Regulatory Properties of Phospho*enol*pyruvate Carboxylase in Crassulacean Acid Metabolism Plants: Diurnal Changes in Phosphorylation State and Regulation of Gene Expression

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Abstract : Regulatory properties of phospho*enol*pyruvate carboxylase (PEPC, EC 4.1.1.31) in three CAM species, *Kalanchoë pinnata*, *K. daigremontiana* and *Ananas comosus* (pineapple) were examined. PEPC activity in the leaves of the three CAM species exhibited diurnal changes peaking during the first 2-h of darkness in *Kalanchoë* species and at midnight in pineapple, and then decreasing drastically until dawn. The oscillations of PEPC activity were far greater in *Kalanchoë* species than in pineapple. In the presence of 2 mM malate, the activity of PEPC decreased in all three CAM species, but the sensitivity of PEPC to malate was markedly different between pineapple and the *Kalanchoë* species. The malate sensitivity was 2- to 3-times higher in pineapple than in the *Kalanchoë* species during the dark period, but it was almost the same during the light period. PEPC in the three CAM species was phosphorylated only during the dark period. PEPC proteins were highly phosphorylated during the first 2-h of darkness in *Kalanchoë* species and at midnight in pineapple, and then they decreased drastically during the latter part of darkness. CAM-specific isoforms of PEPC in the leaves of the three CAM species contained a highly conserved phosphorylation site of Ser-11 at the N-terminus. These PEPC isoforms displayed diurnal changes in transcript abundance, with the peak of transcripts occurring during the dark period. The day/night changes in PEPC transcript abundance were mirrored by changes in the PEPC protein and corresponding enzyme activity over the diurnal cycle. These findings suggest that the diurnal regulation in PEPC activity is determined by the amount of PEPC protein as well as the posttranslational control in these CAM species.

Key words : Crassulacean acid metabolism (CAM), Malate sensitivity, Phospho*enol*pyruvate carboxylase (PEPC), Phosphorylation state, Protein abundance, Transcript abundance.

Phospho*enol*pyruvate carboxylase (PEPC, EC 4.1.1.31) catalyzes the carboxylation of phospho*enol*pyruvate (PEP) in the presence of HCO_3^- to yield oxaloacetate (OAA) and P_i. Besides its cardinal roles in the initial fixation of atmospheric CO_2 during C_4 photosynthesis and Crassulacean acid metabolism (CAM), PEPC functions anaplerotically in non-photosynthetic tissues and the leaf of C_3 plants, and plays specialized roles in carbon metabolism in guard cells during stomata opening and C_4-acid formation in N_2-fixing legume root nodules (Chollet et al., 1996; Vidal and Chollet, 1997; Nimmo, 2000).

The activity of PEPC in CAM plants is regulated at both transcriptional and post-translational levels. Transcriptional control regulates the amount of PEPC protein through changes in the abundance of PEPC mRNA. The day/night changes in transcript abundance of PEPC has also been noted in several CAM species, although their PEPC proteins and activities remain relatively constant over the diurnal

cycle (Boxall et al., 2001; Borland and Taybi, 2004; Taybi et al., 2004). Post-translational control regulates the diel activation/deactivation of PEPC to provide an effective means of fine-tuning the CO_2 uptake that is central to the operation of CAM. PEPC is activated at night by phosphorylation of a single, strictly conserved serine (Ser) residue near the N-terminus of the protein. The phosphorylated PEPC enzyme is denoted to be more active and more sensitive to positive effectors, such as glucose-6-phosphate (Glc-6P) and triose-phosphate, and less sensitive to the allosteric inhibitor, L-malate; whereas the dephosphorylated day-form is more sensitive to malate (Winter, 1982; Buchanan-Bollig and Smith, 1984; Nimmo et al., 1984, 1986; Wu and Wedding, 1985; Chollet et al., 1996; Vidal and Chollet, 1997; Cushman and Bohnert, 1999).

The large difference between apparent Ki for malate of dephosphorylated and phosphorylated PEPC has been reported in many CAM species: the apparent Ki of PEPC for L-malate in *K. fedtschenkoi* increased from

0.3 to 2.5−3.0 mM during the night (Nimmo et al., 1984), compared with the change from 0.5 to 5.0 mM in *K. daigremontiana* (Borland et al., 1999), and 0.9 to 5.8 mM in *K. pinnata* (Shaheen et al., 2002). However, the apparent Ki for L-malate of PEPC in a constitutive CAM pineapple changed only from 0.5 to 1.0 mM. This phenomenon suggests that the phosphorylation process may not be the major regulatory mechanism for the pineapple PEPC (Shaheen et al., 2002). In their study, Shaheen et al. (2002) developed a method to extract and measure the *in vivo* activity of the enzyme, using malate sensitivity as an indicator, to judge the phosphorylation status of PEPC in the two CAM species, *K. pinnata* and pineapple. However, the properties of regulatory phosphorylation site near the N-terminus and the diurnal changes in phosphorylation state of PEPC for these CAM species have not been elucidated.

In the present study, we investigated further the regulatory properties of PEPC phosphorylation in pineapple and two *Kalanchoë* species, *K. pinnata* and *K. daigremontiana*. The purpose of the study was to clarify whether PEPC in pineapple CAM species is regulated by protein phosphorylation/dephosphorylation over the diurnal cycle. The results highlight the diurnal regulation of PEPC activity, phosphorylation state, transcript abundance and protein expression in pineapple, compared to the two *Kalanchoë* species.

Materials and Methods

1. Plant materials

Kalanchoë pinnata, K. daigremontiana and pineapple (*Ananas comosus* (L.) Merr. cv. Smooth-cayenne N67-10) were vegetatively propagated and grown in pots in a heated greenhouse under a natural photoperiod. The plants were transferred to a growth chamber (KG-50 HLK, Koito Industrial Co., Ltd., Japan) and grown there for two weeks, with photoperiod of 10-h (8:00 to 18:00) light and 14-h (18:00 to 8:00) dark before collecting leaf samples. The conditions in the growth chamber were 30ºC during the light period at a photon flux density of 420−450 μmol m^{-2} s^{-1} at the mid-plant height, and 20ºC during the dark period with a relative humidity of 70% during both periods. The fifth to eighth leaf pairs, counting from the apex of *K. pinnata* and *K. daigremontiana*, and the fully expanded mature leaves of pineapple were used for all experiments. Leaf samples collected were immediately immersed in liquid nitrogen, and stored at −80ºC until use. The leaf samples for the isolation of RNA were collected at midnight (01:00), when transcript abundance of CAM-specific isoforms of PEPC should be expressed at a high level.

2. Enzyme extraction

Enzyme extract was prepared according to the methods described by Shaheen et al. (2002) with some modifications of protease inhibitors. Fresh leaf sample (4 cm^2) was ground with a pre-chilled mortar and pestle in a 4 ml ice-cold extraction buffer containing 50 mM Bicine-KOH (pH 8.2), 1 mM EDTA-NaOH (pH 7.0), 5 mM MgCl$_2$, 5 mM DTT, 1% (w/v) Triton X-100, 20% (v/v) glycerol, 1 mM AEBSF, 10 μg ml^{-1} E-64, 10 μg ml^{-1} chymostatin, 100 μM leupeptin and 1 mM PCMB, 2.5% (w/v) insoluble PVP and 0.5 g washed sea sand. The homogenate was passed through one layer of Miracloth (Calbiochem-EMD Biosciences, Inc. La Jolla, CA, USA) and centrifuged for 20 s at 4ºC in a Microcentrifuge (MX-150, Tomy, Japan) at 12,000 rpm to remove the particulate material, and the supernatant was used immediately for enzyme assays. The activity of the enzyme was measured within 2-3 minutes after homogenization of the leaf tissues.

3. Assay of PEPC activity and malate sensitivity

PEPC activity was measured spectrophotometrically at 340 nm and 30ºC in a 3.0 mL assay medium containing 50 mM Tris-HCl (pH 8.2 for pineapple, 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 mL^{-1} BSA, and 10 IU L-MDH. The assays were initiated by the addition of an aliquot of centrifuged crude enzyme extract to the assay medium. Malate sensitivity of PEPC was determined spectrophotometrically at 340 nm in the same assay medium under the same conditions in the presence or absence of 2 mM L-malate. Chlorophyll content was determined according to Arnon (1949). Malate content of the leaf discs was estimated enzymatically as described by Chen et al. (2002).

4. Phosphorylation status of PEPC protein and western blot analysis

To determine changes in the phosphorylation status and expression of PEPC protein, we prepared crude leaf extracts as described above, and estimated the protein content according to Bradford (1976) using BSA as the standard. Three microgram of proteins extracted from leaves at each time point were resolved on 8% (w/v) denatured polyacrylamide gels (Laemmli, 1970). After separation by electrophoresis, one gel was stained with Pro-Q Diamond phosphoprotein gel stain (Molecular Probes, Inc., USA) and subsequently with SYPRO Ruby protein gel stain (Bio-Rad Laboratories, CA. USA) according to the manufacturer's instructions. Another gel was electroblotted onto a nitrocellulose membrane (Bio-Rad Laboratories, CA. USA), and anti-PEPC antiserum (1/40,000 fold diluted rabbit anti-maize leaf PEPC from Rockland, USA) was used to identify PEPC bands. Detection of immunoreactive proteins was achieved by using alkaline phosphatase-link secondary antibody (goat anti-rabbit IgG from Bio-Rad) according to the manufacturer's instruction. Phosphoprotein on the

gels was imaged by scanning with Typhoon image scanner (Typhoon 9000E, Amersham Pharmacia Biotech, USA), and PEPC bands on the blots were captured and quantified using Kodak 1D Image Analysis Software (Kodak, Rochester, USA). SDS-PAGE and western blots were performed in 3 to 4 replicates with consistent results, and representative results are shown.

5. Total RNA isolation and RACE amplification

Total RNA was isolated from leaves collected at midnight (01:00) from the three CAM species, using guanidine hydrochloride or a commercial kit (RNeasy Plant Mini kit, Qiagen, Japan) precipitating with 1-2% (w/v) HMW polyethylene glycol 20,000 (Gehrig et al., 2000). Poly (A)$^+$-enriched RNA was obtained from the total RNA by passing through an oligo (dT)-cellulose spin column (Oligotex^{TM}-dT30 [Super] mRNA Purification kit, TaKaRa, Japan) according to the manufacturer's instruction.

One microgram of poly (A)$^+$-enriched RNA was used for the cDNA synthesis for RACE amplification. The 3' end of PEPC cDNA was amplified with the 3' RACE system (SMART^{TM} RACE cDNA Amplification, BD Biosciences Clontech, USA) according to the manufacturer's instructions, using a degenerate primer, CAAGG(A/T)GA(A/G)GT(C/T)AT(A/T/C)GA(A/G)C, CA(A/G)GG(A/T)GAGGT(T/C)AT(A/T)GA(A/G)C and GAC(A/T/G/C)GT(A/T/G/C)CA(A/G)GG(A/T/G/C)GA(A/G)GT(A/T/G/C)A, corresponding to conserved known partial PEPC sequences for *K. pinnata, K. daigremontiana* and pineapple, respectively. The 5' end of PEPC cDNA was amplified with the 5' RACE system (SMART^{TM} RACE cDNA Amplification, BD Biosciences Clontech, USA) according to the manufacturer's instructions using two internal gene-specific primers, AGATGGAACCTGGTTTGG and TCCAAGGGATGGCACGAAGAGA; TGCGTCCACGCAAATATC and ACGGACGACTCCCCAATGTTCA; and TTGTCTGTGTCCACGCAAAT and GTCGGCTGCCAATGTTCATCCT for *K. pinnata, K. daigremontiana* and pineapple, respectively.

6. Cloning and sequence analysis

PCR products from 3' RACE- and 5' RACE fragments were gel extracted using NucleoTrap (Macherey-Nagel, Germany), and cloned into pGEM-T easy vector system (Promega, Madison, WI) according to the manufacturer's instruction. Plasmids were purified following the protocol of Sambrook and Russell (2001). Positive clones (4-5 independent transformants) of the 3' RACE cDNA fragments were sequenced from both strand directions, while the positive clones of the 5' RACE cDNA fragments (5-6 independent transformants) were partially sequenced at the N-terminus by the ABI Prism 310 Genetic Analyzer, using the Prism BigDye Terminator Ready Reaction Cycle Sequencing kit (ABI Applied Biosystems, 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. The end-to-end PCR was performed and sequenced to validate the 5' region and the 3' end of each PEPC isoform isolated from the same isogene. Multiple sequence alignments were performed with a GENETYX-WIN program (GENETYX-WIN V.5.1, Japan).

7. Northern blot analysis and semi-quantitative RT-PCR

Three microgram of total RNA isolated from leaves collected every 4-h over the time course were fractionated on 1.2% (w/v) agarose gel containing 2% (w/v) formaldehyde, and transferred to nylon membrane (Roche Applied Science, Germany) by capillary transferred method (Sambrook and Russell, 2001). The 3' ends of CAM-specific PEPC cDNA (1.2 kbp fragments from each species) were labeled with DIG RNA labeling kit (Roche Applied Science, Germany). Hybridization was carried out in a high SDS hybridization solution (7% (w/v) SDS, 50% (v/v) deionized formamide, 5x SSC, 50 mM sodium phosphate (pH 7.0), 0.1% (v/v) N-lauroylsarcosine and 2% (w/v) Blocking solution) with the labeled probes at 68ºC overnight. The filters were washed twice under low-stringency conditions (2x SSC, 0.1% SDS) for 5 min at room temperature and twice under high stringency conditions (0.1x SSC, 0.1% SDS) at 68ºC for 15 min. The blots were detected using DIG Luminescent Detection kit (Roche Applied Science, Germany) and exposed to X-ray film (Hyperfilm, Amersham Biosciences, UK) for 5−15 minutes according to the manufacturer's instruction.

Semi-quantitative RT-PCR was performed using 3 μg total RNA and 50U AMV reverse transcriptase in cDNA synthesis. PCR reactions were performed with 0.5 μL of cDNA in a reaction mixture (50 μL) containing 0.5 μM of each primer and 1.25U *Taq*DNA polymerase. A pair of primers for each PEPC isogene from the three CAM species: *K. pinnata* (*KpPpcD-F*, TTCCTGGTTTGGGAGGACA and *KpPpcD-R*, CCATGGAGGTTGATGCAGA; Tm= 63ºC, 25 cycles), *K. daigremontiana* (*KdPpc3-F*, TTGTTGTGGCCTGCAGAA and *KdPpc3-R*, ATGCAAGCCAGTCATGGAG; Tm= 60ºC, 20 cycles) and pineapple (*AcPpc2-F*, GGAGGACCACCCTCATCTTT and *AcPpc2-R*, CAACCTGTGGAATGGGTGTT; Tm=65ºC, 30 cycles) were designed to the 3'UTR using a Primer3 program (Rozen and Skaletsky, 2000). Ubiquitin (*Ubi-F*, GGAGGACCACCCTCATCTTT and *Ubi-R*, CTGCGAAGTCC GTCATCA; Tm= 67ºC, 25 cycles) and actin (*Actin-F*, GGVCGWACWACTGGTATTGT and *Actin-R*, TCYGMBCCAATGGTKATSAC; Tm=
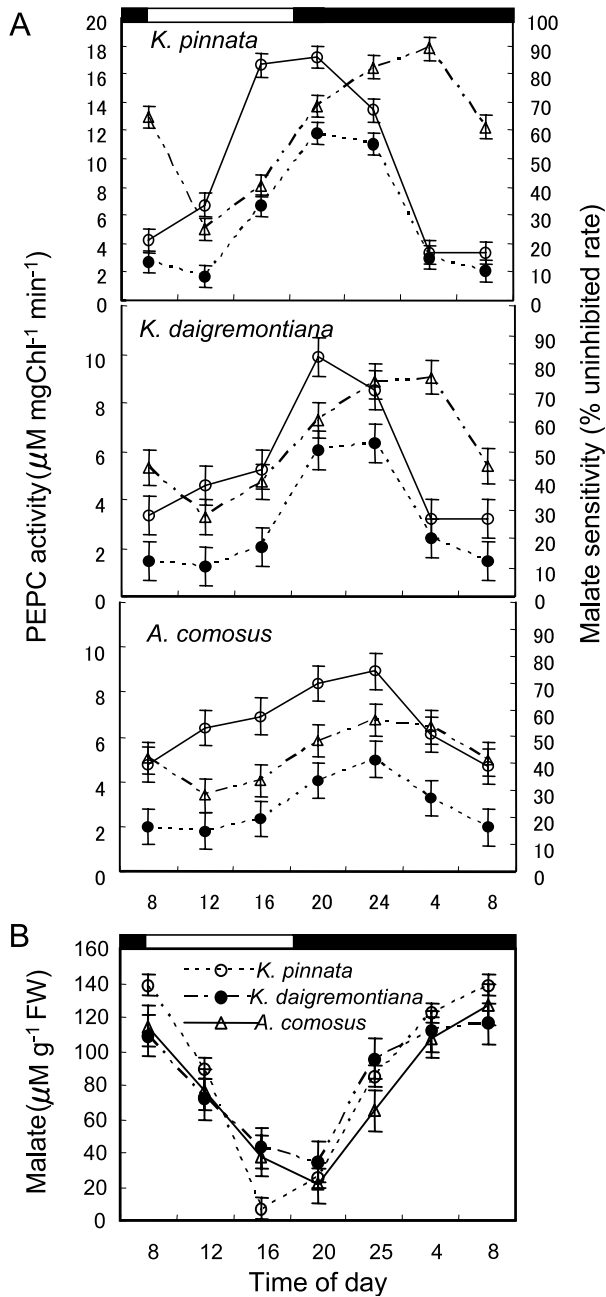
Fig. 1. A. Diurnal changes in extractable PEPC activity in *K. pinnata*, *K. daigremontiana* and *A. comosus*. Specific PEPC activity was measured in the absence (○) and in the presence (●) of 2 mM malate and malate sensitivity of PEPC (△ , % uninhibited rate) was shown with control activity regarded as 100%. Open and closed bars represent day and night, respectively. Values are mean of 3-6 replicates ± SD. B. Malate content of the leaf discs sampled above.

Fig. 2.   Diurnal changes in phosphorylation state of PEPC protein: A. *K. pinnata*, B. *K. daigremontiana* and C. *A. comosus*. Proteins (3 μg) from crude leaf extracts were resolved on SDS-PAGE followed by staining with Pro-Q Diamond Phosphoprotein gel stain (a) and subsequently with SYPRO Ruby protein gel stain (b). The protein bands were visualized with a visible light laser based scanner. Open and closed bars represent day and night, respectively. The black arrowheads show PEPC in the gel. The numbers on the left indicate Mr values × 10^3 of the marker proteins.

63°C, 25 cycle) were used as positive control for pineapple and *Kalanchoë* species, respectively. The PCR conditions were 20-30 cycles, a 94°C denaturing cycle for 30 s, a 60–67°C annealing cycle for 45 s, and a 72°C extension cycle for 80 s. The number of cycles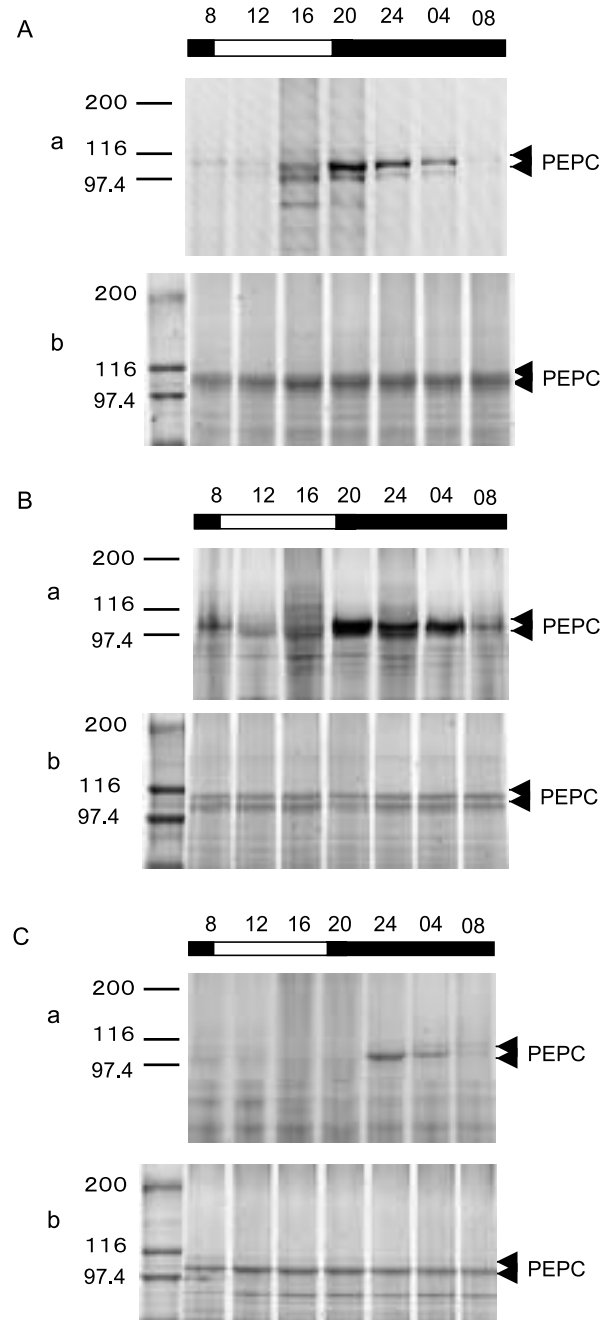 used for each targeted gene was within the linear phase of amplification. The signal of the bands from Northern blots and RT-PCR were captured and quantified by Kodak 1D Image Analysis Software (Kodak, Rochester, USA). Northern blot and semi-quantitative RT-PCR
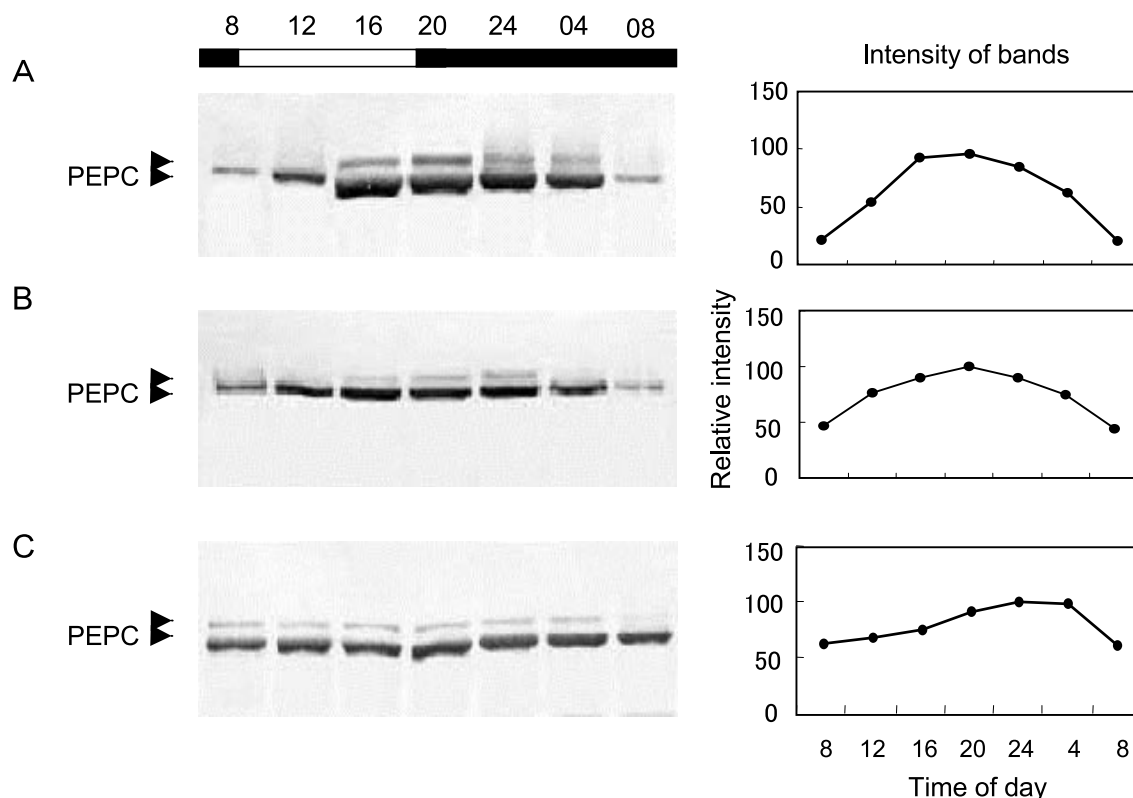
Fig. 3.   Western blot of PEPC protein abundance: A. *K. pinnata*, B. *K. daigremontiana* and C. *A. comosus*. Proteins were probed using rabbit anti-(maize leaf PEPC) antiserum and visualized using alkaline phosphatase link secondary antibody (goat anti-rabbit IgG). The crude proteins used in western blots were the same as the crude leaf extracts used to determine the diurnal changes in phosphorylation state of PEPC in Fig. 2. Open and closed bars represent day and night, respectively. The relative intensities of bands are plotted as a percentage of the maximum level of PEPC protein in each lane. The arrowheads show PEPC protein subunits on the blots.

were repeated twice with consistent results and the representative data are shown.

Results

1. Extraction of PEPC enzyme

When we used an extraction buffer containing protease inhibitors: 200 μM PMSF and 2 mM MIA, western blots of prepared crude extracts using anti-PEPC antiserum revealed two protein subunits in the leaves of *Kalanchoë* species, but only one protein subunit in pineapple. Similar results were obtained when we used the extraction buffer containing 1 mM AEBSF, 10 μg mL^{-1} E-64, 10 μg mL^{-1} chymostatin, 10 μM leupeptin and 2 μg mL^{-1} pepstatin A (data not shown). However, when a protease inhibitor cocktail containing 1 mM PCMB plus 1 mM AEBSF, 10 μg mL^{-1} E-64, 10 μg mL^{-1} chymostatin and 100 μM leupeptin was used in the extraction buffer, the integrity of enzyme was preserved (Fig. 1) and two PEPC protein subunits were detected in all three CAM species (Fig. 2 and 3).

2. Activity and malate sensitivity of PEPC

Diel activities of PEPC in rapidly prepared crude extracts (2–3 min after homogenization) of fresh leaf tissues from the three CAM species, *K. pinnata*, *K. daigremontiana* and pineapple were examined. The trend of diurnal changes in the activity of PEPC measured in the absence of added 2 mM L-malate was basically similar in all three CAM species (Fig. 1A). The PEPC activities rose gradually to the highest levels during the first part of dark period in *Kalanchoë* species, but around midnight in pineapple, and then reduced rapidly in the latter part of darkness and reached minimum levels during the first part of light period in all three species. The day/night oscillations of the PEPC activity were far greater in *Kalanchoë* species than in pineapple, and the oscillations followed the course of the leaf malate content in the opposite direction (Fig. 1). In the presence of 2 mM L-malate, PEPC was inhibited by about 75% when extracted during the light period in all three CAM species. However, the sensitivity of PEPC to malate revealed a marked difference among the species when extracted during the dark period. The absolute levels of the malate sensitivity were far greater in pineapple than that in the two *Kalanchoë* species. As shown in Fig. 1A, PEPC in pineapple was inhibited by about 50%, compared with about 10% and 25% in *K. pinnata* and

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                                                   *
K. pinnata (KpPpc D)           1:MGSRNV EKMASIDAQLR QLVPNKVSEDDKLVEYDTLLLDRFLDILQDLHGEDLREMVQEC 60
K. daigremontiana (KdPpc 3)    1:MGSRNV EKMASIDAQLR QLAPNKVSEDDKLVEYDTLLLDRFLDILQDLHGEELREMVQEC 60
A. comosus (AcPpc 2)           1:MAKSSV ERHQSIDAQLR LLAPAKVSEDDKLIEYDALLVDRFLDILQDLHGEALREFVQEC 60
M. crystallinum (Ppc1, X14587) 1:MSTVKL DRLTSIDAQLR LLAPKKVSEDDKLIEYDALLLDRFLDILQNLHGEDIKETVQEL 60
                                 *     ..   ******* *.* ******** *** **.*******.**** ..* ***.

K. pinnata (KpPpc D)          61:YELSAEYEAKHDPKKLDELGRLLMSLDPGDSI                            92
K. daigremontiana (KdPpc 3)   61:YELSAEYEAKHDPKKLDELGKLLMSLDPGDSI                            92
A. comosus (AcPpc 2)          61:YELSATYESASDFAKLDELGKMLTSLSPGDSIV                           93
M. crystallinum (Ppc1, X14587)61:YEQSAEYERTHDPKKLEELGSMVTSLDAGDSIV                           93
                                **.**.** .*..**.***  . **..****
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Fig. 4. Alignment of partial deduced amino acid sequences of the N-terminus of CAM-specific PEPC isoforms from *K. pinnata* (*KpPpc D*), *K. daigremontiana* (*KdPpc 3*), *A. comosus* (*AcPpc 2*) and *M. crystallinum* (*Ppc1*, X14587). The highly conserved phosphorylation sites near the N-terminus are marked in the square box, and the phosphorylation of Ser residue is noted by asterisk.

K. daigremontiana respectively, when extracted during the latter part of dark period.

3. Phosphorylation status of PEPC

Figure 2 shows the diurnal changes in the phosphorylation status of PEPC protein in the leaves of *K. pinnata*, *K. daigremontiana* and pineapple studied by SDS-PAGE staining with fluorescent phosphoprotein stain. Phosphorylated PEPC protein was observed during the dark, and disappeared during the light period. PEPC protein in the *Kalanchoë* species was highly phosphorylated during the first 2-h of darkness, and then decreased steadily in the latter part of dark and reached a very low level during the early light period. The phosphorylation of PEPC disappeared at midday, but it began to phosphorylate before the darkness at phase IV (Fig. 2A and B, and 3A and B). The phosphorylation status of PEPC in pineapple leaves was delayed until midnight and then decreased dramatically in the latter part of dark period. The PEPC protein was dephosphorylated during the light and the early part of darkness (Fig. 2C and 3C).

4. Abundance of PEPC protein

Figure 3 shows the diel changes in the expression of PEPC protein extracted from the three CAM species over the day/night cycle. The day/night changes in PEPC protein expression were basically similar to the diurnal oscillations in PEPC activities. In the *Kalanchoë* species, the large oscillations in PEPC protein were in agreement with the large changes in PEPC activities, while the small changes of PEPC protein in pineapple was consistent with small changes in PEPC activity in this CAM species (Fig. 1A and 3).

5. Regulatory domain of PEPC N-terminus

The 5'-region of CAM-specific PEPC cDNA from the three CAM species were isolated and partial deduced amino acid sequences were aligned with the PEPC of a model CAM plant of *M. crystallinum* (Fig. 4). Partial amino acid sequences revealed that the N-terminus of CAM-specific PEPC isoforms from these CAM species contained an invariant conserved phosphorylation site of Ser-11. The conserved residues surrounding the target serine (E/DR/KH/MQ/A**S**IDAQLR) were also observed. The partial amino acid sequences revealed a 62% homology for these CAM species (Fig. 4).

6. Cloning and expression of PEPC transcripts

Partial C-terminal cDNA of PEPC isoforms from the three CAM species were isolated and sequenced. The identities of sequenced data were confirmed by BLAST analysis (data not shown). Three designated PEPC isoforms, *KpPpc D*, *KdPpc 3* and *AcPpc 2* were selected for the study of transcript abundance. *KpPpc D* isoform was identical to CAM-specific PEPC isoform D isolated from the leaf of *K. pinnata* currently reported by Gehrig et al. (2005), and *KdPpc 3* and *AcPpc 2* were identical to PEPC isoform 3 (AJ312634) and isoform 2 (AJ312628) in *K. daigremontiana* and pineapple, respectively (Genbank database). The *KdPpc 3* and *AcPpc 2* transcripts appeared to be more abundant during the dark period (see Fig. 5) and were not expressed in the roots (data not shown). Thus, each PEPC isogene was assumed to be a CAM-specific isoform in *K. daigremontiana* and pineapple. The 3' UTRs of three isogenes contained two polyadenylation signals, *aataaa* and *ataaaa* (data not shown); these are typically found in the 3' UTRs of PEPC isolated from other plant species (Cushman and Bohnert, 1989; Honda et al., 1996; Gehrig et al., 1998).

PEPC isoforms characterized by sequencing were studied by Northern blot analyses (Fig. 5). As a probe, a 1.2 kbp fragment of the 3' end of CAM-specific PEPC isogenes of each CAM species was used. The PEPC isoforms in these CAM species showed diurnal changes with higher transcript abundance during the darkness than during the light period. The levels of day/night changes in the transcripts were far greater in the *Kalanchoë* species than in pineapple. In the *Kalanchoë* species, the transcripts of PEPC isoforms increased rapidly to the peak during the first 2-h of dark phase and reduced drastically to very low levels
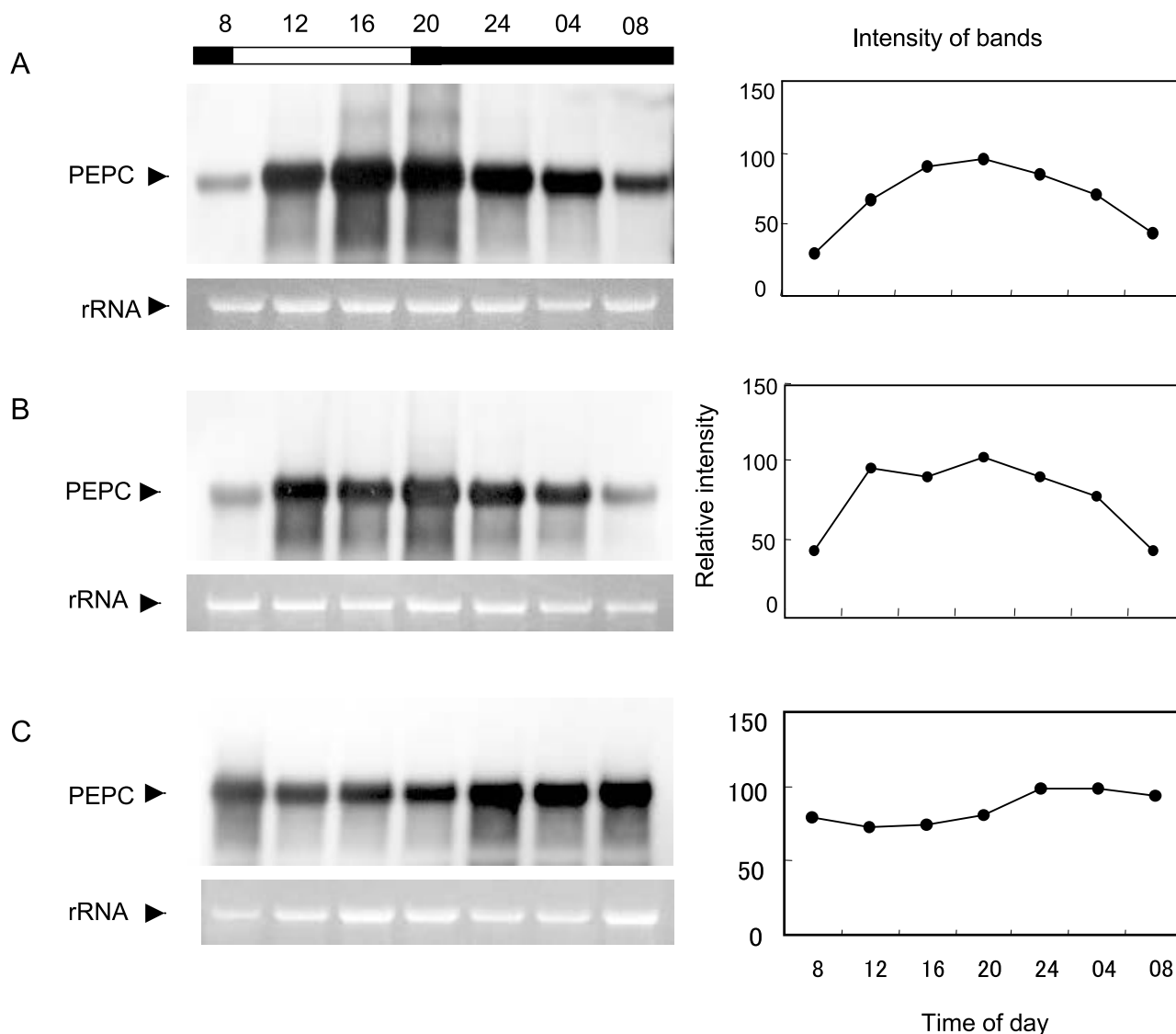
Fig. 5. Northern-blot analysis of PEPC transcripts: A. *K. pinnata*, B. *K. daigremontiana* and C. *A. comosus.* The membranes were hybridized using a DIG RNA labeled 1.2 kpb fragment of the 3' side of CAM-specific PEPC cDNA. Equal volumes of rRNA for each species are shown under the hybridized blots. Open and closed bars represent day and night, respectively. The relative intensities of bands are plotted as a percentage of the maximum level of PEPC mRNA in each lane. The black arrowheads show PEPC and rRNA on the blots and gel respectively.

during the early part of the light period (Fig. 5A and B). The transcript abundance of the PEPC isoform in pineapple rose to the peak at midnight and decreased to the minimum level at midday (Fig. 5C). The large or small day/night changes of the PEPC transcripts were mirrored by large or small changes in PEPC protein amount and corresponding enzyme activities in these species (Fig. 1A, 3 and 5).

To determine which isogenes contribute most to PEPC transcript abundance, semi-quantitative RT-PCR, using 3'UTR primers, was assessed. As shown in Fig. 6, the transcript abundance of CAM-specific PEPC isogene from each CAM species displayed diurnal changes with the patterns similar to those determined by Northern blot analysis, whereas the expression of control amplifications, ubiquitin and actin, remained constant over the day/night period.

Discussion

The previous study by Shaheen et al. (2002) detected only one PEPC protein subunit in pineapple leaves but two subunits in *K. pinnata.* This indicated that proteolysis of PEPC protein in crude extracts from pineapple might not be efficiently prevented, although some protease inhibitors were also added to protein extracts preparation. The results obtained in the present study clearly demonstrated the existence of two PEPC protein subunits in pineapple as in the two *Kalanchoë* species (Fig. 2 and 3). Furthermore, the integrity of enzyme has been preserved, in which PEPC
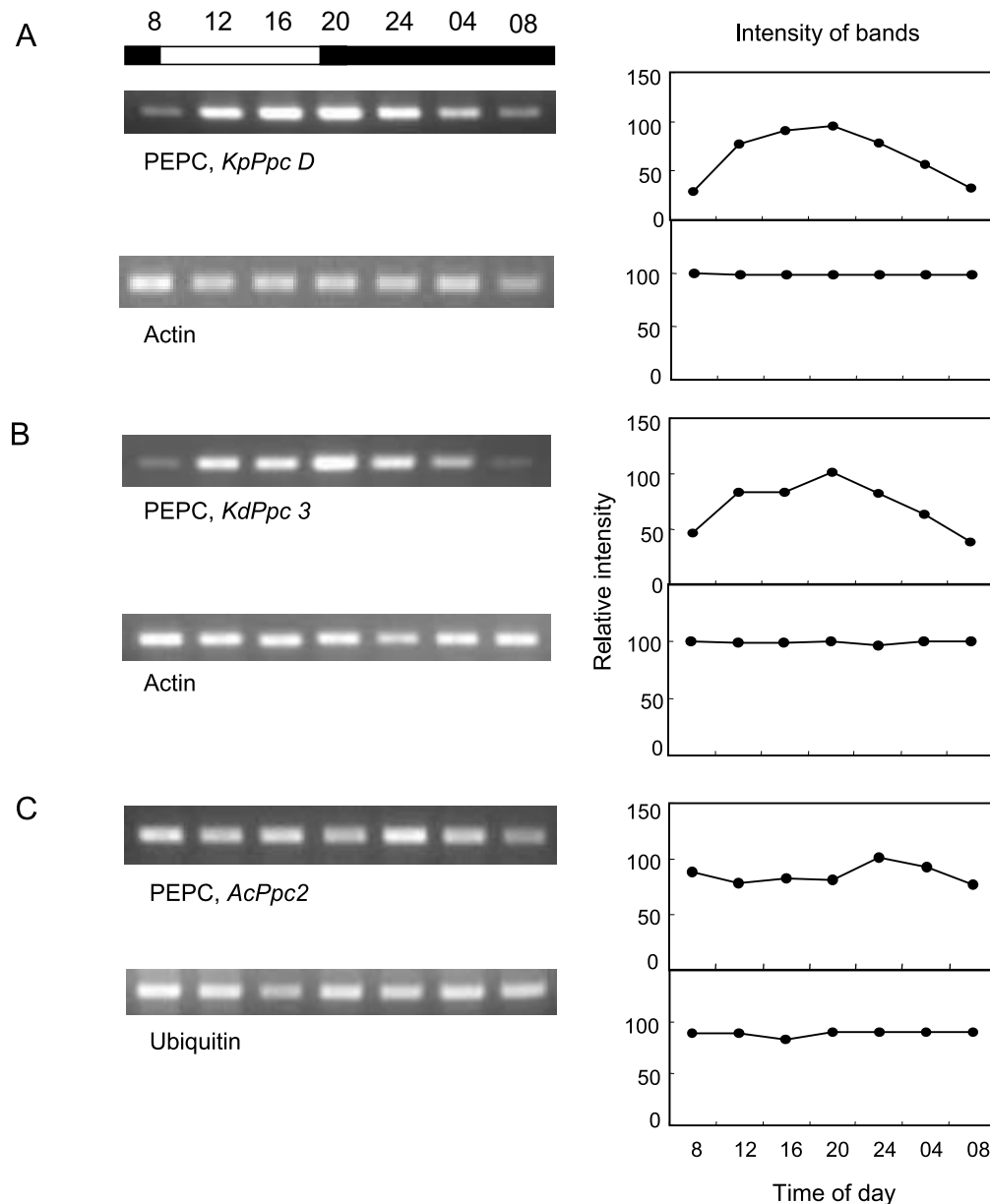
Fig. 6.   RT-PCR of CAM-specific PEPC isogenes: A. *K. pinnata* (*KpPpc D*), B. *K. daigremontiana* (*KdPpc 3*) and C. *A. comosus* (*AcPpc 2*). Ubiquitin and actin are used as control for equal RNA input for pineapple and *Kalanchoë* species, respectively. Open and closed bars represent day and night, respectively. The relative intensities of bands are plotted as a percentage of the maximum level of PEPC mRNA in each lane.

illustrated its sensitivity to malate inhibition (Fig. 1). This means that the proteolysis of PEPC in pineapple was sufficiently inhibited by a better protease inhibitor cocktail in this study.

Shaheen et al. (2002) reported that the activity of PEPC in *K. pinnata* and pineapple was inhibited about 15% and 75%, respectively by 2 mM malate during the night time. This finding suggested that PEPC in *K. pinnata* is phosphorylated, but pineapple PEPC may not be phosphorylated. In the present study, we also found that PEPC in crude extracts prepared from the fresh leaves of pineapple had higher sensitivity to

malate than that from the two *Kalanchoë* species (Fig. 1A). However, PEPC of pineapple was phosphorylated during the dark period (Fig. 2C and 3C).

The activity of PEPC (within 2–3 minutes after homogenization) in the leaves of the three CAM species exhibited diurnal oscillations. The day/night changes in the activity of PEPC were greater in the *Kalanchoë* species than that in pineapple. The PEPC activity was very low from 4:00 to 12:00, and then increased to the peak at 20:00 in the *Kalanchoë* species and midnight in pineapple (Fig. 1A). These findings differed from the results of Nimmo et al. (1984), who

showed that specific PEPC activity of *K. fedtschenkoi* did not change over the diurnal cycle. Winter (1982) also observed no diurnal changes of the PEPC activity in *M. crystallinum* measured at optimum pH 8.0, though at suboptimum pH 7.0 or 7.5 the activity fluctuated. The diurnal changes of PEPC activity in *K. daigremontiana* was similar to that reported by Buchanan-Bollig and Smith (1984).

In the presence of 2 mM malate, PEPC showed similar sensitivity to malate when extracted during the light period, but the sensitivity of PEPC was strikingly different among the CAM species when extracted during the dark period (Fig. 1A). PEPC in the leaves of *K. pinnata* extracted during the latter half of dark period was inhibited by about 10–18%, whereas that of *K. daigremontiana* was inhibited by 25–27% in the presence of 2 mM malate. These results are in agreement with previous reports (Borland et al., 1999; Shaheen et al., 2002). However, pineapple PEPC showed far greater sensitivity to malate than *Kalanchoë* PEPC, which was inhibited about 50% (Fig. 1A). The mechanism causing high malate sensitivity is poorly understood, but this may be related to a number of factors including the relative contribution of tonoplast adenosine triphosphatase (ATPase) and inorganic pyrophosphatase (PPase) to malate transport across the vacuole membrane (Chen and Nose, 2000) and the regulation of PEPC protein kinases in these plants.

In CAM plants, malate synthesized in the cytoplasm via PEPC during the night period is sequestered into the vacuole. This transport is energized by the tonoplast ATPase or PPase, or by a combination of the two (Smith et al., 1996), which establishes the H^+ electrochemical gradient across the membrane since protons are pumped into the vacuole (Cushman and Bohnert, 1999). Chen and Nose (2000) demonstrated that the activities of V-ATPase and PPase (driving force for malic acid accumulation in vacuole) in pineapple leaves were almost two-fold higher than those in *K. pinnata*, indicating that the transport of the nocturnally produced malic acid into the vacuoles could be more efficient in this species. Therefore, it is suggested that the high sensitivity of pineapple PEPC to malate inhibition could be complemented by the effective transport of malic acid into the vacuole; whereas in *K. pinnata*, having very low activities of V-ATPase and PPase, PEPC became less sensitive during the latter part of dark period. In *K. daigremontiana*, the driving force for malic acid accumulation in vacuole was in between those of the pineapple and *K. pinnata*, thus the malate sensitivity of PEPC was also in between them accordingly (Fig. 1A; Chen and Nose, 2000). Winter (1982) also reported that PEPC in *M. crystallinum* was very sensitive to malate with the *Ki* for malate ranging from 0.4–0.9 mM, when extracted during the dark period. The high sensitivity of PEPC to malate was attributed to the efficient sequestration of

nocturnal malic acid into the vacuole.

PEPC in *Kalanchoë* species was highly phosphorylated during the first 2-h of darkness, when PEPC began to lose its sensitivity towards malate. During the remainder of the darkness, the phosphorylation state of PEPC began to decrease while the enzyme became increasingly insensitive to malate (Fig. 1A, 2A and B, and 3A and B). These phenomena were consistent with the observation in *K. uniflora* (Kluge and Brulfert, 1996). In the pineapple, PEPC was highly phosphorylated around the midnight, and then reduced rapidly to the latter part of dark period; but the nocturnal phosphorylation was at a much lower degree than that in the *Kalanchoë* species (Fig. 2 and 3). It is known that the phosphorylation state of PEPC is determined by the presence or absence of Ca^{2+}-independent Ser/Thr protein kinase, which in turn is regulated at the level of gene expression (Carter et al., 1991; Hartwell et al., 1999; Taybi et al., 2000). To determine the involvement of PEPC kinase in the phosphorylation state of PEPC we are presently carrying out the isolation of PEPC kinase transcripts from the three CAM species. The transcript abundance of PEPC kinase in pineapple leaves was about 10-times lower than that in the *Kalanchoë* species (Theng Vuthy, Agarie Sakae, Nose Akihiro, unpublished results). Thus, the lower amount of nocturnal phosphorylation of PEPC in pineapple leaves was associated with the low transcript abundance of PEPC kinase, and this could be attributed to a high sensitivity of PEPC to malate in this plant species.

We found that the conserved phosphorylation site of Ser-11 and the conserved residues surrounding the target Ser (E/DR/KH/MQ/A**S**IDAQLR) are located near the N-terminus of CAM-specific PEPC isoforms of these CAM species (Fig. 4), as reported in other CAM and C_4 plants (Jiao and Chollet, 1991; Chollet et al., 1996; Vidal and Chollet, 1997; Izui et al., 2004). This phosphorylation site has been found to undergo reversible phosphorylation during the light period in C_4 plants, like Ser-8 and Ser-15 of C_4 PEPC from sorghum and maize respectively, or during the darkness in CAM species like Ser-11 of CAM-PEPC in *M. crystallinum* (Chollet et al., 1996; Vidal and Chollet, 1997). From the present study, it is possible to suggest that the Ser-11 of CAM-specific PEPC isoforms from the three species is also involved in the reverse phosphorylation during the dark period (Fig. 2 and 4).

As shown in Fig. 3, the day/night changes in the amount of PEPC protein subunits were observed in the leaves of the three CAM species during the 24-h period. The oscillations of PEPC protein abundance were consistent with that of enzyme activity. In the *Kalanchoë* species, both PEPC activity and protein fluctuated more widely than in pineapple (Fig. 1A and 3). Four PEPC isoforms have been reported to be expressed in the leaves of these CAM species (Gehrig

et al., 2005; Genbank database). In the present study, the PEPC isoforms that we analyzed were suggested to be the major PEPC isogenes contributing mostly to the modulation of the changes in PEPC activity, phosphorylation status and protein expression over the 24-h time course in these CAM species, although the function of other isoforms should be determined.

To determine the levels of expression of CAM-specific PEPC transcripts, we isolated 1.2 kbp fragments of the 3' end of PEPC isogenes of the three CAM species. *KpPpc D*, *KdPpc 3* and *AcPpc 2* isogenes identified to be the CAM-specific PEPC isoforms for *K. pinnata*, *K. daigremontiana* and pineapple, respectively (Gehrig et al., 2005; Genbank database, and reference herein) showed day/night changes in transcript abundance. The PEPC transcript in the leaves of three CAM species increased rapidly to a higher level during the first part of darkness or at midnight, and then decreased dramatically to a lower level during the first part of the light period (Fig. 5). Similarly, the day/night changes in transcript abundance of CAM-specific isoforms of PEPC obtained by semi-quantitative RT-PCR using the specific 3' UTR primers were similar to that of the Northern blot analyses (Fig. 5 and 6). Moreover, the trends of oscillations in PEPC transcripts were in agreement with the diurnal changes in PEPC activity and protein expression (Fig. 1A, 3, 5 and 6), indicating that the oscillation in transcript abundance attributed to changes in PEPC protein amounts and corresponding enzyme activities over the 24-h cycle. These results suggest that only the CAM-specific PEPC isoforms may contribute the most to the modulation of the CAM pathway. These phenomena differed from the recent reports that PEPC isoforms in CAM-performing *Clusia* and *M. crystallinum* showed increased transcript abundance during the day or at the end of the photoperiod, and that their PEPC proteins and extractable enzyme activities remained relatively constant throughout the 24-h diurnal cycle (Boxall et al., 2001; Borland and Taybi, 2004; Taybi et al., 2004). The diurnal changes in PEPC transcript abundances and protein contents (Fig. 3, 5 and 6) may be interspecies-dependent, and the further detailed studies are awaited.

The present study reports the differential regulation of CAM-specific isoforms of PEPC in three constitutive CAM species, *K. pinnata*, *K. daigremontiana* and pineapple. The transcript abundance of CAM-specific PEPC isoforms exhibits diurnal changes, and these changes attribute to changes in PEPC protein expression and extractable enzyme activity over the 24-h diurnal cycle. There are some important differences in the regulatory phosphorylation state of PEPC such as degree and activation time between pineapple and the two *Kalanchoë* species studied. To understand the regulatory phosphorylation of PEPC further, in particular that the pineapple CAM species, studies on

the regulation of PEPC protein kinases responsible for the phosphorylation of PEPC are necessary.

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