction

The cytosolic enzyme phospho*enol*pyruvate carboxylase (EC 4.1.1.31, PEPC) catalyzes the carboxylation of phospho*enol*pyruvate (PEP) in the presence of HCO_3^- to yield oxaloacetate (OAA) and inorganic phosphate (P_i). It plays cardinal roles in the initial fixation of atmospheric CO_2 during C_4 photosynthesis and Crassulacean acid metabolism (CAM). Additional plant PEPC isoforms perform anaplerotic roles in non-photosynthetic tissues and the leaves of C_3 plants, and plays the specialized functions of carbon metabolism in guard cells during stomata opening and C_4-acid formation in N_2-fixing legume root nodules (see Chollet *et al*. 1996, Vidal and Chollet 1997, Nimmo 2000).

Higher plant PEPC is an allosteric enzyme, activated by glucose-6-phosphate (Glc-6-P) and inhibited by malate. Superimposed on this, reversible phosphorylation of a strictly conserved Ser residue near the N-terminal end of the protein causes a change in the allosteric properties of PEPC. This phosphorylation reduces the sensitivity of the enzyme to its inhibitor, malate, but increases its sensitivity to its activator, Glc-6-P. PEPC becomes phosphorylated at night in CAM plants and in response to light in C_4 plants (Chollet *et al.* 1996, Vidal and Chollet 1997, Nimmo 2000).

In CAM and C_4 leaves, PEPC is phosphorylated by a specific Ca^{2+}-independent serine/threonine protein kinase, and dephosphorylated by protein phosphatase 2A (Carter *et al.* 1990, 1991, Li and Chollet 1994, Hartwell *et al.*

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Abbreviations: AEBSF - 4-(2-aminoethyl)benzenesulfonoyl fluoride hydrochloride; BSA - bovine serum albumin; CAM - Crassulacean acid metabolism; CDPK - Ca^{2+}-dependent protein kinase; DIG - digoxigenin; EDTA - ethylenediaminetetraacetic acid; E-64 - *trans*-epoxysuccynil-L-leucylamido (4-guanidino)-butane; Glc-6-P - glucose-6-phosphate; PCMB - *p*-(chloromercurio)-benzoic acid; RACE - rapid amplification of cDNA ends; RT-PCR - reverse transcription-polymerase chain reaction; SSC - sodium chloride sodium citrate solution; UTR - untranslated region; V-ATPase - vacuole adenosine-triphosphatase; V-PPase - inorganic pyrophosphatase.

The nucleotide sequences data reported in this paper have been submitted to DDBJ/EMBL/GenBank databases under the accession number EF157816 and EF157817 for *Kalanchoë pinnata* and *K. daigremontiana,* respectively.

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1999a, Taybi *et al.* 2000, Dong *et al.* 2001). Sequence analysis of PEPC kinase showed that it comprises only a serine/threonine kinase catalytic domain and identifies as a member of plant calcium dependent protein kinases (CDPK) but lacks the C-terminal Ca^{2+}-binding EF hands and the N-terminal extensions of these enzymes (Hartwell *et al.* 1999a). Expression analyses of transcript abundance suggested that PEPC kinase is regulated only at the level of gene expression (Hartwell *et al.* 1999a, Taybi *et al.* 2000). The pharmacological study of various protein synthesis inhibitors showed that a protein synthesis step is required for the appearance of PEPC kinase activity during the dark in CAM and the light in C_4 plants (Carter *et al.* 1991, Jiao *et al.* 1991). Moreover, the day/night changes in transcript abundance of PEPC kinase have been shown to be modulated by the cytosolic metabolite, most probably malate (Borland *et al.* 1999, Nimmo 2000, Taybi *et al*. 2004). Conversely, the other studies have shown that, likely not cytosolic malate, but the increase in cytosolic pH initiates the signal of PEPC kinase expression and the phosphorylation state of PEPC in CAM and C_4 plants (Giglioli-Guivarc'h *et al.* 1996, Bakrim *et al.* 2001). It is now generally accepted that the phosphorylation state of PEPC is largely controlled by changes in the activity of the Ca^{2+}-independent PEPC kinase (Vidal and Chollet 1997). However, some plants containing both Ca^{2+}-dependent/independent protein kinases that can phosphorylate PEPC, have also been reported (Ogawa *et al.* 1992, Bakrim *et al.* 1992, Li and Chollet 1993).

As noted above, CAM PEPC becomes phosphorylated during the dark period, in which the enzyme reversibly changes to an active form and is less sensitive to malate. PEPC from three CAM species, *K. pinnata, K. daigremontiana* and pineapple, underwent the nocturnal regulatory phosphorylation, but the level of phosphorylation and malate sensitivity showed marked difference between pineapple and *Kalanchoë* species (Theng *et al.* 2007). The nature of protein kinase(s) controlling this phosphorylation has not yet been elucidated. Although PEPC kinase activity of *K. daigremontiana* has been reported, using translation products of PEPC kinase translatable mRNA (Borland *et al.* 1999), the transcriptional regulation of PEPC kinase expression in these CAM species is not yet been carried out.

The aim of the present work was to identify the protein kinase(s) responsible for the phosphorylation state of PEPC in the three CAM species, *K. pinnata*, *K. daigremontiana* and *Ananas comosus*. We reported the identification of cDNA clones of PEPC kinase gene and investigated their expression regulation. The results highlight the physiologically significant role of PEPC kinase mRNA abundance in pineapple in the control of phosphorylation state of PEPC compared with other two *Kalanchoë* species.

Materials and methods

Plant materials: *Kalanchoë pinnata, K. daigremontiana* and *Ananas comosus* (L.) Merr. (pineapple cv. Smooth-cayenne N67-10) were vegetatively propagated and grown in pots in a greenhouse with heating under a natural photoperiod. Plants were transferred to a growth chamber (*KG-50 HLK, Koito Industrial Co.,* Tokyo, Japan) for two weeks with a 10-h photoperiod before collecting leaf samples. The conditions in growth chamber were 30 °C during light period (photon flux density of 420 - 450 μmol m^{-2} s^{-1} at the mid-plant height) and 20 °C during the dark period, and a relative humidity of 70 % during both periods. Fifth to eighth leaf pairs counting from the apex of *K. pinnata* and *K. daigremontiana* and fully expanded, mature leaves of *A. comosus* were taken for all experiments. Leaf samples were taken and immediately immersed in liquid nitrogen and stored at -80 °C until use. The leaf samples for isolation of PEPC kinase gene were collected at midnight, when transcripts of PEPC kinase should be expressed at high level.

Extraction and measurement of leaf malate contents: The leaf malate was extracted and measured as described by Chen and Nose (2004), except that we used only 1.2 g of frozen leaf tissue and 5 cm^3 of ice-cold 4 % (v/v) $HClO_4$.

Isolation of total RNA and cDNA fragments for PEPC kinase: Total RNA from leaves collected at midnight from three CAM species was isolated using guanidine hydrochloride or a commercial kit (*RNeasy Plant Mini kit*, *Qiagen*, Japan) precipitating with 10 - 20 mg cm^{-3} polyethylene glycol 20000 (Gehrig *et al.* 2000). Poly(A)$^+$-enriched RNA was obtained from total RNA by a single passage through an oligo (dT)-cellulose spin column (*Oligotex^{TM}-dT30 [Super] mRNA Purification kit*, *TaKaRa*, Ohtsu, Japan) according to the manufacturer's instruction. One microgram of poly (A)$^+$-enriched RNA was used in the cDNA synthesis for the RT-PCR and RACE amplification.

About 150-bp fragments of cDNA for PEPC kinase from the three CAM species were isolated by RT-PCR using PEPC kinase degenerate primers. The forward primer, 5'-A(C/T)(A/C)G(A/T/G/C)GA(C/T)(A/G/C)T (A/T/G/C)AA(A/G)CC(A/T/-G/C)GA-3' corresponded to subdomain VIb (HRDIKPD), and the reverse primer, 5'-TC(A/T/G/C)(A/T/G/C)GC(A/T/G/C)AC(A/G)TA(A/ G)TA(A/T/G/C)GG-3', to subdomain VIII (PYYVAPE). The two motifs in subdomains VIb and VIII are characteristically conserved for both PEPC kinase and calcium-dependent protein kinase in higher plants (Taybi *et al.* 2000, Tsuchida *et al.* 2001). The amplification reaction was carried out for 40 cycles, a 94 °C denaturing cycle for 80 s, a 56 °C annealing cycle for 1 min, and a 72 °C extension cycle for 1 min. PCR products were cloned into pGEM-T easy vector system (*Promega*,

Madison, WI, USA) and sequenced on the *ABI Prism 310 Genetic Analyzer using the Prism BigDye Terminator Ready Reaction Cycle Sequencing kit (ABI Applied Biosystems,* Foster, CA, USA). The identity of the sequence results was confirmed by searching the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov) using *BLAST 2.0* as search tools. The fragments encoded PEPC kinase mRNA was used to design gene specific primers to recover the full-length sequences of PEPC kinase cDNA by RACE amplification.

RACE amplification: The 3' side of cDNA for PEPC kinase of the two *Kalanchoë* species was amplified with 3'RACE system (*SMART™ RACE cDNA Amplification, BD Biosciences Clontech*, Mountain View, USA) according to the manufacturer's instructions using gene specific primers, CGATGATCGTAACAGGTTG and TGAAGCTCGGGGACTTTG for *K. pinnata* and *K. daigremontiana,* respectively. The 5' end of PEPC kinase was amplified with 5'RACE system (*SMART™ RACE cDNA Amplification*) using gene specific primers, CAAGTCTCCCCCACTCATCATCCAT and CAAAGT CCCCGAG-CTTCAACCTGTA for *K. pinnata* and *K. daigremontiana,* respectively. The identity of each of these products was confirmed by DNA sequence analysis. Multiple sequence alignments were performed with *GENTYX-WIN program* (*GENTYX-WIN V.5.1,* Tokyo, Japan).

Northern blot analysis: Three microgram of total RNA isolated from leaves collected every 4 h over the time course were fractionated on 12 mg cm^{-3} agarose gel containing 20 mg cm^{-3} formaldehyde, and then transferred to nylon membranes (*Roche Applied Science,* Nannheim, Germany). The RNA-transferred membranes were hybridised with DIG RNA labeling probe derived from the 3' end of PEPC kinase cDNA (probe I, about 700 bp corresponded to the subdomain VII to poly A tail) from *Kalanchoë* species. The filters were washed twice with 0.1× SSC, 1 mg cm^{-3} SDS at 68 °C for 15 min, and then detected using *DIG Luminescent Detection kit* (*Roche Applied Science*) and exposed to X-ray film (*Hyperfilm*, *Amersham Biosciences*, Chalfont, UK) for 30 - 60 min according to the manufacturer's instruction.

Southern blot analysis: Genomic DNA was isolated as previously described (Stange *et al.*1998). Five μg each of genomic DNA from the three CAM species were digested with *Eco*RI, *Hind*III and *Sac*I, and then separated by electrophoresis on an 8 mg cm^{-3} agarose gel. The DNA-transferred membranes were hybridized with a DIG RNA labeling probe derived from the 3' end (probe I, 700 bp), or a probe from the coding region (probe II, 450 bp) of PEPC kinase cDNA from both *Kalanchoë* species. The former probe was the same probes used in Northern blots and the latter probe corresponded to the start codon to subdomain VII of PEPC kinase cDNA of both *Kalanchoë* species. Washes and detections were performed as described in Northern blots analysis. Northern blot and Southern blot analyses were performed at least in two replicates with similar results, and the representative data are shown.

Protein inhibitors: Cycloheximide (CHX), puromycin (Puro) and W7 were dissolved in ultra-pure water. For experiments on protein kinase(s) activity, a range of concentrations of pharmacological drugs were applied, *via* transpiration stream, to detached leaves 3 - 4 h after the illumination, and whose petioles were retained in the chemical solution until the middle of night. The control leaves were incubated in the distilled water. The leaves were collected and immersed in liquid nitrogen and kept at -80 °C until extraction. The blocking kinase activity was estimated by changes in malate sensitivity and apparent *in vivo* phosphorylation state of PEPC in the treated and control leaves. The treatment was performed at least in two replicates for each dose.

Extraction, activity and phosphorylation status of PEPC: Crude leaf extracts were obtained by grinding, in a prechilled mortar, 4 cm^2 of leaf samples in 3 cm^3 of extraction medium containing 50 mM Bicine-KOH (pH 8.2), 1 mM EDTA-NaOH (pH 7.0), 5 mM $MgCl_2$, 5 mM DTT, 10 mg cm^{-3} Triton X-100, 20 % (v/v) glycerol, 1 mM AEBSF, 10 μg cm^{-3} E-64, 10 μg cm^{-3} chymostatin, 100 μM leupeptin, 1 mM PCMB, 25 mg cm^{-3} insoluble PVP, and 0.5 g of washed sea sand. The homogenates were passed through one layer of *Miracloth* and clarified by centrifugation at 8 060 *g* at 4 °C for 20 s, and the supernatants were used immediately for enzyme assays.

PEPC activity was measured spectrophotometrically at 340 nm and 30 °C in a 3.0 cm^3 assay medium containing 50 mM Tris-HCl (pH 8.2 for *A. comosus*, and pH 8.6 for *Kalanchoë* species), 20 % (v/v) glycerol, 5 mM $MgCl_2$, 2 mM $NaHCO_3$, 0.2 mM NADH, 2 mM PEP, 0.1 mg cm^{-3} BSA, and 166.7 nkat L-MDH. The assays were initiated by the addition of a 0.02 cm^3 aliquot of centrifuged crude enzyme extract. Malate sensitivity of PEPC was determined spectrophotometrically at 340 nm in the same assay and pH conditions with the presence or absence of 2 mM L-malate. Chlorophyll content was determined according to Arnon (1949).

Phosphorylation status of PEPC from the three CAM species was studied by 80 mg cm^{-3} denatured polyacrylamide gels (Laemmli, 1970). The gels were stained with *Pro-Q Diamond Phosphoprotein gel stain* (*Molecular Probes,* Carlsbad, USA) and subsequently with *SYPRO Ruby protein gel stain* (*Bio-Rad Laboratories*, Hercules, CA, USA). Phosphoprotein on the gels was imaged by scanning with *Typhoon image scanner* (*Typhoon 9000E*, *Amersham Pharmacia Biotech*, Piscataway, USA), and analyzed by *Kodak 1D Image Analysis Software* (*Kodak, Rochester,* USA). Protein content was estimated according to Bradford (1976) using BSA as a standard. SDS-PAGE was performed in three replicates with consistent results and representative results are shown.

Results

Diurnal changes in leaf malate: *Kalanchoë* species and pineapple showed typical features of CAM, with malate increasing in the dark and decreasing under irradiance (Fig. 1), being consistent with the previous study (Chen *et al.* 2002).

Isolation of PEPC kinase transcripts: The full-length *KpPpck1* and *KdPpck1* cDNAs contained a single open reading frame of 831 and 819 bp flanked by 5' and 3' untranslated sequences of 39 and 29 bp, and 279 and 304 bp for *K. pinnata* and *K. daigremontiana,* respectively. The 3'-untranslated regions (UTRs) of both PEPC kinase transcripts contained a duplicated (AATA AATAA) polyadenylation signal at 30 and 28 bp from the polyadenylation site for *K. pinnata* and *K. daigremontiana,* respectively, and several canonical

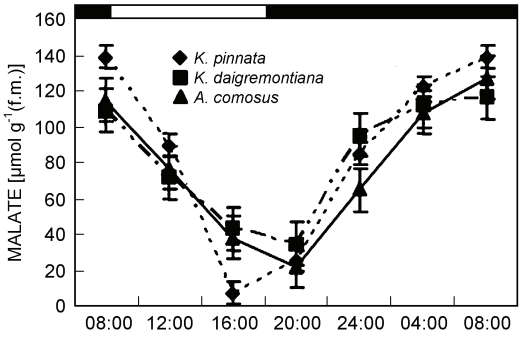

Fig. 1. Malate content in the leaves of *K. pinnata, K. daigremontiana* and pineapple (*A. comosus*) during the 24 h day-night cycle. White- and black-bar in the upper part indicate the light and dark periods, respectively. Values are means ± SD of three to five replicates.

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                          I                                    II          III
Kp-Ppck1     1:MSEALNQNYEICEEIGRGRFGVVYRCVSSASGH--TFAC--KSIDKR-LIANDSID-REC 54
Kd-Ppck1     1:MSEALQQNYEICEEIGRGRFGVVYRCVSSASGQ--TFAC--KSIDKR-LIANDSID-REC 54
Kf-Ppck1     1:MSEALSRVYEVCEEIGRGQFGRVFRCLNLSTN----ELFACKSIDKRVLA--DSID-REC 53
Cm-Ppck2     1:-----------CEEIGRGRFGVVYKCTKLSTNE--TYAL--KSIVKNSLN-DDLLDAR-C 43
Mc-Ppck1     1:MCESFKREYQLCEEIGRGRFGVVYRCYNPSSTEDSDTPLAVKSIDKR-LFLDDETD-REC 58
FtPpck1      1:MKETLNNDYHITEEIGRGRFGTVYRCYSTVSGD--SFAC--KTIDKR-LLNDHT-D-REC 53
               . . .   .  .******.**.*..*    .      .   *.*.*. *   .  * *.*
                                  IV                   V                  VIA
Kp-Ppck1    55:LIKEPKFNQIVAGSPYIVELHDVFEDSDHLHLILEFCGYQTLYEQILA--GTFSEKEAAA 112
Kd-Ppck1    55:LIKEPKFNQIVAGSPYIVELHDVFEDSDHLHLILEFCGYQTLYEQILA--GTFSEKEAAA 112
Kf-Ppck1    54:VEKEPKTMLCLSSHPNIVSIHEAYEDDSHLHLIMDLCEPG-LDLYQLLSGGAVPEQEASV 112
Cm-Ppck2    44:LVTEPKILRQVSPHPHVTQLHDSHEDDCYLHLIIDLCPNGDL-YTLLLSTGPIPEAQSKA 102
Mc-Ppck1    59:LDKEPKILHLLSPHPNILQIHNLFDSDTHLLIVTDLCQEETL-YERIISNGPFSEPDAAA 117
Ft-Ppck1    54:LQKEPKILHILGGNPNIVQIYRLYEDDNYVHMIIDLCDSPDLFDRISNRNGVFSETEAAT 113
               . .***        * ..  .   ... ..... ..*    *    .   * ..* ....
                                VIB                VII
Kp-Ppck1   113:VMEQLVQAVAHCHRRGIAHRDIKPDNVLFDDRNRLKLGDFGSAEWF-GNGE-TMSGLVGT 170
Kd-Ppck1   113:VMEQLVQAVAHCHRRGVAHRDIKPDNVLFDDRNRLKLGDFGSAEWF-GNGE-TMSGLVGT 170
Kf-Ppck1   113:IAASLMEGIAHCHRRGVCHRDVKPENVLFDSVGRLKLADFGSAEWF-GDG-RGMSGVVGT 170
Cm-Ppck2   103:QFVQLMKAVSHCHKSGVVHRDIKPDNILFDSRESLKLADFGSAEVICGKGSDL-NGVVGT 161
Mc-Ppck1   118:IFCQLAEALAHCHRNYVAHRDIKPDNILFDSRNRLKLCDFGSAEWF-GAGDREMRGVVGT 176
Ft-Ppck1   114:VFAPLMSAISYCHRLGIAHRDLKPDNVLFDSRGGLKLADFGSAEWFAMNDRRTMSGIVGT 173
                 .*  . ..**. ...***.**.*.***.. .*** ******.. . .   ..* ***
                VIII                  IX                        X
Kp-Ppck1   171:PYYVAPEVVSGREYTEKVDVWSCGVILYIMLAGIPPFYGESATEIFQAVLRGNLRFPTRP 230
Kd-Ppck1   171:PYYVAPEVVSGREYTEKVDVWSCGVILYIMLAGIPPFYGESATEIFQAVSRGNLRFPTRS 230
Kf-Ppck1   171:PYYVAPEVLQGREYNEKVDVWSAGVILYTMLAGFPPFYGETAQDIFEAVMRGNLRFPFRA 230
Cm-Ppck2   162:PYYVAPEILEGREYGKKVDVWSAGVVLYVMLAGFPPFYGETVVEIFEAVLRGNLRFPSRA 221
Mc-Ppck1   177:PYYVAPEVLSGKDYNEKADVWSAGVILYIMLGGVPPFYGETVEETFEAVLRGNLRFPARI 236
Ft-Ppck1   174:PYYVAPEVLSGMEYNEKVDVWSAGVILYIMLAGVPPFHGDSPADTFEAVLRGNLRFPTRL 233
               *******...*..* .*.****.**.**.**.* ***.*.   ..*.**.******* *
                                XI
Kp-Ppck1   231:FHSVSPAAKDLLKKMLCKDVSRRLSADQVLRHPWMMSGGDLRSMTN--              276
Kd-Ppck1   231:FHSVSPAAKDLLRKMLCKDVSRRLSADQVLRHPWMMSGGELR------              272
Kf-Ppck1   231:FRNVSAGAKDLLRRMLCRDVSRRFSAEQVLRHSWILSAGDDTNF                   274
Cm-Ppck2   222:FCTVSSSVKDLLRRILCRDVSRRFTADQVLRHPWVTSGG---------              260
Mc-Ppck1   237:FRNVSTQARDLLRKMMCKDVSRRFSAEQVLRHPWVTSGGLANM-----              279
Ft-Ppck1   234:FRSVSPEAKDLLRKMLCKDVSRRFSADQVLRHPWVVNGGKTRSMADLT              281
               *  **  ..***....*.*****..*.*****.*  ..*
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Fig. 2. Alignment of amino acid sequences from *K. pinnata* (*KpPpck1*) and *K. daigremontiana* (*KdPpck1*) with other PEPC kinases from three CAM-plants, *K. fedtschenkoi* (Kf, AF162662), *Clusia minor* (Cm, AY478420) and *Mesembryanthemum crystallinum* (Mc, AF158091) and a C_4 plants, *Flaveria trinervia* (Ft, AB065100), where (.), conserved and (*), identical residues. The twelve defined subdomains of the eukaryotic protein kinases are indicated by Roman numerals.

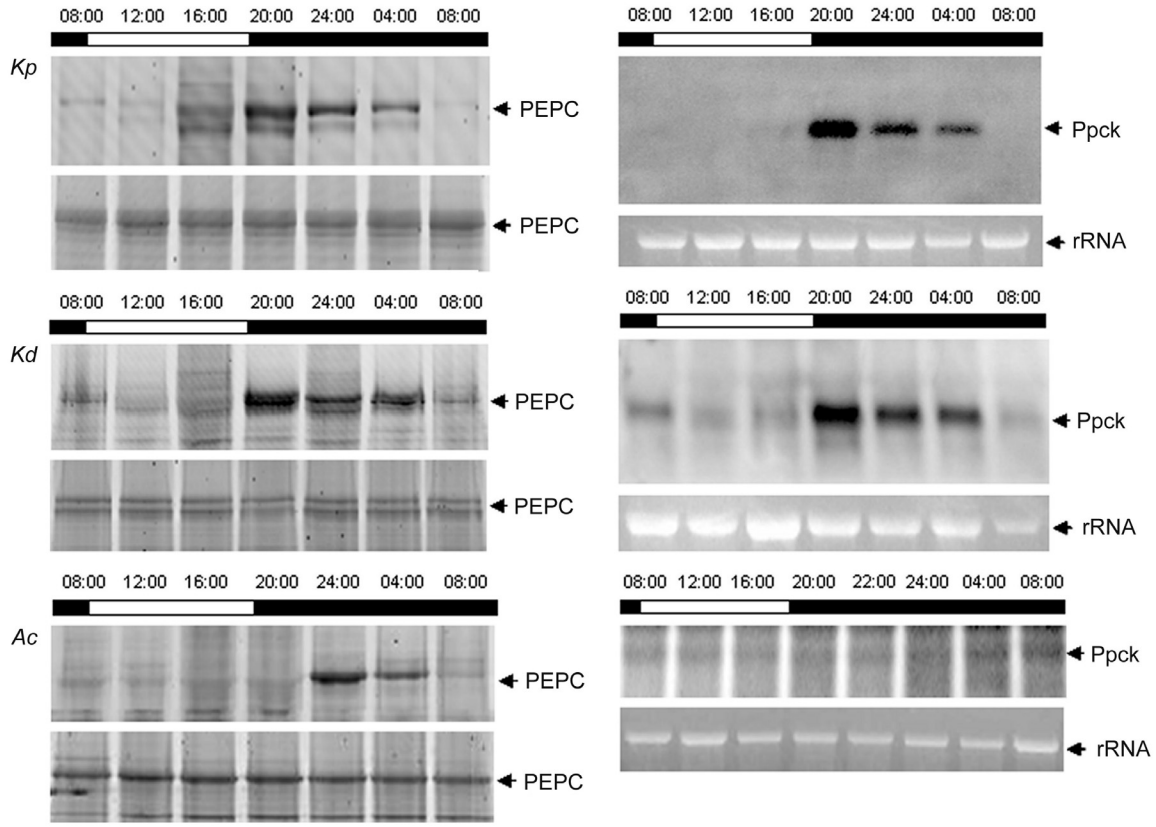

Fig. 3. Diurnal regulation of expression of PEPC kinase (*right*) in parallel with the diurnal changes of phosphorylation state of PEPC (*left*) from *K. pinnata* (*Kp*), *K. daigremontiana* (*Kd*) and pineapple (*Ac*). Transcript abundance of PEPC kinase (*right*, *above panels*) was studied by Northern blots hybridized with probe I for the *Kalanchoë* species, and crossed-hybridized with probe III derived from *Kalanchoë* species for pineapple. Equal volumes of rRNA for each species are shown under the hybridized blots (*right*, *below panels*). The apparent *in vivo* phosphorylation state of PEPC was studied by SDS-PAGE stained with *Pro-Q Diamond* phosphoprotein gel stain (*left*, *above panels*), and subsequently stained with *SYPRO Ruby* (*left*, *below panels*) served as positive control of total PEPC protein. *White-* and *black-bar* indicate the light and dark periods, respectively.

downstream and downstream-like elements (GAA, ATAGATTT and GTA, data not shown). The predicted polypeptides of 276 and 272 amino acids had a calculated molecular mass of 31.0 and 30.6 kD for *KpPpck1* and *KdPpck1*of *K. pinnata* and *K. daigremontiana,* respectively. Analysis of the deduced amino acid sequences indicated that the proteins are mostly close to PEPC kinase as described from CAM and C_4 plants (Hartwell *et al.* 1999a, Taybi *et al.* 2000, 2004, Tsuchida *et al.* 2001). Fig. 2 shows the alignment of the deduced amino acid sequences of *KpPpck1* and *KdPpck1* with other PEPC kinase from CAM species, *K. fedtschenkoi* (AF162662), *Clusia minor* (AY478420) and *Mesembryanthemum crystallinum* (AF158091), and the C_4 plant *Flaveria trinervia* (AB065100). The alignment of the deduced *KpPpck1* and *KdPpck1* showed that they share 58, 61, 63, and 65 % amino acid sequence identity with the *C. minor*, *M. crystallinum, K. fedtschenkoi* and *F. trinervia,* respectively, over the entire length of the kinase catalytic domain (Fig. 2).

Day/night expression of *KpPpck1* and *KdPpck1*: Transcripts were studied by Northern blots using a 3' end of PEPC kinase cDNA as a probe (probe I). The contents of *KpPpck1* and *KdPpck1* transcripts increased and decreased over the diurnal cycle in parallel with the *in vivo* phosphorylation state of PEPC (Fig. 3). In the two *Kalanchoë* species, the transcripts were abundant during the first 2 h of darkness and then decreased until dawn. The transcript abundance of PEPC kinase disappeared during the illumination period. The expression of PEPC kinase transcripts was well consistent with the phosphorylation/ dephosphorylation state of PEPC in these two *Kalanchoë* species. There was no marked difference in the expression pattern of PEPC kinase transcript abundance among the two *Kalanchoë* species examined.

The sequence of PEPC kinase transcript from pineapple could not be isolated in the present study. Thus, to investigate the transcript abundance of PEPC kinase from pineapple we conducted crossed-hybridisation by using PEPC kinase probes from *Kalanchoë* species with total RNA from pineapple. Probe III, corresponding to subdomains VIb to XI, which represent the highly conserved regions of the primary consensus sequence (Taybi *et al.* 2000, Tsuchida *et al.* 2001) of PEPC kinase

transcripts, was used in the hybridisation. The transcript had a very low abundance, about 10-times lower than in the *Kalanchoë* species. The expression of the transcript appeared to be most abundant near the end of the dark period and disappeared during the light period and the first 2 h of dark phase (Fig. 3). The transcript abundance of PEPC kinase did not match with the phosphorylation state of PEPC; it showed a short lag time peaking during the latter dark phase. Similar results of pineapple PEPC kinase expression were obtained by a probe II from *K. pinnata* or from *K. daigremontiana* (data not shown).

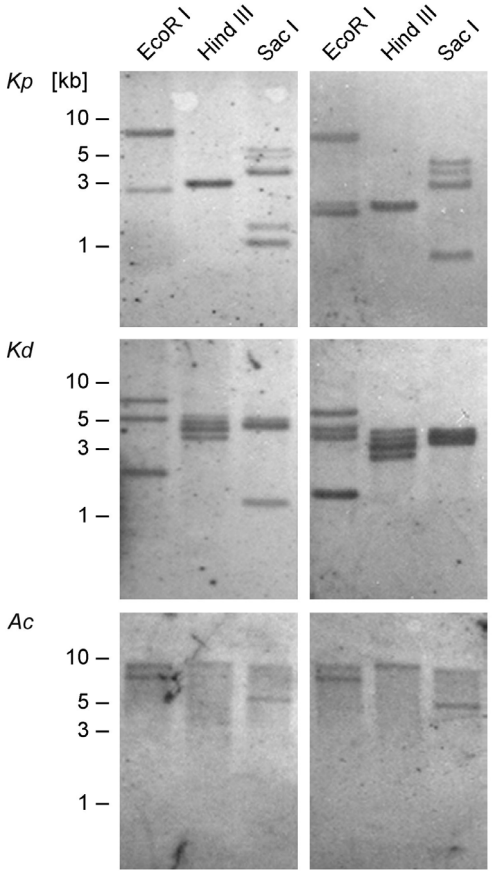

Fig. 4. Genomic Southern blot analysis of PEPC kinase gene. The genomic DNA (5 μg) samples from *K. pinnata* (*Kp*), *K. daigremontiana* (*Kd*) and pineapple (*Ac*) were digested with *Eco*RI, *Hind*III and *Sac*I and separated by electrophoresis. The DNA-transferred membranes from the *Kalanchoë* species were hybridised with a DIG RNA labeling probe derived from the coding region (probe II, *left*), or a probe from the 3' end region (probe I, *right*) of PEPC kinase cDNA, while the pineapple membranes were crossed-hybridized with PEPC kinase probe II (*left*) and probe III (*right*) from *Kalanchoë* species. The molecular mass markers are shown *on the left*.

Genomic DNA Southern blot analysis: On genomic Southern blotting, both probe I and probe II cross-hybridized strongly revealing multiple bands (Fig. 4). The resulting hybridization obtained under high-stringency conditions showed that in the *Kalanchoë* species both probe I and probe II gave at least two bands on each lane. In *K. pinnata,* lane *Hind* III gave one strong band for both probes, these bands might be double bands. Probe I could not make crossed-hybridization to genomic

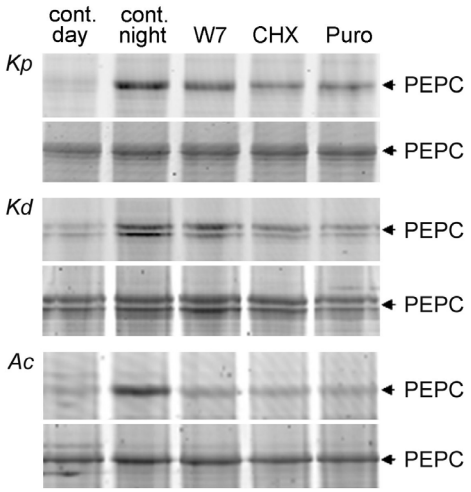

Fig. 5. Effects of W7, CHX and Puro on the apparent *in vivo* phosphorylation state of PEPC in the leaves of *K. pinnata* (*Kp*), *K. daigremontiana* (*Kd*) and pineapple (*Ac*). Leaves were excised 3 - 4 h after the illumination and fed with water (control) and with W7 (500 μM), CHX (250 μM for *Kp* and *Ac*, and 100 μM for *Kd*) and puromycin (Puro, 1 mM). Crude extracts were prepared from these leaves at the midday and midnight. The proteins were resolved by SDS-PAGE and stained with *Pro-Q Diamond* phosphoprotein gel stain (*above panels*) and subsequently stained with *SYPRO Ruby* (*below panels*) served as positive control of total PEPC protein.

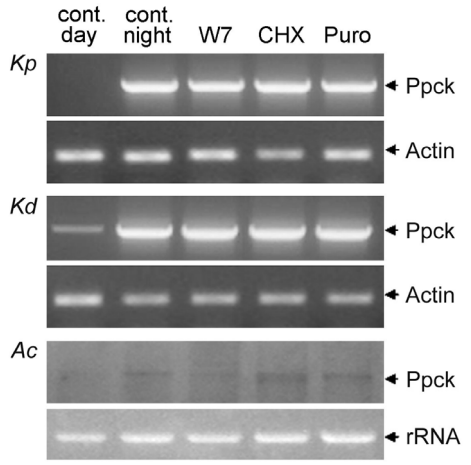

Fig. 6. PEPC kinase transcripts in the leaves of *K. pinnata* (*Kp*), *K. daigremontiana* (*Kd*) and pineapple (*Ac*) fed with water (control) and with W7 (500 μM), CHX (250 μM for *Kp* and *Ac*, and 100 μM for *Kd*) and puromycin (Puro, 1 mM). RT-PCR was performed using gene specific primers for the *KpPpck1* and *KdPpck1* for *K. pinnata* and *K. daigremontiana*, respectively (*above panels*) and control gene of actin (*below panels*). Northern blot of total RNA isolated from pineapple was crossed-hybridised with PEPC kinase probe III from *Kalanchoë* species, where above panel is PEPC kinase transcript and the below is rRNA.

Table 1. Effects of W7, cycloheximide (CHX) and puromycin (Puro) on malate sensitivity of PEPC in excised, darkened leaves of *K. pinnata* (*Kp*), *K. daigremontiana* (*Kd*) and pineapple (*Ac*). Leaves were excised 3 - 4 h after the illumination and fed with water (control) or W7, CHX, and Puro. Control activity (without malate) = 100 %. Values are means ± SD (*n* = 4 - 6) of separate experiments. ND - not determined.

Plants	Relative decrease in malate sensitivity of PEPC [%]								
	control		W7		CHX			Puro	
	day	night	250 μM	500 μM	50 μM	100 μM	250 μM	500 μM	1000 μM
Kp	29.0 ± 0.6	71.5 ± 1.2	64.3 ± 2.6	48.2 ± 1.3	69.8 ± 3.1	52.2 ± 2.3	34.1 ± 2.2	63.6 ± 3.0	35.7 ± 3.0
Kd	24.7 ± 2.7	66.1 ± 4.1	61.5 ± 2.8	43.8 ± 1.5	39.1 ± 2.5	26.3 ± 1.2	ND	62.5 ± 6.1	26.7 ± 4.9
Ac	28.6 ± 0.4	56.1 ± 4.6	38.9 ± 1.8	28.6 ± 2.1	65.1 ± 2.3	63.2 ± 1.3	30.6 ± 2.7	49.5 ± 2.3	31.4 ± 2.4

DNA from pineapple. Thus, the probe II and III were used in the crossed-hybridization and gave at least two bands on each restriction enzyme (Fig. 4).

Protein inhibitors: Cycloheximide (CHX) and puromycin, and W7 are well-known protein synthesis inhibitors and calcium/calmodulin antagonist for PEPC kinase and CDPK, respectively. At low concentrations, 50 μM CHX had no effect to block PEPC kinase activity, as judged by its sensitivity to malate, except that in *K. daigremontiana* (Table 1). At 100 μM, CHX had no effect in pineapple, it had no clear effect in *K. pinnata* but clear effect in *K. daigremontiana* (Table 1, Fig. 5). However, in both *K. pinnata* and pineapple, 250 μM CHX blocked the activity of PEPC kinase (Table 1) and the apparent *in vivo* phosphorylation state of PEPC disappeared (Fig. 5). 1000 but not 500 μM puromycin, had clear effect in blocking the PEPC kinase activity and no phosphorylation of PEPC was detected in the leaves from the three CAM species. The W7 had no clear effect on the reduction of malate sensitivity and the apparent phosphorylation state of PEPC in both *Kalanchoë* species even at a high concentration (500 μM), but it had significant effect in the pineapple. Control leaves, allowed to take up water, contained kinase activity and the apparent phosphorylation state of PEPC (Table 1, Fig. 5).

To determine whether the chemicals had an effect on PEPC kinase transcripts, RT-PCR using total RNA isolated from the control and treated leaves were performed for the *Kalanchoë* species, whereas in pineapple was crossed-hybridized with PEPC kinase probe III from *K. pinnata* or *K. daigremontiana*. These chemicals neither increased nor decreased the PEPC kinase transcripts (translatable mRNA) in the three CAM species (Fig. 6).

Discussion

Purification of PEPC kinase protein and cloning the gene has been proved very difficult because of the very low abundance of the protein, with 1.4 million-fold enrichment (1 μg of the enzyme from 2.6 kg of leaf tissue) (Chollet *et al.* 1996, Echevarria and Vidal 2003). In this paper we have used conventional RT-PCR, using degenerate primers corresponding to the conserved catalytic subdomains of PEPC kinase, to clone PEPC kinase gene from three CAM species, *K. pinnata, K. daigremontiana* and pineapple. We have satisfactorily cloned this gene from the two *Kalanchoë* species, but unsatisfactorily cloned from pineapple. Although many cDNA fragments generated from different primers that have been designed to different catalytic subdomains of PEPC kinase, we have still unsatisfied to clone PEPC kinase from pineapple; only CDPK and/or Ser/Thr kinase candidates were obtained, indicating that these proteins may be more abundant than PEPC kinase protein. The physiological role of these unidentified proteins is unknown for this CAM species.

Analysis of sequences of PEPC kinase gene, *KpPpck1 and KdPpck1* isolated from *K. pinnata* and *K. daigremontiana,* respectively, showed that they are mostly close to PEPC kinase cloned from CAM and C_4 plants. The predicted molecular mass of protein encoded by these genes is similar to those of other plant species ranging from 30.6 to 31.0 kD and comprise a protein kinase catalytic domain (Fig. 2) with minimal insertions or extensions at the N- or C-terminal ends (Hartwell *et al.* 1999a, Taybi *et al.* 2000, Tsuchida *et al.* 2001). Their sequences are almost closely to CDPK, but lack the autoinhibitory region and the Ca^{2+}-binding EF hands of this enzyme (Hartwell *et al.* 1999a, Hardie 1999). The UTRs of both *KpPpck1* and *KdPpck1* contain a duplicated (AATAAATAA) polyadenylation signal at 30 and 28 bp from the polyadenylation site for *K. pinnata* and *K. daigremontiana,* respectively. In addition, the 3' UTRs of the two *Ppck* genes contain several canonical downstream and downstream-like elements: GAA, GTA, and ATAGATTT (the last motif is not present in the 3' UTR of *K. pinnata*, data not shown), which is similar to those described in *M. crystallinum*, *C. minor* and C_4 plant *F. trinervia* (Taybi *et al.* 2000, 2004, Tsuchida *et al.*

2001). These elements are suspected to contribute to mRNA instability in plants (Gutiérrez *et al.* 1999) and suggest the rapid turnover of PEPC kinase transcripts. The rapid turnover of PEPC kinase transcripts is an important aspect for the day/night regulation of PEPC kinase and PEPC phosphorylation and is thought to be regulated by the 3' UTR (Taybi *et al.* 2004).

In the C_3, C_4 and CAM plants, PEPC kinase is encoded by a small gene family (Taybi *et al.* 2000, Nimmo *et al.* 2001, Tsuchida *et al.* 2001). To determine whether the similar phenomena occur in the three CAM species, Southern blot analyses of genomic DNA were performed using probe I and probe II for the *Kalanchoë* species, and probe II and probe III for the pineapple. In the *Kalanchoë* species, even under highly stringent conditions, probe I gave more than one band on each lane (Fig. 4), suggesting that the *KpPpck1* and *KdPpck1* exist as a multi-copy gene for *K. pinnata* and *K. daigremontiana,* respectively. Similar results were obtained using probe II of the 5' coding regions, which contained no intron in PEPC kinases isolated from many plant species (Nimmo 2003), indicating that several genes, being close to *KpPpck1* or *KdPpck1*, were present in both *Kalanchoë* species. In the pineapple, though the transcript of PEPC kinase could not be isolated, PEPC kinase in this CAM species is also encoded by a small gene family of two members because the products of each restriction enzyme digestion reaction hybridized to two bands (Fig. 4) using *Kalanchoë* probe II and/or probe III.

Northern blot analysis showed that the transcript of *KpPpck1* and *KdPpck1* was abundant during the first 2 h of darkness, and then decreased its transcript abundance until dawn. It has been shown that the transcript abundance of PEPC kinase matches the track of the PEPC kinase activity and the phosphorylation state of PEPC in *K. fedtschenkoi* (Hartwell *et al.* 1999a, Carter *et al.* 1991). The expression of PEPC kinase transcript from the two *Kalanchoë* species tracked the apparent *in vivo* phosphorylation state of PEPC, in which the transcripts/phosphorylation peaked during the first dark period (Fig. 3). These data suggest that the major factor controlling the phosphorylation of CAM PEPC is the abundance of PEPC kinase transcript in the two CAM *Kalanchoë* species. In pineapple, the expression pattern of PEPC kinase, obtaining by crossed-hybridisation with PEPC kinase probes from *K. pinnata* or *K. daigremontiana*, did not match the apparent *in vivo* phosphorylation state of PEPC. The abundance of PEPC phosphorylation was observed around the midnight but that of PEPC kinase mRNA was during the end of dark phase (Fig. 3). The factors accounting for this lack of correlation may possibly be attributed from the metabolite control that modulate the expression of PEPC kinase and/or from other protein kinase (possibly CDPK) responsible for the activation of PEPC *in vivo* in pineapple (see below).

The nocturnal transcript abundance of PEPC kinase in pineapple was strikingly lower than that in the two *Kalanchoë* species, attributing in a markedly low nocturnal phosphorylation state of PEPC in this CAM species (Fig. 3). The lower amount of nocturnal phosphorylation state of PEPC associated with the high activity of V-ATPase and PPase for the accumulation of malate in the vacuole of the pineapple leaf (Chen and Nose 2000) is reflected in a higher malate sensitivity of PEPC for this particular CAM species (Theng *et al.* 2007, Theng *et al.* unpublished).

Experiments involving the use of protein synthesis inhibitors in higher plants were performed to understand the mechanism underlying the nocturnal appearance of PEPC kinase activity. Treatment of detached leaves with CHX or puromycin (Table 1) during the day inhibited the appearance of PEPC kinase activity during the night for the three CAM species, as judged by malate sensitivity and the apparent *in vivo* phosphorylation state of PEPC (Table 1, Fig. 5). The effective concentration of CHX and puromycin varied among CAM species, this variation is not yet known. The effective concentrations used to block PEPC kinase activity in this study are similar to those in the previous studies in CAM and C_4 plants (Carter *et al.* 1991, Bollig and Wilkins 1979, Hartwell *et al.* 1999b). However, the treatments did not affect the expression of PEPC kinase transcripts as observed in the three CAM species (Fig. 6). These data indicate strongly that the protein synthesis inhibitors prevented the translation of PEPC kinase mRNA, and thereby blocked the nocturnal appearance of PEPC kinase activity and maintained PEPC in the dephosphorylated state *in vivo*. The finding is consistent with the hypothesis that *de novo* synthesis of a protein is required for the appearance of PEPC kinase activity, and that the kinase activity turns over rapidly (Carter *et al.* 1991, Jiao *et al.*1991, Bakrim *et al.* 1993).

Although PEPC kinase transcription/translation is a major controlling mechanism, other factors may be susceptible to modulate its activity. Malate has been proposed to be one component altering the PEPC kinase gene expression in CAM plants (Borland *et al.* 1999, Nimmo 2000). Malate inhibits PEPC kinase activity through its interaction with PEPC and reduces the phosphorylation state of PEPC (Carter *et al.* 1991, Wang and Chollet 1993, Li and Chollet 1994). In addition, malate modulates the changes in cytosolic pH, which has been shown to increase during the late day/early dark phase and decrease during the late night/early illumination phase (Hafke *et al.* 2001). Subsequently, the rapid fluctuations of cytosolic pH modulate the expression of PEPC kinase mRNA levels and phosphorylation state of PEPC in the leaves of C_4 and CAM plants (Bakrim *et al.* 2001, Echevarria and Vidal 2003). The present study, which is consistent with this view, illustrated that the low malate content (Fig. 1), and increased cytosolic pH during the first dark phase for the *Kalanchoë species*, caused increase in PEPC kinase transcripts and phosphorylation state of PEPC *in vivo* (Fig. 3). During the remainder of dark phase, the leaf malate content increased *via* PEPC phosphorylation, but the malate sequestration into vacuoles was very slow and/or was not completely removed from the cytoplasm

because the low activity of V-ATPase and V-PPase activity for the two *Kalanchoë* species (Chen and Nose 2000). This caused decrease in cytosolic pH that attributed to reduce the expression of PEPC kinase mRNA and PEPC phosphorylation accordingly (Bakrim *et al.* 2001). The data of *K. daigremontiana* in the present study differed from previous study by Borland *et al.* (1999), having shown that the PEPC kinase mRNA accumulation was abundant during the 9 h dark phase. The differences in the timing of transcript abundance of PEPC kinase mRNA observed in this study and those in the previous study can be attributed, in part, to the difference in methods and/or the growth conditions used.

As mentioned above in the pineapple, the expression of PEPC kinase mRNA was abundant at late night but the phosphorylation of PEPC had a peak around midnight (Fig. 3). Thus, abundance of phosphorylation state of PEPC occurred before the abundance of PEPC kinase mRNA, indicating that CAM regulation in pineapple did not follow the mechanism by which the PEPC kinase mRNA expression/translation appears concomitantly with the PEPC phosphorylation. The cause of PEPC kinase mRNA expressed increasingly during the late dark phase in pineapple could result from the increase in cytosolic pH because the malate removal from the cytoplasm could be more efficient due to the markedly high activity of V-ATPase and PPase in this plant species (Chen and Nose 2000, Bakrim *et al.* 2001).

The calcium/calmodulin antagonist W7 blocked the apparent phosphorylation state of PEPC in CAM *M. crystallinum* and C_4 *Digitaria sanguinalis* (Bakrim *et al.* 2001, Giglioli-Guivarc'h *et al.* 1996). The signal transduction of PEPC phosphorylation cascade in these plants involve the activation of calcium channel in the tonoplast membrane and calcium release into the cytosol, and hence activate Ca^{2+}-dependent protein kinase, possibly CDPK. In addition, it is noteworthy that C_4 plants, maize and sorghum, contain both Ca^{2+}-dependent and independent protein kinases that can phosphorylate PEPC (Ogawa *et al.* 1992, Bakrim *et al.* 1992, Li and Chollet 1993). In this study, the W7 also showed the significant effect on the pineapple and decreased the nocturnal phosphorylation state of PEPC, and maintained the enzyme in the dephosphorylated state as judged by the malate sensitivity, but this drug had no significant effect on the two *Kalanchoë* species even at higher concentration than that in *M. crystallinum* (Table 1, Fig. 5, Bakrim *et al.* 2001). Moreover, as noted above, the expression of PEPC kinase mRNA matched the apparent *in vivo* phosphorylation state of PEPC in the *Kalanchoë* species, but did not match in pineapple (Fig. 3). Therefore, it is possible to suggest that phosphorylation state of PEPC in the *Kalanchoë* species is largely controlled by PEPC kinase, but that in pineapple may possibly be involved by both PEPC kinase and CDPK. The signal transduction pathways together with the purification or isolation of both Ca^{2+}-dependent and independent protein kinases, particularly from pineapple, require further clarification. Ogawa *et al.* (1998) has suggested that the regulatory phosphorylation of PEPC in C_4 plant maize is controlled by two different signal transductions, at which the basal level is controlled by the authentic Ca^{2+}-independent, and the additional level is controlled by Ca^{2+}-dependent protein kinase.

In conclusion, several different features in PEPC phosphorylation process have been observed between *Kalanchoë* species and pineapple. In the two *Kalanchoë* species, the phosphorylation state of PEPC is largely controlled by PEPC kinase transcript abundance, while in pineapple besides the PEPC kinase, CDPK may possibly be involved in the activation of PEPC phosphorylation. The expression of PEPC kinase mRNA may be modulated by codependence on malate fluxes and alkalinisation of cytosolic pH during the dark phase for the three CAM species. Moreover, the PEPC kinase mRNA expression and the phosphorylation state of PEPC are of significantly lower degree in pineapple than in the two *Kalanchoë* species examined. Therefore, to gain further insight into the phosphorylation cascade of PEPC in pineapple, the signal transduction pathway and the purification or isolation of both Ca^{2+}-dependent and independent protein kinases are necessary.

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