CSIRO PUBLISHING

www.publish.csiro.au/journals/fpb *Functional Plant Biology*, 2005, **32**, 467–472

***Research note*:**

Large gene family of phospho*enol*pyruvate carboxylase in the crassulacean acid metabolism plant *Kalanchoe pinnata* (Crassulaceae) characterised by partial cDNA sequence analysis

Hans H. Gehrig[A], *Joshua A. Wood*[B], *Mary Ann Cushman*[B], *Aurelio Virgo*[A], *John C. Cushman*[B,C] *and Klaus Winter*[A]

[A]Smithsonian Tropical Research Institute, P.O. Box 2072, Balboa, Ancón, Republic of Panama.
[B]Department of Biochemistry and Molecular Biology, University of Nevada, Reno, NV 89557-0014, USA.
[C]Corresponding author. Email: jcushman@unr.edu

This paper originates from a presentation at the IVth International Congress on Crassulacean Acid Metabolism, Tahoe City, California, USA, July–August 2004

Abstract. Clones coding for a 1100-bp cDNA sequence of phospho*enol*pyruvate carboxylase (PEPC) of the constitutive crassulacean acid metabolism (CAM) plant *Kalanchoe pinnata* (Lam.) Pers., were isolated by reverse transcription-polymerase chain reaction (RT–PCR) and characterised by restriction fragment length polymorphism analysis and DNA sequencing. Seven distinct PEPC isogenes were recovered, four in leaves and three in roots (EMBL accession numbers: AJ344052–AJ344058). Sequence similarity comparisons and distance neighbour-joining calculations separate the seven PEPC isoforms into two clades, one of which contains the three PEPCs found in roots. The second clade contains the four isoforms found in leaves and is divided into two branches, one of which contains two PEPCs most similar with described previously CAM isoforms. Of these two isoforms, however, only one exhibited abundant expression in CAM-performing leaves, but not in very young leaves, which do not exhibit CAM, suggesting this isoform encodes a CAM-specific PEPC. Protein sequence calculations suggest that all isogenes are likely derived from a common ancestor gene, presumably by serial gene duplication events. To our knowledge, this is the most comprehensive identification of a PEPC gene family from a CAM plant, and the greatest number of PEPC isogenes reported for any vascular plant to date.

Keywords: crassulacean acid metabolism, evolution, gene family, isogenes, *Kalanchoe pinnata*, phospho*enol*pyruvate carboxylase.

Introduction

The cytosolic enzyme phospho*enol*pyruvate carboxylase (EC 4.1.1.31; PEPC) catalyses the β-carboxylation of phospho*enol*pyruvate, with oxaloacetate and inorganic phosphate as products, and serves various functions in plants (Chollet *et al*. 1996; Nimmo 2000). In C_4 photosynthesis and crassulacean acid metabolism (CAM) PEPC catalyses the initial fixation of atmospheric CO_2 into C_4-dicarboxylic acids. PEPC also performs anapleurotic roles in leaves and non-photosynthetic tissues, as well as specialised functions in stomatal guard cells (Kopka *et al*. 1997), legume root nodules (Hata *et al*. 1997; Pathirana *et al*. 1997), developing and germinating seeds (Golombek *et al*. 1999), extension of cotton fibres (Smart *et al*. 1998), and fruit ripening (Guillet *et al*. 2002). Photosynthetic isoforms of PEPC are distinguished by their elevated mRNA and protein expression in leaf tissues in C_4 (Crétin *et al*. 1991; Schaffner and Sheen 1992; Lepiniec *et al*. 1994; Rao *et al*. 2002) and CAM plants (Gehrig *et al*. 1995, 1998; Cushman and Bohnert 1999). Evidence from comparative analysis of C_3, C_3–C_4 intermediates, and C_4 *Flaveria* species suggests that C_4 photosynthetic PEPC isoforms have evolved from ancestral non-photosynthetic or C_3 isoforms and acquired distinct kinetic and regulatory properties (Bläsing *et al*. 2002) mediated by discrete amino acid changes (Engelmann *et al*. 2002). A similar evolutionary progression is also likely to have occurred in CAM plants as demonstrated by the existence of C_3–CAM intermediates (Winter and Smith 1996;

Abbreviations used: CAM, crassulacean acid metabolism; PEPC, phospho*enol*pyruvate carboxylase; RT–PCR, reverse transcriptase-coupled polymerase chain reaction.

 10.1071/FP05079 1445-4408/05/050467

Winter and Holtum 2002), however, detailed comparative analysis of the molecular genetic and biochemical properties of photosynthetic PEPC isoforms is lacking. Amongst CAM species, up to four PEPC isogenes have been described for a given plant and before the current analysis, CAM specific PEPC isoforms have been described in three species (Cushman *et al*. 1989; Gehrig *et al*. 1995, 2001). Here we attempt a comprehensive characterisation of PEPC isogenes in one of the classic study objects of CAM research (Edwards and Walker 1983): *Kalanchoe pinnata* (syn. *Bryophyllum calycinum*). Such analyses are an essential first step in our understanding of the molecular evolution of CAM.

Materials and methods

Plant materials

Kalanchoe pinnata (Lam.) Pers. (Crassulaceae) was grown in a screenhouse at the Tupper building of the Smithsonian Tropical Research Institute, Panama City, Republic of Panama. Experimental plants had a height of 32–36 cm and had eight leaf pairs. One leaf of each pair was cleaned, frozen in liquid nitrogen immediately after harvesting, and stored at −80°C for isolation of total RNA. Root, stem, petal, and anther tissues used for RNA isolation were treated similarly.

Plants used for measurements of titratable acidity measurements at dawn and dusk, were as described above except were grown in a growth chamber on a 12-h light (28°C, 300 µmol m^{-2} s^{-1}) / 12-h dark (18°C) cycle at the University of Nevada, Reno.

Titratable acidity measurements

The first through eighth leaf pairs were harvested at dawn (0600 h) and dusk (1800 h), immersed in liquid nitrogen immediately and stored at −80°C until analysis. The frozen tissue (1 g for leaf pairs 2–8; 0.07–0.08 g for leaf pair 1, the apical leaf pair) was ground completely with a mortar and pestle. Methanol (10 mL, 50% v/v) was added and boiled for 10 min at 80°C. Distilled water was added to each sample to restore the original volume. After centrifugation at 12 000 *g* for 10 min, the clarified supernatant was titrated with 10 mM KOH to pH 7.0. The data were the average of three independent biological replicates and were expressed as µmol of acid g^{-1} fresh weight. The small amount of tissue available for the apical leaf pair may have led to an underestimation of absolute titratable acidity levels in these samples.

Nucleic acid extraction

Total RNA was extracted after Gehrig *et al*. (2000) except that tissue amount was limited to 30–40 mg and RLC (RNeasy lysis buffer C containing guanidine hydrochloride)-buffer [containing 20 mg polyethylene glycol (PEG) 20 000 mol wt] volume was increased to 0.6 mL. RNA quality was assessed by formaldehyde gel electrophoresis and samples were stored at −80°C.

RT–PCR amplification and cloning

RT–PCR amplification was performed as described by Gehrig *et al*. (1998). The degenerate primers used for initial isoform amplification were: Forward 5′-TC(A / T / C)GA(C / T)TC(A / T / C) GG(A / C)AA(A / G)GA(C / T)GC-3′ and Reverse 5′-GC(G / A / T) GC(G / A / T)AT(G / C / A)CC(C / T)TTCAT(G / T)G-3′ as described previously (Gehrig *et al.* 2001).

Amplified PCR products were subcloned into the TA-TOPO cloning pCR2.1 vector system (Invitrogen™ Life Technologies Corporation, Carlsbad, CA) and transformed into TOP10 competent cells following the manufacturer's instructions. Plasmid DNA was isolated by the rapid boiling method (Holmes and Quigley 1981) from 2–4 randomly selected transformants for each isoform. Complementary DNA clones containing the anticipated PEPC fragment size were analysed by *Eco*RI digestion and electrophoresis on 0.8% agarose gels. Clones containing ~1100 bp PEPC fragments were then digested with three restriction enzymes (*Hin*dIII, *Bam*HI, *Pst*I) and analysed on 1.2% agarose gels (see Fig. 2).

Sequence analysis

Plasmid DNA from 2–4 positive clones for each isoform was prepared with a QIAprep™ spin miniprep kit (Qiagen Inc., Valencia, CA). Plasmids were sequenced in both directions using the T7- or M13 reverse primer on a Perkin-Elmer Applied Biosystems 3700 automated DNA sequencing system (Nevada Genomics Center, University of Nevada, Reno) with the Prism™ Ready Reaction Dyedeoxy™ Terminator Cycle Sequencing kit (Applied Biosystems, Foster City, CA).

Raw sequence data were edited manually to remove vector sequences and aligned with PILEUP. The alignment obtained by the program was then optimised manually with LINEUP. Gaps were treated as missing data. The resulting matrix was analysed by the distance method Neighbour-Joining (NJ, as a tool in the program package PHYLIP, version 3.4; Felsenstein 1991). Sequence divergence values (NJ) between species were calculated after bootstrapping (1000 replicates) by the two-parameter method (Kimura 1980) with the DNADIST program of PHYLIP. This method allows for the correction of multiple substitutions and differential transition / transversion probabilities based on empirical observation from the data. The ratio was set at 1.0, based on the actually observed frequencies in the maximum parsimony tree. As a result, it was assumed that there is an equal probability of independent change at all sites. Trees were oriented with PEPC amino acid sequences from *Escherichia coli* and *Chara fragilis* as functional outgroups.

Results and discussion

Nocturnal acidification

Measurements of titratable acidity content at 0600 h and 1800 h showed that CAM was absent from the youngest leaf pair (no nocturnal increase in H^+). In contrast leaves 2–8 showed nocturnal increases in nocturnal H^+, the magnitude of which increased from leaf 2 to leaf 4 and then decreased from leaf 4 to leaf 8 (Fig. 1). Such leaf-age-related changes

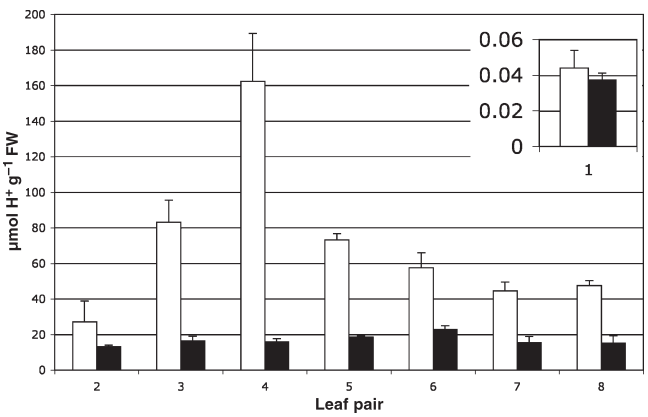

Fig. 1. Titratable acidity of *Kalanchoe pinnata* leaves at various developmental stages. Samples collected from leaf pairs 2–8 at 0600 h, reflecting CAM nocturnal acidification, are shown as open bars. Samples collected at 1800 h are shown as black bars. Inset presents data for leaf pair 1, the apical leaf pair. Data represent the average of three biological replicates. Error bars represent standard error.

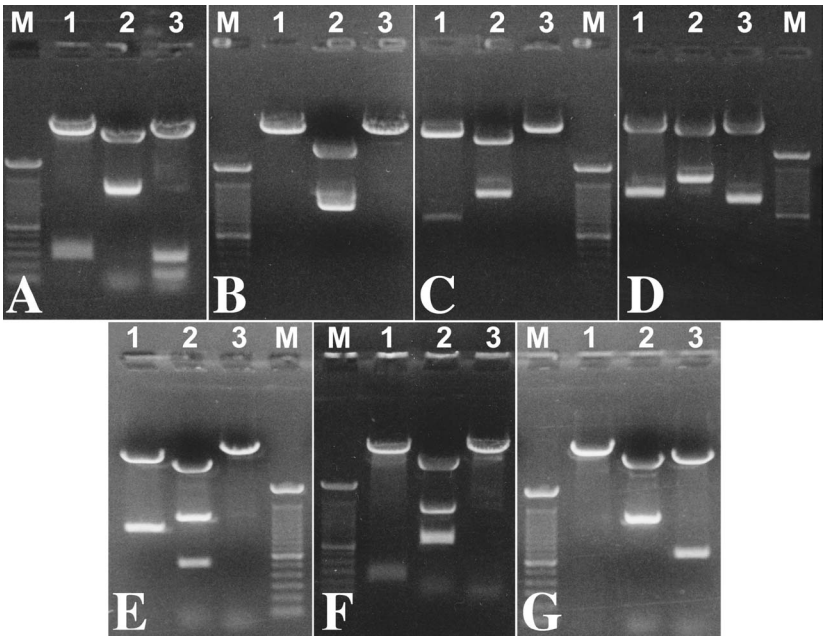

Fig. 2. Agarose gel electrophoresis analyses of seven PEPC isoforms of *Kalanchoe pinnata*. Lane 1 shows the restriction pattern with *Bam*HI; lane 2 with *Hin*dIII; and lane 3 with *Pst*I. M is the molecular standard (100 bp ladder).

in the degree of CAM are well known from species of *Kalanchoe* (Jones 1975; Nishida 1978; Deleens and Queiroz 1984; Winter *et al*. 1997).

Isolation of PEPC isoforms

After degenerate RT–PCR and cloning, 24–38 independent clones were characterised from each tissue and a total of seven PEPC isoforms were distinguishable by restriction analysis, four in leaves, stems, petals, anthers (Fig. 2*A–D*, Table 1) and three in roots (Fig. 2*E–G*, Table 1). The frequency with which each isoform was recovered from each tissue is summarised in Table 1. Isoforms A and D were most often recovered from leaves and stems, whereas isoforms E and F were recovered exclusively from roots. Isoform A was also frequently recovered from petal and anther tissues. Isoform G was recovered from roots (and petals). Isoform D was most readily detectable in leaves 2–8, which perform CAM, but not in leaf pair 1, the apical leaf pair, which does not perform CAM. In contrast to isoform A, isoform D was not detected in anthers. Based on its high relative abundance and specific expression in CAM-performing leaves, we conclude that isoform D is the CAM-specific isoform in *K. pinnata*.

Sequence characterisation of PEPC isoforms

Clones representing each of the seven isoforms were then analysed by DNA sequencing and the aligned sequences were compared (Fig. 3). The four PEPC isoforms found in leaves were clearly distinguishable from the root isoforms by the insertion of a protein motif of 16 amino acid residues in position 331-SSSSSSSSSTSNPAYE-346. These four isoforms could be further separated into two groups (Fig. 3) based on their pair-wise amino sequence identities: 97% between A and D and 98% between B and C. The three root isoforms (E, F, and G) were closely related to one another sharing 99% amino sequence identity. The two pairs [A / D] and [B / C] shared an average of 81% amino acid sequence identity between them. The A and D isoforms shared the highest amino acid sequence identity (97%) with the

Table 1. Summary of frequency and total number of PEPC isoforms recovered from various tissues of *Kalanchoe pinnata*

Numbers of PEPC-isoforms (A–G) identified by RT–PCR with degenerate primers followed by molecular cloning and restriction enzyme analyses described in Materials and methods (LP = leaf pair; ST = stem; PE = petal; AN = anthers; R = root)

	PEPC-isoforms							
Tissue	A	B	C	D	E	F	G	Total
LP1	19	2	7	–	–	–	–	28
LP2	10	2	6	18	–	–	–	36
LP3	11	1	4	16	–	–	–	32
LP4	12	2	6	18	–	–	–	38
LP5	10	2	2	16	–	–	–	30
LP6	12	1	3	16	–	–	–	32
LP7	14	1	2	15	–	–	–	32
LP8	9	2	5	14	–	–	–	30
ST	18	–	2	8	–	–	–	28
PE	19	–	3	2	–	–	–	24
AN	19	2	3	–	–	–	1	25
R	1	–	–	–	9	18	4	32

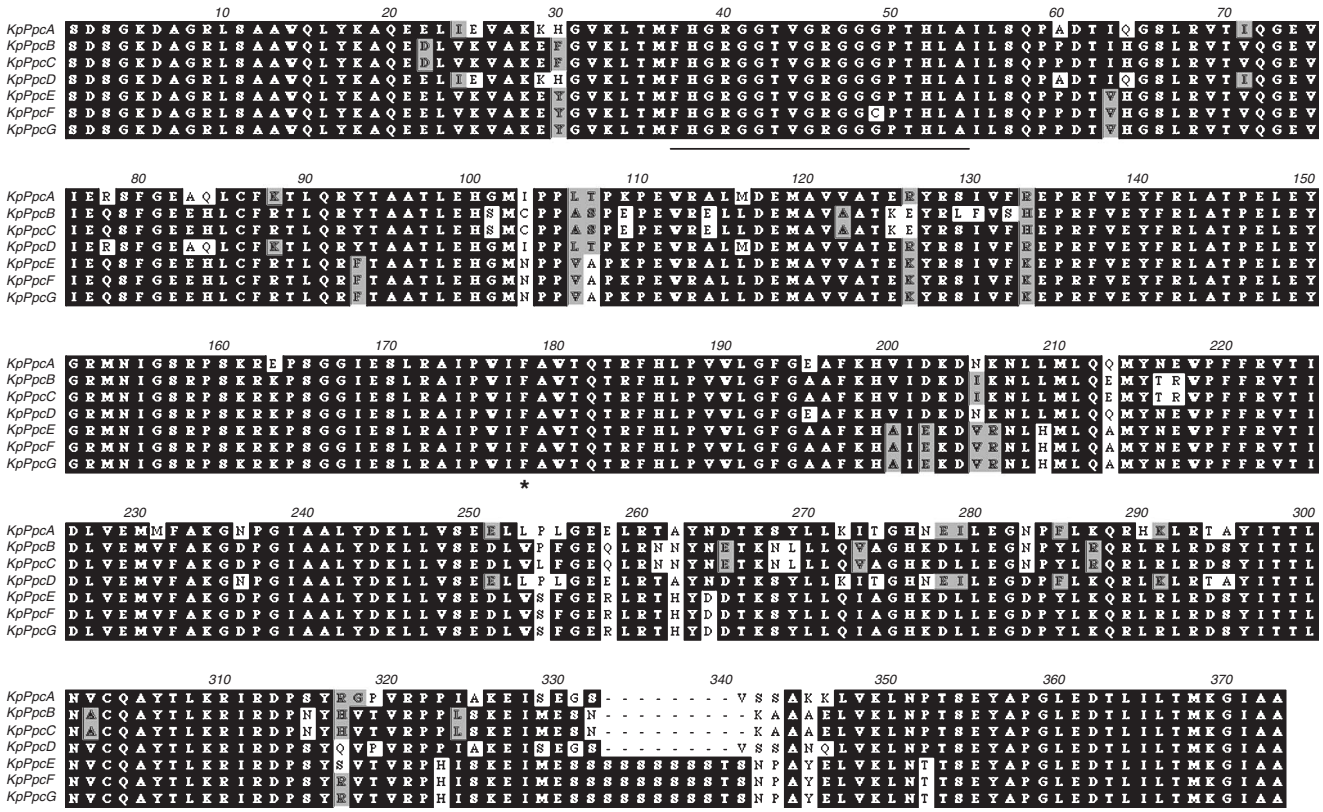

Fig. 3. Aligned partial sequences of seven PEPC isoforms of *Kalanchoe pinnata.* Gaps are indicated by –. Dark shading indicates identical residues and light shading indicates conserved residues. Solid line below the aligned sequences indicates highly conserved amino acid sequences of the catalytic subdomain that participate in PEP / HCO_3^- binding (Izui *et al.* 2004). The * at position 179 indicates the conserved Ala residue at position 774 that when changed to Ser comprises a necessary, but not sufficient determinant of C_4-specific kinetics of C_4 PEPCs (Engelmann *et al.* 2002).

CAM-specific isoforms of PEPC from *Kalanchoe blossfeldiana* (Gehrig *et al*. 1995). The two pairs [A / D] and [B / C] shared an average of 82% and 85% amino acid identity, respectively, with the three root isoforms.

To discern the relationships of these isoforms among themselves and other selected plant PEPC amino acid sequences, a neighbour-joining tree was created (Fig. 4). Neighbour-joining nucleotide sequence calculation shows that all isogenes are probably derived from a common ancestor gene, presumably by a series of gene duplication events. The A and D isoforms grouped with two CAM-specific isoforms from *Kalanchoe blossfeldiana*, whereas the B and C isoforms grouped with non-CAM *K. blossfeldiana* leaf isoforms (Gehrig *et al*. 1995). The root-derived isoforms (E, F, G) clustered with C_3 isoforms from *Arabidopsis* and *Mesembryanthemum crystallinum*. This nucleotide sequence similarity analysis suggests that both isoforms A and D may participate in CAM, however, the relatively high abundance and relatively specific expression of isoform D in CAM performing leaves suggest that only this isoform is likely to serve a dedicated CAM-specific functional role.

Because our study was based on 452 plasmids containing the 1100-bp cDNA PEPC fragment, we are confident that we detected all higher plant type PEPC isogenes expressed in *K. pinnata*. However, higher plant genomes also encode a non-phosphorylatable type of PEPC gene that is more closely related to bacterial and algal types of PEPC genes (Sanchez and Cejudo 2003). Because the nucleotide sequences of such bacterial / algal PEPC genes are quite distinct from the other higher plant forms, we did not isolate this type of PEPC gene using the degenerate priming strategy outlined in this study. Nonetheless is the most comprehensive characterisation of a PEPC isogene family in a CAM plant if not in any vascular plant. In an evolutionary context, it will be interesting to describe PEPC gene families in plant families such as Bromeliaceae, Clusiaceae, and Orchidaceae that contain species that exhibit the full range of carbon assimilation patterns from C_3 photosynthesis to weak CAM

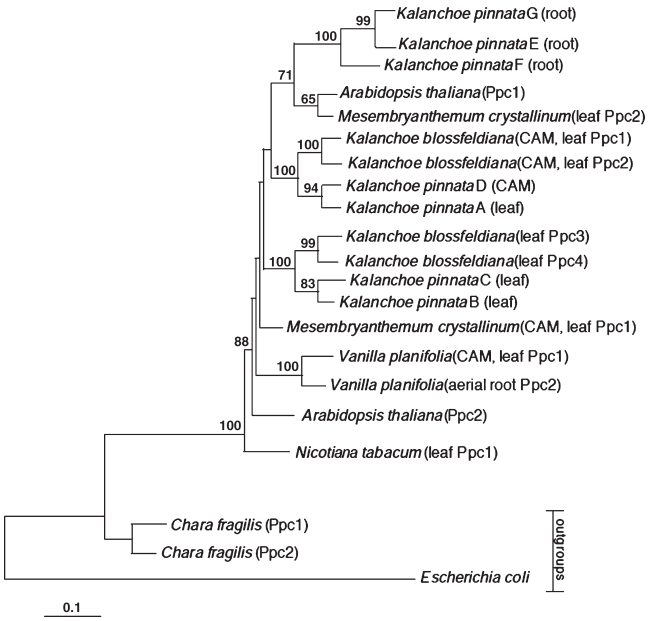

Fig. 4. Phylogenetic tree derived from neighbour-joining analysis of 1100-bp alignment of Ppc nucleotide sequences from selected C_3 (*Arabidopsis thaliana* and *Nicotiana tabacum*) and various CAM plants, and rooted with the PEPC sequence from *E. coli* and *Chara fragilis* as outgroups. Bootstrap values (1000 replicates branch and bound Wagner analyses) below 50% were not shown. CAM-specific isoforms are indicated with CAM. Organ specific designations (e.g. leaf) do not necessarily preclude the localisation of isoforms to other tissues or organs. Accession numbers obtained from GenBank were: *Kalanchoe pinnata* A–G (AJ344052–AJ344058), *Arabidopsis thaliana* Ppc1 (AC008007) and Ppc2 (AC007087), *Nicotiana tabacum* (X59016), *Mesembryanthemum crystallinum* Ppc1 (X14587) and Ppc2 (X14588), *Kalanchoe blossfeldiana* Ppc1 (X87818), Ppc2 (X87819), Ppc3 (X87820), and Ppc4 (X87821); *Vanilla planifolia* Ppc1 (AJ312624) and Ppc2 (AJ312625); *Chara fragilis* Ppc1 (X95851) and Ppc2 (X95857); *Escherichia coli* (NC_000913).

to strong CAM (Winter *et al*. 1992; Gehrig *et al*. 2003; Crayn *et al*. 2004; Holtum *et al*. 2004; Silvera *et al*. 2005). This would enable us to examine whether species, in which the CAM cycle is only weakly expressed to merely recycle respiratory CO_2 (Holtum and Winter 1999), also possess a CAM-specific PEPC and are thus distinctly different from regular C_3 plants.

Acknowledgements

This research was supported by grants from the Andrew W. Mellon Foundation through the Smithsonian Tropical Research Institute to JCC and KW, and by funds from the Smithsonian Tropical Research Institute to KW. Additional support was provided by the Nevada Agricultural Experiment Station and this article is published as publication No. 03055524 of the University of Nevada Agricultural Experiment Station.

References

Bläsing OE, Ernst K, Streubel M, Westhoff P, Svensson P (2002) The non-photosynthetic phosphoenolpyruvate carboxylases of the C_4 dicot *Flaveria trinervia* — implications for the evolution of C_4 photosynthesis. *Planta* **215**, 448–456. doi: 10.1007/s00425-002-0757-x

Chollet R, Vidal J, O'Leary MH (1996) Phosphoenolpyruvate carboxylase: a ubiquitous, highly regulated enzyme in plants. *Annual Review of Plant Physiology and Plant Molecular Biology* **47**, 273–298. doi: 10.1146/annurev.arplant.47.1.273

Crayn DM, Winter K, Smith JAC (2004) Multiple origins of crassulacean acid metabolism and the epiphytic habit in the neotropical family Bromeliaceae. *Proceedings of the National Academy of Sciences USA* **101**, 3703–3708. doi: 10.1073/pnas.0400366101

Crétin C, Santi S, Keryer E, Lepiniec L, Tagu D, Vidal J, Gadal P (1991) The phosphoenolpyruvate carboxylase gene family of *Sorghum*: promoter structures, amino acid sequences, and expression of genes. *Gene* **99**, 87–94. doi: 10.1016/0378-1119(91)90037-C

Cushman JC, Bohnert HJ (1999) Crassulacean acid metabolism: molecular genetics. *Annual Review of Plant Physiology and Plant Molecular Biology* **50**, 305–332. doi: 10.1146/annurev.arplant.50.1.305

Cushman JC, Meyer G, Michalowski CB, Schmitt JM, Bohnert HJ (1989) Salt stress leads to the differential expression of two isogenes of phospho*enol*pyruvate carboxylase during crassulacean acid metabolism induction in the common ice plant. *The Plant Cell* **1**, 715–725. doi: 10.1105/tpc.1.7.715

Deleens E, Queiroz O (1984) Effects of photoperiod and aging on the carbon isotope composition of *Bryophyllum daigremontianum* Berger. *Plant, Cell & Environment* **7**, 279–283.

Edwards G, Walker DA (1983) 'C_3, C_4: mechanisms, and cellular and environmental regulation of photosynthesis.' (Blackwell: Oxford)

Engelmann S, Bläsing OE, Westhoff P, Svensson P (2002) Serine 774 and amino acids 296 to 437 comprise the major C_4 determinants of the C_4 phosphoenolpyruvate carboxylase of *Flaveria trinervia*. *FEBS Letters* **524**, 11–14. doi: 10.1016/S0014-5793(02)02975-7

Felsenstein J (1991) Counting phylogenetic invariants in some simple cases. *Journal of Theoretical Biology* **152**, 357–376.

Gehrig HH, Taybi T, Kluge M, Brulfert J (1995) Identification of multiple PEPC isogenes in leaves of the facultative crassulacean acid metabolism (CAM) plant *Kalanchoe blossfeldiana* Poelln. cv. Tom Thumb. *FEBS Letters* **377**, 399–402. doi: 10.1016/0014-5793(95)01397-0

Gehrig HH, Faist K, Kluge M (1998) Identification of phosphoenolpyruvate carboxylase isoforms in leaf, stem and roots of the obligate CAM plant *Vanilla planifolia* Salib. (Orchidaceae): a physiological and molecular approach. *Plant Molecular Biology* **38**, 1215–1223. doi: 10.1023/A:1006006331011

Gehrig H, Heute V, Kluge M (2001) New partial sequences of phosphoenolpyruvate carboxylase as molecular phylogenetic markers. *Molecular Phylogenetics and Evolution* **20**, 262–274. doi: 10.1006/mpev.2001.0973

Gehrig HH, Winter K, Cushman J, Borland AM, Taybi T (2000) An improved RNA isolation method for succulent plant species rich in polyphenols and polysaccharides. *Plant Molecular Biology Reporter* **18**, 369–376.

Gehrig HH, Aranda J, Cushman MA, Virgo A, Cushman JC, Hammel BE, Winter K (2003) Cladogram of Panamanian *Clusia* based on nuclear DNA: implications for the origins of crassulacean acid metabolism. *Plant Biology* **5**, 59–70. doi: 10.1055/s-2003-37983

Golombek S, Heim U, Horstmann C, Wobus U, Weber H (1999) Phosphoenolpyruvate carboxylase in developing seeds of *Vicia faba* L.: gene expression and metabolic regulation. *Planta* **208**, 66–72. doi: 10.1007/s004250050535

Guillet C, Just D, Bénard N, Destrac-Irvine A, Baldet P, Hernould M, Causse M, Raymond P, Rothan C (2002) A fruit-specific phosphoenolpyruvate carboxylase is related to rapid growth of tomato fruit. *Planta* **214**, 717–726. doi: 10.1007/s00425-001-0687-z

Hata S, Izui K, Kouchi H (1997) Expression of a soybean nodule-enhanced phosphoenolpyruvate carboxylase gene that shows striking similarity to another gene for a housekeeping isoform. *The Plant Journal* **7**, 198–201.

Holmes DS, Quigley M (1981) A rapid boiling method for the preparation of bacterial plasmids. *Analytical Biochemistry* **114**, 193–197. doi: 10.1016/0003-2697(81)90473-5

Holtum JAM, Winter K (1999) Degrees of crassulacean acid metabolism in tropical epiphytic and lithophytic ferns. *Australian Journal of Plant Physiology* **26**, 749–757.

Holtum JAM, Aranda J, Virgo A, Gehrig HH, Winter K (2004) $\delta^{13}C$ values and crassulacean acid metabolism in *Clusia* species from Panama. *Trees — Structure and Function* **18**, 658–668. doi: 10.1007/s00468-004-0342-y

Izui K, Matsumura H, Furumoto T, Kai Y (2004) Phosphoenolpyruvate carboxylase: a new era of structural biology. *Annual Review of Plant Biology* **55**, 69–84. doi: 10.1146/annurev.arplant.55.031903.141619

Jones MB (1975) The effect of leaf age on leaf resistance and CO_2 exchange of the CAM plant *Bryophyllum fedtschenkoi. Planta* **123**, 91–96. doi: 10.1007/BF00388063

Kimura M (1980) A simple model for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences. *Journal of Molecular Evolution* **16**, 111–120.

Kopka J, Provart NJ, Muller-Rober B (1997) Potato guard cells respond to drying soil by a complex change in the expression of genes related to carbon metabolism and turgor regulation. *The Plant Journal* **11**, 871–882. doi: 10.1046/j.1365-313X.1997.11040871.x

Lepiniec L, Vidal J, Chollet R, Gadal P, Crétin C (1994) Phosphoenolpyruvate carboxylase: structure, regulation, and evolution. *Plant Science* **99**, 111–124. doi: 10.1016/0168-9452(94)90168-6

Nimmo HG (2000) The regulation of phosphoenolpyruvate carboxylase in CAM plants. *Trends in Plant Science* **5**, 75–80. doi: 10.1016/S1360-1385(99)01543-5

Nishida K (1978) Effect of leaf age on light and dark $^{14}CO_2$ fixation in a CAM plant, *Bryophyllum calycinum. Plant & Cell Physiology* **19**, 935–941.

Pathirana MS, Samac DA, Roeven R, Yoshioka H, Vance CP, Gantt JS (1997) Analyses of phosphoenolpyruvate carboxylase gene structure and expression in alfalfa nodules. *The Plant Journal* **12**, 293–304. doi: 10.1046/j.1365-313X.1997.12020293.x

Rao SK, Magnin NC, Reiskind JB, Bowes G (2002) Photosynthetic and other phosphoenolpyruvate carboxylase isoforms in the single-cell, facultative C(4) system of *Hydrilla verticillata. Plant Physiology* **130**, 876–886. doi: 10.1104/pp.008045

Sanchez R, Cejudo FJ (2003) Identification and expression analysis of a gene encoding a bacterial-type phospho*enol*pyruvate carboxylase from *Arabidopsis* and rice. *Plant Physiology* **132**, 949–957. doi: 10.1104/pp.102.019653

Schaffner AR, Sheen J (1992) Maize C4 photosynthesis involves differential regulation of phosphoenolpyruvate carboxylase genes. *The Plant Journal* **2**, 221–232.

Silvera K, Santiago LS, Winter K (2005) Distribution of crassulacean acid metabolism in orchids of Panama: evidence of selection for weak and strong modes. *Functional Plant Biology* **32**, 397–407. doi: 10.1071/FP04179

Smart LB, Vodjani F, Maeshima M, Wilkins TA (1998) Genes involved in osmoregulation during turgor-driven cell expansion of developing cotton fibers are differentially regulated. *Plant Physiology* **116**, 1539–1549. doi: 10.1104/pp.116.4.1539

Winter K, Holtum JAM (2002) How closely do the $\delta^{13}C$ values of crassulacean acid metabolism plants reflect the proportion of CO_2 fixed during day and night? *Plant Physiology* **129**, 1843–1851. doi: 10.1104/pp.002915

Winter K, Smith JAC (1996) 'Crassulacean acid metabolism: biochemistry, ecophysiology and evolution.' Ecological Studies Vol. 114. (Springer-Verlag: Berlin)

Winter K, Zotz G, Baur B, Dietz KJ (1992) Light and dark CO_2 fixation in *Clusia uvitana* as affected by plant water status and CO_2 availability. *Oecologia* **91**, 47–51. doi: 10.1007/BF00650323

Winter K, Richter A, Engelbrecht B, Posada J, Virgo A, Popp M (1997) Effect of elevated CO_2 on growth and crassulacean-acid-metabolism activity of *Kalanchöe pinnata* under tropical conditions. *Planta* **201**, 389–396. doi: 10.1007/s004250050081

Manuscript received 4 April 2005, accepted 3 May 2005