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A comparative study on diurnal changes in metabolite levels in the leaves of three crassulacean acid metabolism (CAM) species, *Ananas comosus*, *Kalanchoë daigremontiana* and *K. pinnata*

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Abstract

A comparative study on diurnal changes in metabolite levels associated with crassulacean acid metabolism (CAM) in the leaves of three CAM species, *Ananas comosus* (pineapple), a hexose-utilizing species, and *Kalanchoë daigremontiana* and *K. pinnata*, two starch-utilizing species, were made. All three CAM species showed a typical feature of CAM with nocturnal malate increase. In the two *Kalanchoë* species, isocitrate levels were higher than citrate levels; the reverse was the case in pineapple. In the two *Kalanchoë* species, a small nocturnal citrate increase was found and *K. daigremontiana* showed a small nocturnal isocitrate increase. Glucose 6-phosphate (G-6-P), fructose 6-phosphate (F-6-P) and glucose 1-phosphate (G-1-P) levels in the three CAM species rose rapidly during the first part of the dark period and decreased during the latter part of the dark period. The levels of the metabolites also decreased during the first 3 h of the light period, then, remained little changed through the rest of the light period. Absolute levels of G-6-P, F-6-P and G-1-P were higher in pineapple than in the two *Kalanchoë* species. Fructose 1,6-bisphosphate (F-1,6-P_2) levels in the three CAM species increased during the dark period, then dramatically decreased during the first 3 h of the light period and remained unchanged through the rest of the light period. The extent of nocturnal F-1,6-P_2 increase was far greater in the two *Kalanchoë* species than in pineapple. Absolute levels of F-1,6-P_2 were higher in the two *Kalanchoë* species than in pineapple, especially during dark period. Diurnal changes in oxaloacetate (OAA), pyruvate (Pyr) and phospho*enol*pyruvate (PEP) levels in the three CAM species were similar.

Key words: *Ananas comosus*, crassulacean acid metabolism, *Kalanchoë daigremontiana*, *K. pinnata*, metabolite level.

Introduction

A central characteristic of crassulacean acid metabolism (CAM) is that it allows massive uptake of atmospheric CO_2 via phospho*enol*pyruvate (PEP) carboxylase (PEPC, EC 4.1.1.31), using PEP produced in glycolysis, into organic acid (mainly malic acid) at night in the cytosol of photosynthetic cells. The organic acid formed is then transported into the vacuole, where it is accumulated during the night. During the day, the organic acids are released from the vacuoles. The major organic acid is malic acid, which is usually decarboxylated by malic enzyme (ME) or PEP carboxykinase (PEPCK, EC 4.1.1.49) in the cytoplasm to form CO_2 for photosynthetic assimilation via the Calvin cycle plus either pyruvate (Pyr) or PEP. Then, the Pyr or PEP produced from malic acid is recycled via gluconeogenesis and accumulated as the storage carbohydrate pool (Kenyon *et al.*, 1985; Lüttge, 1998).

CAM plants can be divided into two groups, starch-former and extrachloroplastic carbohydrate-former, based on the major carbohydrate reservoir used in their daily cycle. They both use polysaccharides (starch) stored in the chloroplast and soluble hexose stored in the extrachloroplast, respectively, as the precursor for glycolytic PEP formation (Fahrendorf *et al.*, 1987;

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Christopher and Holtum, 1996). In some starch-formers, such as *K. pinnata* and *K. daigremontiana*, fructose 2,6-bisphosphate (F-2,6-P_2) levels and the ratio of pyrophosphate-dependent phosphofructokinase (PPi-PFK, EC 2.7.1.90) activity to fructose 1,6-bisphosphatase activity (FBPase, EC 3.1.3.11) are low (Osmond *et al*., 1999). ATP-dependent phosphofructokinase (ATP-PFK, EC 2.7.1.11) activity is higher than that of PPi-PFK, and fructose 6-phosphate (F-6-P) is phosphorylated to fructose 1,6-bisphosphate (F-1,6-P_2) by ATP-PFK (Carnal and Black, 1983, 1989). However, in extrachloroplastic carbohydrate-formers, such as pineapple, F-2,6-P_2 levels and the ratio of PPi-PFK activity to FBPase activity are high (Fahrendorf *et al*., 1987; Osmond *et al*., 1999). PPi-PFK activity is 10–20 times higher than ATP-PFK activity and PPi-PFK could function in the glycolytic direction and substitute for ATP-PFK (Carnal and Black, 1983, 1989; Trípodi and Podestá, 1997). In starch-formers, such as *K. pinnata*, and *K. daigremontiana*, malic acid is decarboxylated by malic enzyme (ME) to Pyr and CO_2. Pyr is phosphorylated to PEP by catalysis of Pyr orthophosphate dikinase (PPDK, EC 2.7.9.1), then is recycled via gluconeogenesis. In extrachloroplastic carbohydrate-formers like pineapple, oxaloacetate (OAA) produced from malate is decarboxylated by PEP carboxykinase (PEPCK, EC 4.1.1.49) to PEP and CO_2 (Holtum and Osmond, 1981; Kondo *et al*., 1998). Recently, Chen and Nose found that the tonoplast PPase activity of *K. pinnata*, and *K. daigremontiana* was higher than their tonoplast ATPase activity, while the reverse was the case in pineapple (Chen and Nose, 2000). These results suggest that there are many important differences among the various CAM groups. Therefore, the diurnal changes in metabolite levels associated with CAM may be different among various CAM groups.

The diurnal changes of some metabolite levels associated with CAM have been investigated (Sideris *et al*., 1948; Vickery, 1952; Milburn *et al*., 1968; Cockburn and McAulay, 1977; Pierre and Queiroz, 1979; Kenyon *et al*., 1981). However, the majority of the studies were performed with *Kalanchoë* species, which stored carbon as starch in the chloroplast (Christopher and Holtum, 1996). In addition, these experiments were performed in different laboratories with very different growth conditions for plant materials. The data from these experiments are complex and very difficult to compare.

In some species, there may be oscillations of citric acid in addition to malic acid (Vickery, 1952; Milburn *et al*., 1968; Ting *et al*., 1985; Lüttge, 1988; Winter and Smith, 1996; Borland and Griffiths, 1997). However, it is unclear whether isocitrate levels change during the day–night CAM cycle in some CAM species. Wolf suggested that isocitrate did not change through the day–night rhythm (Wolf, 1960) and early work suggesting day–night oscillations of isocitrate might be due to analytical problems. Based on former data (Vickery, 1952; Milburn *et al*., 1968; Wolf, 1960), Lüttge also suggested that citric acid accumulated during the dark period in some CAM species and isocitrate did not accumulate (Lüttge, 1988). However, in a comparative study of eight species of *Sedum*, it was found that, during the dark period, malate and isocitrate levels increased (Knopf and Kluge, 1979) and citrate remained unchanged in the leaves of *S. telephium* and *S. praealtum*. Nocturnal isocitrate increase was also found in *K. daigremontiana* and *S. telephium* leaves (Kenyon *et al*., 1985).

In this paper, diurnal changes in the levels of malate, citrate, isocitrate, glucose 6-phosphate (G-6-P), F-6-P, glucose 1-phosphate (G-1-P), F-1,6-P_2, OAA, PEP, and Pyr were determined in the leaves of three CAM species: pineapple, a hexose-utilizing species, and *K. pinnata* and *K. daigremontiana*, two starch-utilizing species. The objective was to investigate the differences in diurnal changes in metabolite levels associated with CAM among various CAM groups.

Materials and methods

Plant materials

Pineapple (*Ananas comosus* cv. Smooth-cayenne N67-10), *K. pinnata* and *K. daigremontiana* were vegetatively propagated and grown in pots in a greenhouse with heating under a natural photoperiod. Fifteen days before the experiments, all plants were transferred to a growth chamber with a photoperiod of 12 h (08.00 h to 20.00 h) light and 12 h (20.00 h to 08.00) dark. Conditions in the growth chamber were 30 °C during the light period at a photon flux density at the mid-plant height of 420–450 μmol m^{-2} s^{-1}, and 20 °C during the dark period, and a relative humidity of 65% during both periods. Fourth to eighth leaf pairs, counting from the apex of *K. pinnata* and *K. daigremontiana* and fully expanded, mature leaves of pineapple were used in each experiment. Leaf samples were taken and immediately immersed in liquid nitrogen until extraction.

Extraction and measurement of pyrophosphate (PPi)

About 1.5 g frozen pineapple leaves were ground in liquid nitrogen with a pestle and mortar and 5 ml of ice-cold 5% (w/v) trichloroacetic acid (TCA) containing 250 mg polyvinylpolypyrrolidone (PVPP) was added to the powder and gently pulverized. The mixture was allowed to thaw slowly on ice. The resulting suspension was kept on ice for 30 min to allow binding of phenolic compounds on the PVPP, then centrifuged for 10 min at 20 000 *g*. Four ml of supernatant was added to 35 mg charcoal (activated, washed with HCl) and the mixture was vortexed at least three times for 15 min. The charcoal was precipitated by 10 min centrifugation at 20 000 *g*. The supernatant was used for the measurement of PPi as described previously (Chen and Nose, 2001).

Extraction and measurement of other metabolites

About 1.7 g frozen tissue was ground in liquid nitrogen with a pestle and mortar, 6.5 ml of ice-cold 4% (v/v) $HClO_4$ was added to the powder and gently pulverized. The mixture was allowed

to thaw slowly on ice. The resulting suspension was kept on ice for 30 min, then centrifuged for 10 min at 20 000 *g*. One ml of the supernatant extracted from pineapple leaves was used for measurement of inorganic phosphate (Pi), which was measured spectrophotometrically at 820 nm (Ames, 1966).

Five ml of supernatant was neutralized at 4 °C with 5 M K_2CO_3, and the resulting potassium chlorate removed by 10 min centrifugation at 20 000 *g*. Fifty mg charcoal (activated, washed with HCl) was added to the supernatant, and after 15 min at 4 °C, removed again by 10 min centrifugation at 20 000 *g*. The supernatant was used for measurement of metabolites.

Malate was measured in 1 ml reaction mixture containing 50 mM 3-amino-1-propanol-HCl, pH 10, 30 mM glutamate-Na-NaOH, pH 10, 2.7 mM NAD, 1 unit glutamate-oxaloacetate transaminase (GOT, EC 2.6.1.1), and 10 units malate dehydrogenase (MDH, EC 1.1.1.37) (Möllering, 1974).

OAA was measured in 1 ml reaction mixture containing 150 mM 2-amino-2-(hydroxymethyl)-1,3-propanediol (Tris)-HCl, pH 7.6, 10 mM ethylenediaminetetraacetic acid (EDTA)-NaOH, pH 7.0, 0.15 mM NADH, and 2.0 units MDH (Wahlefeld, 1974).

Pyr was measured in 1 ml reaction mixture containing 100 mM Tris-HCl, pH 7.6, 3 mM EDTA-NaOH, pH 7.0, 0.2 mM NADH, and 2 units lactate dehydrogenase (LDH, EC 1.1.1.27). The reaction was started by the addition of the latter. The blank contained the extract and all the reagents given above except LDH, which was replaced by water (Du *et al*., 1998).

The assay of PEP followed the procedure of Pistelli *et al*. with some modifications. PEP was measured in 1 ml mixture containing 100 mM HEPES-KOH, pH 7.6, 10 mM $MgSO_4$, 100 mM KCl, 1 mM ADP, 1.5 mM EDTA, 0.2 mM NADH, 14 units LDH, and 2 units Pyr kinase (EC 2.7.1.40). The reaction was started by the addition of the latter. The blank contained the extract and all the reagents given above except the Pyr kinase, which was replaced by water (Pistelli *et al*., 1987).

G-6-P, F-6-P and G-1-P content was measured in 1 ml reaction mixture containing 100 mM HEPES adjusted to pH 7.6 with KOH, 0.2 mM NADP, 4 mM $MgCl_2$, 1 unit glucose-6-phosphate dehydrogenase (EC 1.1.1.49) for G-6-P, then 1 unit glucosephosphate isomerase (GPI, EC 5.3.1.9) for F-6-P, and finally 1 unit phosphoglucomutase (PGM, EC 5.4.2.2) for G-1-P (Mohanty *et al*., 1993).

F-1,6-P_2 was measured in 1 ml reaction of 100 mM HEPES adjusted to pH 7.6 with KOH, 4 mM $MgCl_2$, 0.2 mM NADH, 1.7 units glycerol-3-phosphate dehydrogenase (GDH, EC 1.1.1.8), 3.4 units triose-phosphate isomerase (TPI, EC 5.3.1.1) and 0.45 units aldolase (EC 4.1.2.13) (Mohanty *et al*., 1993).

Citrate was measured in 1 ml reaction mixture of 100 mM Tris adjusted to pH 7.6 with HCl, 0.2 mM NADH, 7 units LDH, 14 units MDH, and 0.5 units citrate lyase (EC 4.1.3.6) (Delhaize *et al*., 1993; Dagley, 1974).

Isocitrate was measured in 1 ml reaction of 100 mM TRIS adjusted to pH 7.6 with HCl, 3.3 mM $MnSO_4$, 0.15 mM NADP, and 0.1 unit isocitrate dehydrogenase (EC 1.1.1.42) (Siebert, 1974).

The recoveries of metabolites were between 90–100% in all cases.

Results

Diurnal changes in the levels of malate, citrate and isocitrate

All the three CAM species showed a typical feature of CAM with malate increase during the dark period and malate decrease during the light period. Malate accumulation during the dark period in pineapple, *K. pinnata* and *K. daigremontiana* was of 119, 120 and 92 μmol g^{-1} FW, respectively. In the two *Kalanchoë* species, isocitrate levels were high and citrate levels were low, while the reverse was the case in pineapple. In the two *Kalanchoë* species, nocturnal citrate increase of 8 μmol g^{-1} FW was found, while in the pineapple, citrate levels remained unchanged through the day–night cycle (Fig. 1). As shown in Table 1, 1 mol malate synthesized via PEPC and MDH only required 0.5 mol hexose, while 1 mol citrate produced via glycolysis, Pyr dehydrogenase complex, and citrate synthase (EC 4.1.3.7) required 1 mol hexose. Therefore, the hexoses used in citrate accumulation in *K. pinnata* and *K. daigremontiana* are 11.8% and 14.8%, respectively, of the total hexoses used in malate and citrate accumulation. A nocturnal isocitrate increase of 11 μmol g^{-1} FW in the leaves of *K. daigremontiana* was found. Like citrate, 1 mol citrate produced via glycolysis, Pyr dehydrogenase complex, citrate synthase, and aconitase (EC 4.2.3.7) required 1 mol hexose (Table 1). Thus, the hexoses used in isocitrate accumulation are 16.9% of the total hexoses used in malate, citrate and isocitrate accumulation.

Diurnal changes in the levels of G-6-P, F-6-P, G-1-P and F-1,6-P_2

Figure 2 shows the patterns of diurnal changes in the levels of four kinds of hexose-phosphates (hexose-P) in the leaves of pineapple, *K. pinnata* and *K. daigremontiana*. The trends of diurnal changes in the levels of G-6-P, F-6-P and G-1-P in the leaves of the three CAM species were basically similar. G-6-P, F-6-P and G-1-P levels in the leaves of the three CAM species rose rapidly to high levels during the first part of the dark period, and decreased during the latter part of the dark period and during the first 3 h of the light period. Then, G-6-P, F-6-P and G-1-P levels remained little changed throughout the rest of the light period. However, the absolute levels of G-6-P, F-6-P and G-1-P were always higher in pineapple than in the two *Kalanchoë* species. In the three CAM species, the sequence of the absolute levels of the three kinds of hexose-P was always G-6-P > F-6-P > G-1-P. As shown in Fig. 2, F-1,6-P_2 levels in the leaves of the three CAM species increased during the dark period, then dramatically decreased to very low levels during the first 3 h of the light period and remained unchanged throughout the rest of the light period. But the extent of F-1,6-P_2 increase was far greater in the two *Kalanchoë* species than in pineapple during the dark period. In addition, the absolute levels of F-1,6-P_2 were higher in the two *Kalanchoë* species than in pineapple, especially during the dark period.

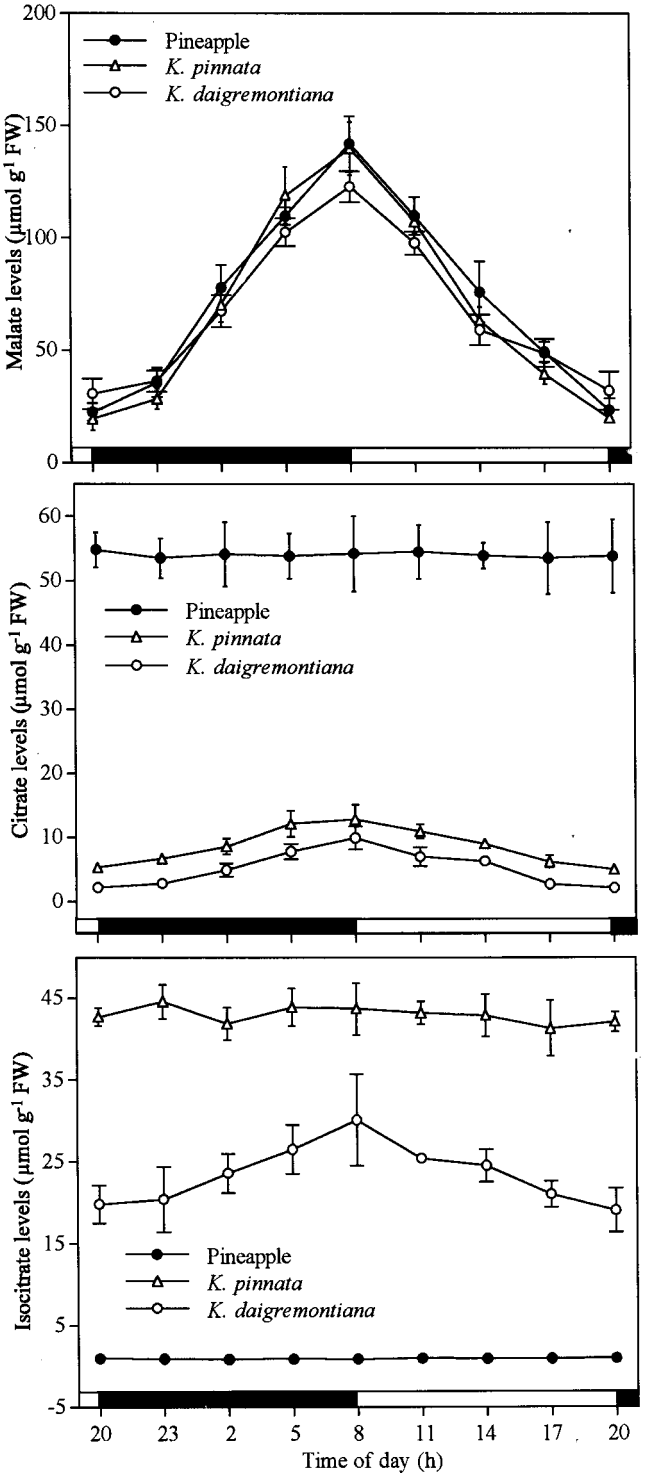

Fig. 1. Levels of malate, citrate and isocitrate in the leaves of pineapple, *K. pinnata* and *K. daigremontiana* during the 24 h day–night cycle. The closed and open horizontal bars indicate the dark and light periods, respectively. Values are means of six experiments ±SD.

Diurnal changes in the levels of OAA, PEP and Pyr

OAA levels in the leaves of the three CAM species increased during the dark period, then decreased dramatically to very low levels during the first 3 h of the light period and remained little unchanged throughout the rest of the light period (Fig. 3). PEP levels in the leaves of the three CAM species increased rapidly during the first 3 h of the light period, then remained little changed in the leaves of the two *Kalanchoë* species and decreased slightly in the leaves of pineapple throughout the rest of the light period. During the first 3 h of the dark period, PEP levels in the leaves of the three CAM species decreased drastically to very low levels, then remained little changed throughout the rest of the dark period. As shown in Fig. 3, diurnal changes in Pyr levels in the leaves

Table 1. *Energy requirement of nocturnal isocitric acid accumulation during the dark phase of* K. daigremontiana

It is assumed that OAA, malate and isocitrate across the inner mitochondrial membrane is transported by tricarboxylate carrier and the transport of the three organic acids may be driven by concentration gradients maintained during metabolism and may not require extra ATP energy. Pyr is cotransported with proton (Heldt, 1976) and the potential cost may be 1 mol ATP mol^{-1} Pyr transported (Lüttge, 1988). Three mol ATP are formed from the oxidation of 1 mol $[NADH + H^+]$ in the mitochondria and 2 mol ATP are formed from the oxidation of 1 mol $[NADH + H^+]$ in the cytosol in the respiratory chain (Heldt, 1976). G3P, glyceraldehyde 3-phosphate; DHAP, dihydroxyacetone phosphate; 1,3-PGA, 1,3-bisphosphoglycerate; 3-PGA, 3-phosphoglycerate; 2-PGA, 2- phosphoglycerate; Cyt, cytoplasm; Mit, mitochondria; Vac, vacuole.

$$\text{Starch} + \text{Pi} \leftrightarrow \text{Hexose-P}$$

$$\text{Hexose-P} + \text{ATP} \leftrightarrow \text{F-1,6-P}_2 + \text{ADP}$$

$$\text{F-1,6-P}_2 \leftrightarrow \text{G3P} + \text{DHAP}$$

$$\text{DHAP} \leftrightarrow \text{G3P}$$

$$2\ \text{G3P} + 2\ \text{Pi} + 2\ \text{NAD}^+_{\text{Cyt}} \leftrightarrow 2\ \text{1,3-PGA} + 2\ [\text{NADH} + \text{H}^+]_{\text{Cyt}}$$

$$2\ \text{1,3-PGA} + 2\ \text{ADP} \leftrightarrow 2\ \text{3-PGA} + 2\ \text{ATP}$$

$$2\ \text{3-PGA} \leftrightarrow 2\ \text{2-PGA}$$

$$2\ \text{2-PGA} \leftrightarrow 2\ \text{PEP} + 2\ \text{H}_2\text{O}$$

$$\text{PEP} + \text{ADP} \leftrightarrow \text{Pyr} + \text{ATP}$$

$$\text{Pyr}_{\text{Cyt}} + \text{ATP} \leftrightarrow \text{Pyr}_{\text{Mit}} + \text{ADP} + \text{Pi}$$

$$\text{Pyr} + \text{NAD}^+_{\text{Mit}} \leftrightarrow [\text{NADH} + \text{H}^+]_{\text{Mit}} + \text{AcCoA} + \text{CO}_2$$

$$\text{PEP} + \text{CO}_2 \leftrightarrow \text{OAA} + \text{Pi}$$

(a) $$\text{OAA}_{\text{Cyt}} \leftrightarrow \text{OAA}_{\text{Mit}}$$

Alternatively:

(b) $$\text{OAA} + [\text{NADH} + \text{H}^+]_{\text{Cyt}} \leftrightarrow \text{Malate} + \text{NAD}^+_{\text{Cyt}}$$

(b) $$\text{Malate}_{\text{Cyt}} \leftrightarrow \text{Malate}_{\text{Mit}}$$

(b) $$\text{Malate} + \text{NAD}^+_{\text{Mit}} \leftrightarrow \text{OAA} + [\text{NADH} + \text{H}^+]_{\text{Mit}}$$

$$\text{AcCoA} + \text{OAA} \leftrightarrow \text{Citric acid}$$

$$\text{Citric acid} \leftrightarrow \text{Isocitric acid}$$

$$\text{Isocitric acid}_{\text{Mit}} \leftrightarrow \text{Isocitric acid}_{\text{Cyt}}$$

$$\text{Isocitric acid}_{\text{Cyt}} + 1.5\ \text{ATP} \leftrightarrow \text{Isocitric acid}_{\text{Vac}} + 1.5\ \text{ADP} + 1.5\ \text{Pi}$$

Net energy gain per mol isocitric acid accumulated: with reaction (a)

$$[\text{NADH} + \text{H}^+]_{\text{Mit}} + 2\ [\text{NADH} + \text{H}^+]_{\text{Cyt}} - 0.5\ \text{ATP} = 6.5\ \text{ATP}$$

Alternatively: with reaction (b)

$$2\ [\text{NADH} + \text{H}^+]_{\text{Mit}} + [\text{NADH} + \text{H}^+]_{\text{Cyt}} - 0.5\ \text{ATP} = 7.5\ \text{ATP}$$

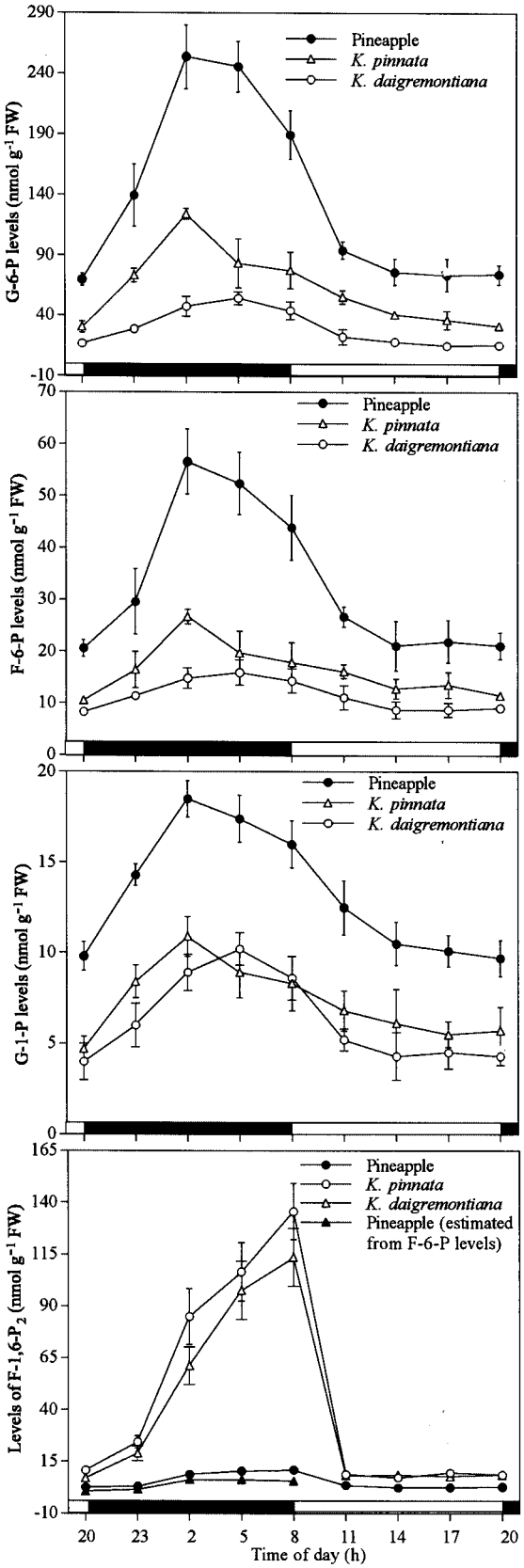

Fig. 2. Levels of G-6-P, F-6-P, G-1-P, and F-1,6-P_2 in the leaves of pineapple, *K. pinnata* and *K. daigremontiana* during the 24 h day–night cycle. The closed and open horizontal bars indicate the dark and light periods, respectively. Values are means of six experiments ±SD. F-1,6-P_2 levels estimated from F-6-P levels in pineapple leaves are from Table 4.

of the three CAM species were similar. Pyr levels in the leaves of the three CAM species increased during the dark period and decreased during the light period.

Discussion

The present work, like that of previous workers (Vickery, 1952; Milburn *et al.*, 1968; Knopf and Kluge, 1979; Popp *et al.*, 1987; Borland and Griffiths, 1997) indicates that besides malate, citrate also plays an important role in CAM (Lüttge, 1988). Although citrate levels in the leaves of pineapple were rather high, they remained constant throughout the day–night cycle (Fig. 1). This is inconsistent with the result of Kenyon *et al.* that citrate levels increased during the dark in the leaves of pineapple grown in a growth chamber with 30/15 °C, 15/9 h day/night thermoperiod and 70–80% relative humidity (Kenyon, 1985). In addition, Medina *et al.* investigated the diurnal changes in citrate levels in two pineapple cultivars, cv. Brecheche and cv. Spanish Red (Medina *et al.*, 1991). The former is originally cultivated under partial shade in palm swamps, while the latter is cultivated under full sun exposure. Under high light intensity (325–400 μmol m^{-2} s^{-1}), both Brecheche and Spainsh Red had a small and significant nocturnal citrate increase, while under low light intensity (25–50 μmol m^{-2} s^{-1}), only Brecheche had a small and significant nocturnal citrate increase. Therefore, it is suggested that the difference in diurnal changes in citrate levels may be related to the relative requirement of different cultivars for light intensity and light period.

As shown in Fig. 1, isocitrate levels in the leaves of pineapple and *K. pinnata* did not fluctuate throughout the day–night rhythm. This is consistent with the results obtained earlier in *K. pinnata* (Vickery, 1952) and in pineapple (Kenyon *et al.*, 1985). Isocitrate levels in *K. daigremontiana* leaves increased during the dark period and decreased during the light, which agrees with the results obtained earlier in *S. telephium* and *K. daigremintiana* (Kenyon *et al.*, 1985) and in *S. telephium* and *S. praealtum* (Knopf and Kluge, 1979). These results suggest that nocturnal isocitrate accumulation may occur in some CAM species, at least in some starch-utilizing species. Further study is needed to answer the question whether nocturnal isocitrate increase also occurs in some hexose-utilizing species.

It has been suggested that nocturnal citrate accumulation is more advantageous to balance the energy budget

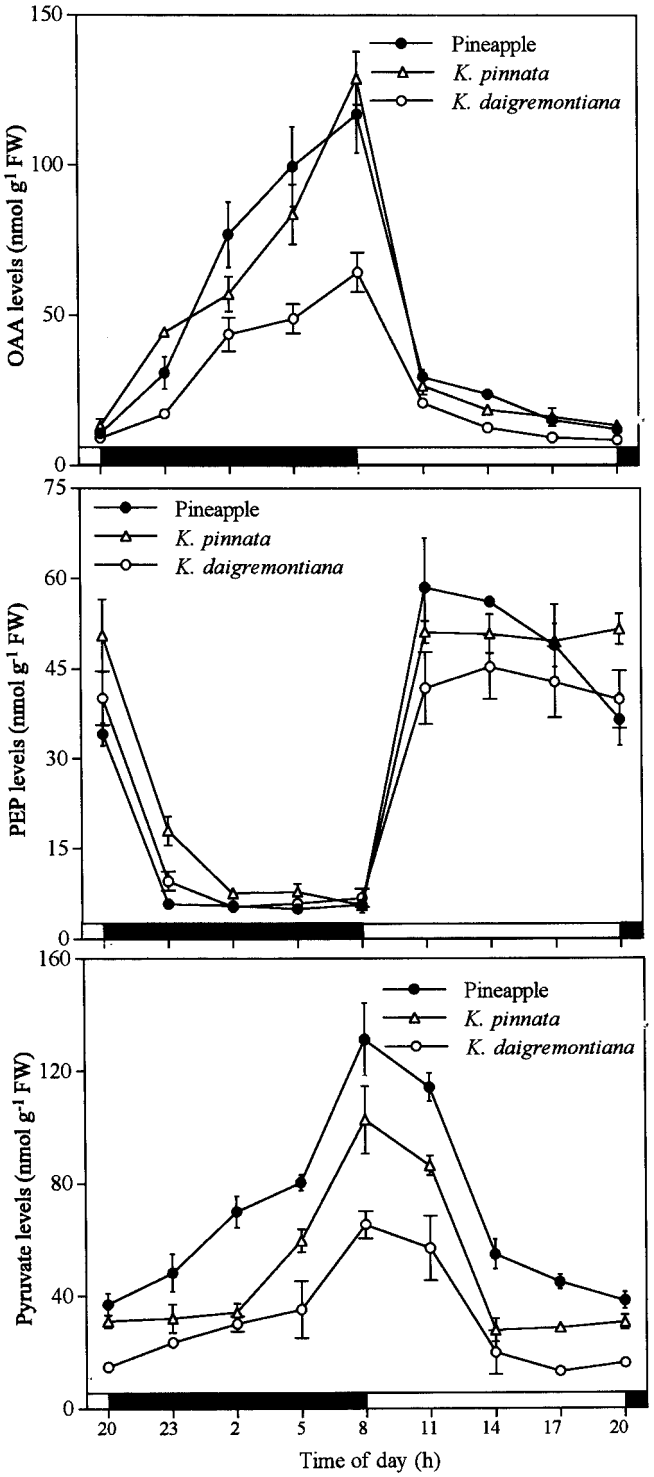

Fig. 3. Levels of OAA, PEP and Pyr in the leaves of pineapple, *K. pinnata* and *K. daigremontiana* during the 24 h day–night cycle. The closed and open horizontal bars indicate the dark and light periods, respectively. Values are means of six experiments ±SD.

during the dark period than nocturnal malate accumulation (Lüttge, 1988; Winter and Smith, 1996). Nocturnal isocitrate increase may also be energetically more favourable than nocturnal malate accumulation. As shown in Table 1, a net of 7 mol ATP is gained when 1 mol hexose-P is used for the vacuolar storage of 1 mol isocitric acid, compared with a net loss of 1 mol ATP when 1 mol hexose-P is used for the vacuolar storage of 2 mol malate.

As shown in Table 2, the net energy requirement for 1 mol malate accumulation in the vacuoles of *K. pinnata* and *K. daigremontiana*, which was 0.03 and −0.946 mol ATP, respectively, was less than that of pineapple, which was a net loss of 0.5 mol ATP (Carnal and Black, 1989). This is consistent with the results that the extent of nocturnal ATP increase was far greater in the *Kalanchoë* species than in the pineapple (data not shown).

As shown in Fig. 2, G-6-P, F-6-P and G-1-P levels in the leaves of the three CAM species increased rapidly during the first part of the dark period and decreased during the latter part of the dark period. These results suggest that hexose-P produced in glycolysis may be more than that required for malate accumulation during the first part of the dark period, while the reverse may be the case during the latter part of the dark period. This is consistent with the results that F-2,6-P_2 levels in the leaves of pineapple and *Bryophyllum tubiflorum* remain relatively stable and high during the first several hours of the dark period and decrease towards the end of the dark period (Fahrendorf *et al.*, 1987). It is known that F-2,6-P_2 activates PPi-PFK and inhibits cytosolic FBPase (Carnal and Black, 1989; Trípodi and Podestá, 1997). Rising F-2,6-P_2 levels are believed to be a signal that hexose-P is being produced rapidly (Stitt, 1990). However, F-1,6-P_2 levels in the two *Kalanchoë* species always increased during the dark period (Fig. 2). As shown in Fig. 2, G-6-P, F-6-P and G-1-P levels were always higher in pineapple leaves than in the two *Kalanchoë* species leaves. The higher G-6-P, F-6-P and G-1-P levels (when all the three species studied showed very similar levels of CAM activity in terms of malate accumalation) may be related to high F-2,6-P_2 levels in the pineapple leaves (Fahrendorf *et al.*, 1987). Truesdale *et al.* found that F-2,6-P_2 played a major role in regulating partitioning between sucrose and starch synthesis during photosynthesis but genetic alterations in F-2,6-P_2 levels had little effect on malate mobilization during CAM fluxes (Truesdale, 1999). F-2,6-P_2 is synthesized and degraded by two specific enzymes, called fructose 6-phosphate 2-kinase (F-6-P 2-kinase, EC 2.7.1.105) and fructose 2,6-bisphosphatase (F-2,6-P_2ase, EC 3.1.3.46), and its concentration can be altered by changing the activity of these enzymes (Stitt, 1990). F-6-P stimulates F-6-P 2-kinase and inhibits F-2,6-P_2ase, favouring an increase of F-2,6-P_2 concentration (Stitt *et al.*, 1987). As described here, it is obviously advantageous for pineapple to have higher F-1,6-P levels.

Dennis and Greyson showed that G-1-P, G-6-P and F-6-P were at equilibrium through the action of GPI

Table 2. *ATP requirement for malate, citrate and isocitrate accumulation in the vacuole of* K. pinnata *and* K. daigremontiana *during the dark period*

The net ATP requirement is 0.5 mol ATP for 1 mol malate accumulation and −7 mol ATP for 1 mol citrate (or isocitrate) accumulation in the vacuoles (Table 1; Carnal and Black, 1989; Lüttge, 1988).

Content	*K. pinnata*	*K. daigremontiana*
Total daily Δ-malate (μmol g^{-1} FW)	120	92
Total daily Δ-citrate (μmol g^{-1} FW)	8	8
Total daily Δ-isocitrate (μmol g^{-1} FW)		11

Net ATP requirement for malate and citrate accumulation in the vacuole of *K. pinnata* 120×0.5 ATP-8×7 ATP$=4$ ATP for 120 malate and 8 citrate accumulation i.e. 0.03 ATP for 1 malate and 0.07 citrate accumulation

Net ATP requirement for malate, citrate and isocitrate accumulation in the vacuole of *K. daigremontiana* 92×0.5 ATP-8×7 ATP-11×7 ATP$=-87$ ATP for 92 malate, 8 citrate and 11 isocitrate accumulation i.e. −0.946 ATP for 1 malate, 0.09 citrate and 0.12 citrate accumulation

and PGM (Dennis and Greyson, 1987). The equilibrium constant (K_{eq}) for PGI and PGM are 0.36 to 0.47 and 17.0, respectively (Kubota and Ashihara, 1990). In Table 3, the calculated mass-action ratios (MARs) of the two enzymes, except the MARs of GPI in *K. daigremontiana*, were somewhat lower than K_{eq}, respectively. Especially MARs of PGM were low in *K. daigremontiana*. Kubota and Ashihara found the MARs for PGI and PGM in suspension-cultured cell of *Catharanthus roseus* were 0.18 and 9.7, respectively (Kubota and Ashihara, 1990) and were similar to the present results. This suggests that the three hexose-P are near equilibrium at the three CAM species *in vivo*. MARs of PGI and GPI in the three CAM species showed a diurnal change and higher MARs were observed at midnight and early morning for GPI and PGM, respectively. As shown here, while the first and terminal reactions of the glycolysis and the gluconeogenesis in three CAM species are similar to the results obtained in the non-CAM plants, the diurnal changes of GPI and PGM suggest that there are specific regulations around GPI and PGM in CAM.

Although F-1,6-P_2 levels in pineapple increased during the dark period, the extent of the nocturnal increase was far less than that in the two *Kalanchoë* species. In addition, the absolute levels of F-1,6-P_2 in pineapple were far less than these in the two *Kalanchoë* species during the dark period (Fig. 2; Table 4). This may be related to the fact that pineapple leaves have high PPi- PFK activity (Carnal and Black, 1989; Trípodi and Podestá, 1997). Unlike ATP-PFK, PPi-PFK catalyses a readily reversible reaction, which is close to equilibrium *in vivo* (Trípodi and Podestá, 1997). PPi-PFK in pineapple leaves was considered to function in the glycolytic direction, and could substitute for the ATP-PFK (Carnal and Black, 1989; Trípodi and Podestá, 1997). In addition, the enzyme was also considered to function in the gluconeogenic direction (Fahrendorf *et al*., 1987). If so, F-1,6-P_2 levels could be estimated from F-6-P levels according to Pi and PPi levels in the cytosol of pineapple leaves, equilibrium constant ($K_{eq}=$[F-1,6-P_2][Pi]/[F-6-P][PPi]) for PPi-PFK in pineapple leaves, subcellular compartment of F-6-P and F-1,6-P_2 in the pineapple leaves. K_{eq} for PPi-PFK in pineapple leaves is not known, yet it is known that K_{eq} for PPi-PFK in other plants is 3.3 (Weiner *et al*., 1987; Stitt, 1990). To calculate F-1,6-P_2 levels, PPi and Pi levels in pineapple leaves were determined (Table 4). Former studies suggested that PPi was located predominantly in the cytosol (Weiner *et al*., 1987; Takeshige and Tazawa, 1989). Black *et al*. found that the cytosol was 3.3% of the cell volume in pineapple leaves (Black *et al*., 1982). In addition, they also found that the vacuoles of *Sedum telephium* contain a transient pool of Pi and one-third of the vacuole Pi moved into the cytosol during the dark period. F-6-P and F-1,6-P_2 in various subcellular compartments of CAM plants are not known. Gerhardt *et al*. found that hexose-P was almost located in the cytosol of spinach leaves during the dark period (Gerhardt *et al*., 1987). According to the results mentioned above, F-1,6-P_2 levels were estimated from F-6-P levels in pineapple leaves. Here, the assumptions are as follows: (1) K_{eq} for PPi-PFK in pineapple leaves is 3.3; (2) PPi is located predominantly in the cytosol of pineapple leaves; (3) one-third of Pi is in the cytosol of pineapple leaves during the dark period; (4) the cytosol is 3.3% of the cell volume in the leaves of pineapple; and (5) the subcellular compartments of F-6-P and F-1,6-P_2 in the cells of pineapple leaves are similar during the dark, which may be located mainly in the cytosol. As shown in Table 4, F-1,6-P_2 levels in pineapple leaves estimated from F-6-P levels are similar to the levels determined here. This suggests that PPi-PFK could be involved in cytoplasmic F-6-P and F-1,6-P_2 transformations and PPi, Pi, F-6-P and F-1,6-P levels are close to the dynamics equilibrium in the cytosol of pineapple leaves. This also supports the view that the reaction catalysed by PPi-PFK is close to equilibrium *in vivo* (Trípodi and Podestá, 1997).

In addition, the F-1,6-P_2 levels determined here were somewhat higher that those calculated from F-6-P levels (Table 4); this suggests that F-1,6-P_2 produced in glycolysis is more than required for malate accumulation during the dark period, and that PPi-PFK activity is

Table 3. *Mass-action ratios for the reactions catalysed by glucosephosphate isomerase and phosphoglucomutase in the leaves of pineapple,* K. pinnata *and* K. daigremontiana

It is assumed that the subcellular compartments of G-1-P, G-6-P and F-6-P in the cells are similar. Mass-action ratios were calculated from the concentrations of G-1-P, G-6-P and F-6-P shown in Fig. 2. The dark and light period is 20.00 h to 08.00 h and 08.00 h to 20.00 h, respectively. The equilibrium constant (K_{eq}) for glucosephosphate isomerase and phosphoglucomutase is 0.36–0.47 and 17, respectively (Kubota and Ashihara, 1990).

Time of day (h)	Glucosephosphate isomerase			Phosphoglucomutase		
	Pineapple	*K. pinnata*	*K. daigremontiana*	Pineapple	*K. pinnata*	*K. daigremontiana*
20	0.29	0.35	0.49	7.1	6.4	4.2
23	0.21	0.22	0.40	9.7	8.7	4.8
2	0.22	0.22	0.31	13.7	11.4	5.3
5	0.21	0.24	0.29	14.1	9.4	5.3
8	0.23	0.23	0.33	11.8	9.3	5.1
11	0.28	0.29	0.50	7.5	8.1	4.3
14	0.28	0.31	0.48	7.3	6.7	4.2
17	0.30	0.37	0.58	7.3	6.6	3.3
20	0.28	0.37	0.59	7.6	5.6	3.6

Table 4. *F-1,6-P_2 levels estimated from F-6-P levels in pineapple leaves during the dark period*

It is assumed that K_{eq} for PPi-PFK in pineapple leaves is 3.3; PPi is located predominantly in the cytosol of pineapple leaves; one-third of Pi is in the cytosol of pineapple leaves during the dark period; the subcellular compartments of F-6-P and F-1,6-P_2 in the cells of pineapple leaves are similar during the dark and cytosol is 3.3% of the cell volume in pineapple leaves.

Time of day (h)	20	23	2	5	8
Pi levels (μmol g^{-1} FW)	6.78	7.21	7.54	8.35	8.21
PPi levels (nmol g^{-1} FW)	23	39	85	110	106
Pi concentration in cytosol (mM)	68.5	72.9	76.1	84.3	83.0
PPi concentration in cytosol (mM)	0.69	1.19	2.59	3.33	3.21
F-6-P levels (nmol g^{-1} FW)	20.6	29.6	56.6	52.4	43.9
F-1,6-P_2 levels calculated from F-6-P (nmol g^{-1} FW)	0.7	1.6	6.3	6.3	5.6
F-1,6-P_2 levels (nmol g^{-1} FW)	2.7	3.1	8.9	10.5	11.0
Estimated F-1,6-P_2 levels/F-1,6-P_2 levels (%)	26	52	71	60	51

sufficient for the rates of hexose turnover required for malic acid accumulation or that some limitations for using F-1,6-P_2 may have existed in the later parts of glycolysis. As described above, although the levels of F-1,6-P_2 in pineapple leaves were significantly lower than those of two *Kalanchoë* species, the results in pineapple were almost similar to the levels calculated with related observations.

Compared with malate, citrate and isocitrate absolute levels, OAA, Pyr and PEP did not accumulate in large amounts. However, large diurnal variations in OAA, Pyr and PEP concentrations in the leaves of the three CAM species were observed (Fig. 3). This suggests that the compounds must be involved in metabolic regulation. OAA levels in the three CAM species increased during the dark period and decreased rapidly to very low levels during the first 3 h of the light period and remained little changed throughout the rest of the light period (Fig. 3). Similar results have been obtained with *K. daigremontiana* (Kenyon *et al*., 1981) and *Bryophyllum crenatum* (Milburn *et al*., 1968) leaves. Nocturnal OAA increase suggests that PEPC is active and OAA generated by carboxylation is more than the requirement for malate synthesis during the dark period. That OAA generated by malate decarboxylation is less than the OAA requirement for hexose synthesis via gluconeogenesis may also be the cause for the OAA level decrease in pineapple leaves during the light period. As shown in Fig. 3, PEP declined drastically to very low levels during the first 3 h of the dark period, then remained unchanged through the rest of the dark period, suggesting that PEP is in limited supply at night and may limit PEPC activity. During the light period, both PEP carboxylation decline and conversion of Pyr to PEP via gluconeogenesis may be the cause for PEP increase. PEP is a compound with 1 energy-rich ~P-bond in the molecule. It was found that the levels of ATP in *Kalanchoë* leaves increased during the dark period (Smith *et al*., 1982; Pistelli *et al*., 1987). Nocturnal ATP increase and PEP decrease may be a regulation of total balance of energy-rich phosphate. During the light period, Pyr level decrease may be due to the conversion of Pyr into starch or soluble hexose via gluconeogenesis. During the dark period, Pyr level increase may be due to the conversion of some PEP into Pyr.

In conclusion, there are many important differences in metabolite levels, especially in major organic acids

and hexose-P, associated with CAM among pineapple, a hexose-utilizing species, and *K. daigremontiana* and *K. pinnata*, two starch-utilizing species. To understand the metabolic regulation of CAM further, studies on subcellular compartments of metabolites associated with CAM and diurnal changes of metabolite levels in various organelles are necessary.

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References

Ames BN. 1966. Assay of inorganic phosphate, total phosphate and phosphatase. *Methods in Enzymology* **8,** 115–118.

Black CC, Carnal NW, Kenyon WH. 1982. Compartmentation and the regulation of CAM. In: Ting IP, Gibbs M, eds. *Crassulacean acid metabolism*. Baltimore, Maryland: Waverly Press, 51–68.

Borland AM, Griffiths H. 1997. A comparative study on the regulation of C_3 and C_4 carboxylation processes in the constitutive crassulacean acid metabolism (CAM) plant *Kalanchoë daigremontiana* and the C_3-CAM intermediate *Clusia minor*. *Planta* **201,** 368–378.

Carnal NW, Black CC. 1983. Phosphofructokinase activities in photosynthetic organisms: the occurrence of pyrophosphate-dependent 6-phosphofructokinase in plants and algae. *Plant Physiology* **71,** 150–155.

Carnal NW, Black CC. 1989. Soluble sugars as the carbohydrate reserve for CAM in pineapple leaves: implications for the role pyrophosphate:6-phosphofructokinase in glycolysis. *Plant Physiology* **90,** 91–100.

Chen L-S, Nose A. 2000. Characteristics of adenosine-triphosphatase and inorganic pyrophosphatase in tonoplast isolated from three CAM species, *Ananas comosus*, *Kalanchoë pinnata* and *K. daigremontiana*. *Plant Production Science* **3,** 24–31.

Chen L-S, Nose A. 2001. An improved method for extraction and measurement of the inorganic pyrophosphate in leaves of crassulacean acid metabolism (CAM) plants. *Plant Production Science* **4,** 15–19.

Christopher JT, Holtum JAM. 1996. Patterns of carbon partitioning in leaves of crassulacean acid metabolism species during deacidification. *Plant Physiology* **112,** 393–399.

Cockburn W, McAulay A. 1977. Changes in metabolic levels in *Kalanchoë daigremontiana* and the regulation of malic accumulation in crassulacean acid metabolism. *Plant Physiology* **59,** 455–458.

Dagley S. 1974. Citrate: UV spectrophotometric determination. In: Bergmeyer HU, ed. *Methods of enzymatic analysis*, Vol. 3. New York: Academic Press, 1562–1565.

Delhaize E, Ryan PR, Randall PJ. 1993. Aluminum tolerance in wheat (*Triticum aestivum* L.). II. Aluminum-stimulated excretion of malic acid from root apices. *Plant Physiology* **103,** 695–702.

Dennis DT, Greyson MF. 1987. Fructose 6-bisphosphate metabolism in plants. *Physiologia Plantrum* **69,** 395–404.

Du Y-C, Nose A, Wasano K, Uchida Y. 1998. Responses to water stress of enzyme activities and metabolite levels in relation to sucrose and starch synthesis, the Calvin cycle and the C_4 pathway in sugarcane (*Saccharum* sp.) leaves. *Australian Journal of Plant Physiology* **25,** 253–260.

Fahrendorf T, Holtum JAM, Mukherjee U, Latzko E. 1987. Fructose 2,6-bisphosphate, carbohydrate partitioning and crassulacean acid metabolism. *Plant Physiology* **84,** 182–187.

Gerhardt R, Stitt M, Heldt HW. 1987. Subcellular metabolite levels in spinach leaves: regulation of sucrose synthesis during diurnal alterations in photosynthetic partitioning. *Plant Physiology* **83,** 399–407.

Heldt HW. 1976. Transport of metabolites between cytoplasm and the mitochondrial matrix. In: Stocking CR, Heber U, eds. *Encyclopedia of plant physiology*, New series, Vol. 3. Berlin: Springer-Verlag, 235–254.

Holtum JAM, Osmond CB. 1981. The gluconeogenic metabolism of pyruvate during deacidification in plants with crassulacean acid metabolism. *Australian Journal of Plant Physiology* **8,** 31–44.

Kenyon WH, Holaday AC, Black CC. 1981. Diurnal changes in metabolite levels and crassulacean acid metabolism in *Kalanchoë daigremontiana* leaves. *Plant Physiology* **68,** 1002–1007.

Kenyon WH, Severson RF, Black Jr CC. 1985. Maintenance carbon cycle in crassulacean acid metabolism plant leaves: source and compartmentation of carbon for nocturnal malate synthesis. *Plant Physiology* **77,** 183–189.

Knopf O, Kluge M. 1979. Properties of phospho*enol*pyruvate carboxylase in *Sedum* species in relation to crassulacean acid metabolism (CAM). *Plant, Cell and Environment* **2,** 73–78.

Kondo A, Nose A, Ueno O. 1998. Leaf inner structure and immunogold location of some key enzymes involved in carbon metabolism in CAM plants. *Journal of Experimental Botany* **49,** 1953–1961.

Kubota K, Ashihara H. 1990. Identification of non-equilibrium glycolytic reactions in suspension-cultured plant cells. *Biochimica et Biophyusica Acta* **1036,** 138–142.

Lüttge L. 1988. Day–night changes of citric-acid levels in crassulacean acid metabolism: phenomenon and ecophysiological significance. *Plant, Cell and Environment* **11,** 445–451.

Lüttge U. 1998. Crassulacean acid metabolism. In: Raghavendra AS, ed. *Photosynthesis: a comprehensive treatise*. Cambridge University Press, 136–149.

Medina E, Popp M, Lüttge U, Ball E. 1991. Gas exchange and acid accumulation in high and low irradiance grown pineapple cultivars. *Photosynthetica* **25,** 489–498.

Milburn TR, Pearson DJ, Ndegwe NA. 1968. Crassulacean acid metabolism under natural tropical conditions. *New Phytologist* **67,** 883–897.

Mohanty B, Wilson PM, ap Ress T. 1993. Effects of anoxia on growth and carbohydrate metabolism in suspension cultures of soybean and rice. *Phytochemistry* **34,** 75–82.

Möllering H. 1974. L-Malate: determination with malate dehydrogenase and glutamate-oxaloacetate transaminase. In: Bergmeyer HU, ed. *Methods of enzymatic analysis*, Vol. 3. New York: Academic Press, 1589–1593.

Osmond B, Maxwell K, Popp M, Robinson S. 1999. On being thick: fathoming apparently futile pathways of photosynthesis and carbohydrate metabolism in succulent CAM plants. In: Bryant JA, Burrell MM, Kruger NJ, eds. *Plant carbohydrate biochemistry*, Oxford: Bios Scientific Publishers Ltd, 183–200.

Pierre JN, Queiroz O. 1979. Regulation and level of the crassulacean acid. *Planta* **144,** 143–151.

Pistelli L, Marigo G, Ball E, Lüttge U. 1987. Day–night changes in the levels of adenine nucleotides, phospho*enol*pyruvate and inorganic pyrophosphate in leaves of plants having crassulacean acid metabolism. *Planta* **172,** 479–486.

Popp M, Kramer D, Lee H, Diaz M, Ziegler H, Lüttge U. 1987. Crassulacean acid metabolism in tropical dicotyledonous trees of the genus *Clusia*. *Tree* **1,** 238–247.

Sideris CP, Young HY, Chun HHQ. 1948. Diurnal changes and growth rates as associated with ascorbic acid, titratable acidity, carbohydrate and nitrogenous fractions in the leaves of *Ananas comosus* (L.) Merr. *Plant Physiology* **23,** 38–69.

Siebert G. 1974. Isocitrate: UV spectrophotometric determination. In: Bergmeyer HU, ed. *Methods of enzymatic analysis*, Vol. 3. New York: Academic Press, 1570–1573.

Smith JAC, Marigo G, Lüttge U, Ball E. 1982. Adenine-nucleotide levels during crassulacean acid metabolism and the energetics of malate accumulation in *Kalanchoë tubiflora*. *Plant Science Letters* **26,** 13–21.

Stitt M. 1990. Fructose-2,6-bisphosphate as a regulatory molecule in plants. *Annual Review of Plant Physiology and Plant Molecular Biology* **41,** 153–185.

Stitt M, Gerhardt R, Whilke I, Heldt HW. 1987. The contribution of fructose 2,6-bisphosphate to the regulation of sucrose synthesis during photosynthesis. *Physiologia Plantrum* **69,** 377–386.

Takeshige K, Tazawa M. 1989. Determination of the inorganic pyrophosphate level and its subcellular localization in *Chara corallina*. *Journal of Biology Chemistry* **25,** 3262–3266.

Ting IP, Lord EM, Deniro MJ. 1985. Crassulacean acid metabolism in the strangler *Clusia rosea* Jacq. *Science* **229,** 969–971.

Trípodi KEJ, Podestá FE. 1997. Purification and structural and kinetic characterization of the pyrophosphate:fructose-6-phosphate 1-phosphotransferase from the crassulacean acid metabolism plant, pineapple. *Plant Physiology* **113,** 779–786.

Truesdale MR, Toldi O, Scott P. 1999. The effect of elevated concentrations of fructose 2,6-bisphosphate on carbon metabolism during deacidification in the crassulacean acid metabolism plant *Kalanchoë daigremontiana*. *Plant Physiology* **121,** 957–964.

Vickery HB. 1952. The behavior of isocitric acid in excised leaves of *Bryophyllum calycium* during culture in alternating light and darkness. *Plant Physiology* **27,** 9–27.

Wahlefeld AW. 1974. Oxaloacetate: UV spectrophotometric determination. In: Bergmeyer HU, ed. *Methods of enzymatic analysis*, Vol. 3. New York: Academic Press, 1604–1608.

Weiner H, Stitt M, Heldt HW. 1987. Subcellular compartment of pyrophosphate and alkaline pyrophosphatase in leaves. *Biochimica et Biophysica Acta* **13,** 13–21.

Winter K, Smith JAC. 1996. Crassulacean acid metabolism: current status and perspectives. In: Winter K, Smith JAC, eds. *Crassulacean acid metabolism: biochemistry, ecophysiology and evolution*. Berlin: Springer-Verlag, 389–426.

Wolf J. 1960. Der diurnal Särerhythmus. In: Ruhland W, ed. *Encyclopedia of plant physiology*, Vol. 12, Berlin: Springer-Verlag, 809–889.