Effects of High Night Temperature on Crassulacean Acid Metabolism (CAM) Photosynthesis of *Kalanchoë pinnata* and *Ananas comosus*

Qin Lin1,2, Syunsuke Abe3, Akihiro Nose2, Akira Sunami2 and Yoshinobu Kawamitsu3

1The United Graduate School of Agriculture Science, Kagoshima University, Kagoshima 890-0065, Japan; 2Faculty of Agricultural, Saga University, 1 Honjo-machi, Saga 840-8502, Japan; 3Faculty of Agriculture, University of the Ryukyus, 1 Seibaru, Okinawa 903-0213, Japan

Abstract: The effects of the night temperature on CO2 exchange rate and organic acid accumulation in the leaves of two crassulacean acid metabolism (CAM) plants, *Kalanchoë pinnata* and *Ananas comosus* (pineapple), were examined under a fixed day-temperature condition of 30°C. With the increase of the night temperature, the CO2 exchange rate decreased in both species, and *K. pinnata* completely lost nocturnal CO2 uptake under a high night temperature (30/37°C in day/night) condition (HNT). Malate accumulation in the leaves of pineapple and *K. pinnata* in the morning decreased with increasing night temperature, but that in the afternoon was not influenced by the night temperature. Diurnal changes of ten kinds of metabolites were investigated under HNT. Pineapple accumulated a large amount of nocturnal malate under HNT, but *K. pinnata* did not. Four kinds of hexose-phosphate (hexose-P) were accumulated at the same levels during the day/night cycle under HNT in both plant species. Nocturnal accumulation of oxaloacetate (OAA) was observed but phosphoenolpyruvate (PEP) was kept at a high level both in day and night under HNT in both plant species. The concentrations of malate required for 50% inhibition of the activities of day and night forms of PEP carboxylase (PEPC) from the pineapple leaves were 1.2 and 0.7 mM, respectively, whereas those from the *K. pinnata* leaves were 3.7 and 2.0 mM, respectively. In both plants, NAD-MDH activity in vitro increased with increasing temperature. It is therefore suggested that under HNT, phosphorylation may not be the major factor controlling PEPC activity in pineapple, and therefore CAM mode in pineapple was maintained under HNT. The nighttime phosphorylation of PEPC in *K. pinnata* would disappear under HNT leading to the loss of nocturnal malate accumulation.

Key words: CO2, Crassulacean acid metabolism (CAM), Malate, Night temperature, Phosphoenolpyruvate carboxylase (PEPC).

Crassulacean acid metabolism (CAM) is one of three metabolic pathways for assimilation of atmospheric CO2 in vascular plants. During the night, CO2 fixation of CO2 is catalyzed by phosphoenolpyruvate carboxylase (PEPC). This results in the formation of malate, which is stored in a large central vacuole. During the following daytime, the malate released from the vacuole is decarboxylated by malic enzymes (ME, EC 1.1.1.39 and EC 1.1.1.40) or phosphoenolpyruvate carboxykinase (PCK, EC 4.1.1.49), leading to the liberation of CO2 and the formation of three carbon compounds, pyruvate or PEP. The liberated CO2 is refixed by ribulose 1,5-bisphosphate carboxylase/oxygenase (Rubisco) and assimilated via C3 photosynthetic carbon reduction cycle, with regeneration of storage carbohydrate largely from pyruvate and PEP in gluconeogenesis (Winter and Smith, 1996).

CAM plants can be divided basically into two groups, starch formers and extrachloroplastic carbohydrate formers, based on the major final carbohydrate reservoir used in their daily cycle (Christopher and Holtum, 1996). Previous studies (Chen et al., 2002) indicated that both groups showed typical characters of the diurnal change in glycolytic metabolites under lower night temperatures with relatively higher day temperature conditions. Sekizuka et al. (1992) investigated the effects of the combination of day/night temperature on CO2 exchange in a CAM plant, *Dendrobium Ekapol Panda No.1*, and found that when the night temperature was higher than the daytime temperature, the CO2 efflux occurred at the beginning of the dark period, and the CO2 balance in the dark period is disturbed drastically. Thomas et al. (1954) observed that the uptake of CO2 including respiratory CO2 in the CAM plants was increased by a...
high night temperature, depressing malate gain. In the leaves of *Bryophyllum fedtschenkoi*, the nighttime CO₂ fixation was restrained by a high temperature (40°C), due to the inhibition of the activity of PEPC by malate (Anderson et al., 1989). Carter et al. (1995a, b) also reported the temperature-dependent properties of PEPC in *B. fedtschenkoi*, such as the influence of temperature on the activity of PEPC kinase (PCK) and the allosteric properties of PEPC. Thus, the change of night temperature significantly influences on the PEPC activity and CO₂ fixation, resulting in the reinitiation of the CAM rhythm. In other words, activation of PEPC is regulated by the transcription level of PEPC kinase and is a crucial control step in CAM. The day/night regulation of carbon flux through PEPC is achieved by reversible phosphorylation that reduces the sensitivity of the enzyme to L-malate; malate insensitive (active) form of PEPC presented at night (Nimmo et al., 1984, 1986, 1987, 2000). However, Shaheen et al. (2002) indicated that PEPC is not phosphorylated in pineapple, but is phosphorylated in *K. pinnata*. On the other hand, from the biochemical and molecular analysis, and model simulation study, Lüttge (2000) strongly suggested that the master switch for circadian regulation of CAM was the tension/relaxation mechanism of tonoplast. Wilkins (1983) investigated temperature-related phase shifts of the endogenous CO₂-exchange rhythms in *B. fedtschenkoi*, and concluded that alterations of temperature could influence the permeability of the tonoplast, thus controlling malate transport across the membrane (Nimmo, 2000).

Recent studies on the malate accumulation in the CAM have been focused on the sensitivity to malate of PEPC and the fluidity of the tonoplast (Lüttge and Smith, 1984; Friemert et al., 1988; Behzadipour et al., 1998; Nimmo et al., 2000). However, there could be other metabolic systems which might control CAM, such as glycolysis and gluconeogenesis. Although it is known that a high night temperature can significantly affect CAM, the effects of high night temperature on the CO₂ exchange and accumulation of some metabolites are not yet clear. The influence of a high temperature on the CO₂ exchange in pineapple was noticed by Neales et al. (1980) and Zhu et al. (1999). However, their results did not clarify sufficiently the effects a high night temperature on the controlling mechanism of CAM photosynthesis. Kluge and Ting (1978) demonstrated that no starch loss could be measured in succulent CAM plants at 35°C. Sutton (1975) found that very low activities of pyruvate kinase and glycolytic enzyme 6-phosphofructokinase (PFK) were the most rate-limiting factors of PEP supply for night-time CAM. Thus, the glycolytic metabolites as well as other metabolites involved in CAM photosynthesis must also be considered as the possible factors for the temperature-induced change in the CO₂ gas exchange pattern. Furthermore, Cuevas and Podesta (2000) indicated that NAD-MDH is responsible for the nighttime malate synthesis in pineapple, but not sufficient to support the malate conversion to OAA in the daytime. Based on these findings, it is suggested that possible factors involved in the response of CAM to temperature stress are 1) the levels of metabolites in glycolysis and gluconeogenesis, 2) the properties of NAD-MDH, 3) the sensitivity of PEPC to inhibition by malate and 4) the characteristics of tonoplast.

In this study, we examined the properties of PEPC and NAD-MDH, which are involved in glycolysis and gluconeogenesis to understand the relationship between CAM and high-night temperature stress. For this purpose, the effects of high night temperature on CO₂ exchange, organic acid levels and the diurnal changes in the levels of 10 kinds of glycolytic metabolites were examined. These metabolites were related to the rate-limiting enzymes of PEP supply in the two CAM plants, pineapple and *K. pinnata* under a high night temperature condition at 30/37°C (day/night). The *in vitro* sensitivity to PEPC inhibition by malate and the temperature response of NAD-MDH were also investigated, and the processes and shift of photosynthesis mode a high-temperature condition in *K. pinnata* were discussed. The findings in this study may be helpful in understanding the influence of the high-temperature stress on CAM, and improvement of the production of crops such as pineapple in tropical areas.

**Materials and methods**

**Plant materials**

Pineapple (*Ananas comosus* cv. Smmoth-cayenne N67-10) and *Kalanchoë pinnata* were vegetatively propagated and grown in pots in a greenhouse with heating under a natural photoperiod. The plants were irrigated two or three times every week depending on the soil moisture state, and fertilized with a 500-fold diluted solution of mixed fertilizers (No.1 and No.2 of Otsuka House) once a month. The plants were transferred to a growth chamber (KG-50 HLA, Koiro Industrial Co., Ltd., Japan) before the experiment. The photoperiod was11-h light (6:00-17:00) and 13-h darkness (17:00-6:00). In the growth chamber, the temperature was kept at 30°C during the light period at a photosynthetic active radiation (PAR) of 420 to 450 μmol m⁻² s⁻¹ at the mid-plant height, and the relative humidity was 65% in the daytime. Fourth to eighth leaf pairs, counting from the apex of *K. pinnata* and fully expanded mature leaves of pineapple were used for each experiment.

**Measurement of CO₂ exchange rate**

Leaves were placed in an acrylic assimilation chamber (L×W×H: 28×24×11.5 cm) maintained 30°C in the light period and 16 to 37°C in the dark...
period using a heat-exchanger. The photoperiod was 10.5-h light (7:30-18:00) and 13.5-h darkness (18:00-7:30). PAR on the leaf surface of pineapple and *K. pinnata* were 280-340 and 325-380 µmol m⁻² s⁻¹, respectively. The relative humidity in the light and dark periods was 65% and 75%, respectively. CO₂ exchange was measured by the ventilation-type assimilation box method, using the absolute value type-CO₂ IRGA (LI-6251, Li-Cor, USA). The measurements were made after plants were set in respective night temperature regime for three to four days.

**Extraction and Measurement of metabolites**

The metabolites were extracted and measured 7 days after the transfer of plants to the growth chamber by the method described by Chen et al. (2002).

**Extraction and assay of PEPC and MDH**

The leaf samples 4 cm² in area for malate inhibition of PEPC were collected at 11:30 and 23:30 in the growth chamber kept at 30/37°C in the light/dark period. Then, the sensitivity of PEPC to malate was assayed by the same method as that of Shaheen et al. (2002).

Leaf samples (4 cm²) for assay of NAD-MDH activity were collected at 11:00 and 23:00 in the growth chamber kept at 30/20°C in the light/dark period. NAD-MDH activity was assayed according to the method of Pastore et al. (2001) with some modification. The samples were frozen in liquid nitrogen and stored at −80°C until analysis. The frozen tissue was homogenized in a mortar with a pestle with 0.2 g sea sand and 40 mg of polyvinylpolypyrrolidone in 4 mL of ice-cold extraction buffer, which was composed of 50 mM Tris-HCl (pH 7.8), 1 mM EDTA-KOH (pH 7.0), 5 mM DTT, 0.2% BSA and 8 mM MgCl₂. The homogenate was filtered through one layer of Miracloth, and the filtrate was centrifuged at 2000g for 20s at 4°C in a micro centrifuge (CFM-100, IWAKI Glass Co., Ltd, Japan). The supernatant was used immediately for the enzyme assay. The temperature-dependent activity of NAD-MDH was measured after the oxidation of NADH spectrophotometrically at 340 nm using a UV-visible spectrophotometer (V-550, Jasco, Japan) with the cell holder (STR 458, Jasco, Japan). It was connected to a circulator (UA-100, Tokyo Rikakikai Co., Ltd, Japan), kept at the designated temperature. The reaction mixture (1 ml) containing 25 mM Hepes-KOH (pH 7.4), 10 mM KH₂PO₄, 10 mM KCl, 0.1% BSA, 5 mM MgCl₂ and 2 mM NADH, adjusted to the required temperature before the reaction was started by adding 600 µM OAA.

**Results**

1. **Influence of night temperature on CO₂ exchange**

Figure 1 shows the influences of night temperature on CO₂ exchange in the leaves of *K. pinnata* and pineapple. The light period was maintained at 30°C. In *K. pinnata*, the pattern of CO₂ exchange was almost the same at the night temperature of 16°C to 31°C, while the amount of CO₂ in dark was significantly decreased as the temperature was raised. When the night temperature over 28°C, the onset time of CO₂ uptake in the phase 4 became early. When the night temperature was raised to 37°C, the nocturnal CO₂ absorption was lost completely. Thus, the CO₂.
Lin et al. — High Temperature Effects on CAM Photosynthesis

High Temperature Effects on CAM Photosynthesis

exchange pattern in *K. pinnata* was converted from CAM to C₃ mode at the dark temperature of 37 °C with loss of nocturnal CO₂ absorption. In pineapple, at the night temperature between 16 °C and 25 °C, the onset time of CO₂ uptake in phase 1 was almost the same. At the temperature of 34 °C and 37 °C, the CO₂ exchange in phase 1 showed two maximum peaks. When the night temperature was between 16 °C and 25 °C, the maximum value of CO₂ fixation rate in the night was nearly the same. However, this value dropped gradually as the temperature rose from 28 °C to 37 °C. When the night temperature was higher than the day temperature (30 °C), the CO₂ uptake rate decreased substantially, and the onset time of CO₂ uptake in the afternoon (phase 4) became early. We found a clearer effect of temperature on the diurnal changes of CO₂ exchange than that reported by Neales et al. (1980) and Zhu et al. (1999). The difference between pineapple and *K. pinnata* in the response to temperature was also clear.

2. Influence of night temperature on organic acid levels

Figure 2 shows the effect of night temperature on organic acid levels in the leaves of pineapple and *K. pinnata*. The organic acid levels were measured after the plants were transferred to the growth chamber for 3 days.

![Fig. 2](image)

**Fig. 2** The effects of night temperature on malate (A,B) and citrate (C,D) levels in the pineapple (left) and *K. pinnata* (right) leaves. Temperature in the light period was 30 °C. Samples were taken at 6:00 (■) and 16:30 (○). The organic acid levels were measured after the plants were transferred to the growth chamber for 3 days.

temperature, while light temperature was kept at 30 °C. The malate level was highest at the night temperature of 20 °C in both species. Compared with the each level of 20 °C in two species, the malate level at the end of the dark period at dark temperature of 25, 30, 35, and 37 °C was reduced by 3, 12, 35 and 50%, respectively, lower than that at 20 °C in pineapple leaves, and 3, 44, 76 and 90%, respectively, lower than that at 20 °C in *K. pinnata*. However, the malate levels in the afternoon were not influenced by the night temperature.

Influences of night temperature on citrate levels in the leaves of pineapple were not so clear either in the morning or afternoon, although the levels were somewhat lowered at high night temperature. However, the citrate levels in *K. pinnata* decreased gradually when the night temperature was raised from 20 °C to 37 °C.

3. Diurnal changes in the levels of malate, citrate, and isocitrate

In this study, the levels of three kinds of organic acids, namely, malate, citrate and isocitrate, in the leaves of *K. pinnata* and pineapple were measured.
every 3 to 4 hours during a 24-hour light/dark period at 30/37 °C (Fig. 3). Pineapple maintained the diurnal change of malate level as a typical CAM plant. Nocturnal malate accumulation reached about 39 µmol g\(^{-1}\) FW. On the other hand, *K. pinnata* showed almost no diurnal change of malate level, ranging from 21 to 24 µmol g\(^{-1}\) FW. This shows that the pattern of diurnal change of malate in *K. pinnata* was converted from CAM to C\(_3\) mode under the high night temperature condition (HNT, 30/37 °C in day/night), whereas pineapple maintained a CAM mode even under HNT. The isocitrate level in pineapple leaves and citrate level in *K. pinnata* leaves were very low during the light-dark cycle. These results differ from those in the plants under NNT, in which G1P, G6P and F6P increased rapidly to high levels during the first half part of the dark period, and decreased during the latter half part of the dark period (Chen et al., 2002).

**4. Diurnal changes in the levels of G1P, G6P, F6P, and FBP**

The levels of four kinds of hexose-P did not significantly change throughout the light-dark cycle under HNT (Fig. 4). The levels of G1P, G6P, and F6P under HNT were higher in pineapple than in *K. pinnata*. However, the level of FBP was higher in *K. pinnata* than in pineapple. These results were different from those in the plants under NNT, in which G1P, G6P and F6P increased rapidly to high levels during the first half part of the dark period, and decreased during the latter half part of the dark period (Chen et al., 2002).

**5. Diurnal changes in the levels of OAA, PEP and pyruvate**

Figure 5 shows the diurnal changes in the levels of OAA, PEP and pyruvate under HNT. OAA levels in pineapple and *K. pinnata* leaves were very low during the light-dark cycle. These results differ from those in the plants under NNT, in which G1P, G6P and F6P increased rapidly to high levels during the first half part of the dark period, and decreased during the latter half part of the dark period. These patterns are consistent with those found in the plants under NNT (Chen et al., 2002). However, compared with the 58.8 nmol g\(^{-1}\) FW nocturnal
accumulation of OAA in pineapple, only 13.4 nmol g\(^{-1}\) FW nocturnal accumulations was observed in *K. pinnata*. In pineapple, the PEP level increased during the first three hours of the light period, and kept a stable high level during the remaining light period. Then it decreased during the first 6 hours of the dark period, by about 36 nmol g\(^{-1}\) FW. However, in *K. pinnata* the PEP level increased by 18 nmol g\(^{-1}\) FW during the last four hours of the dark periods and the same level was maintained for six hours in the following light periods. The diurnal pattern of pyruvate level clearly differed between pineapple and *K. pinnata*. The pyruvate level in the leaves of pineapple increased during the dark period and decreased during the light period in *K. pinnata*, however, the pyruvate level did not change so much.

6. Comparison of metabolite levels between normal and high night temperature condition

Table 1 shows the metabolite levels in the plants kept under the normal (NNT) and high night temperature conditions (HNT). In *K. pinnata*, the nocturnal malate level was about 70% lower than that under NNT. The citrate and isocitrate levels in the leaves of pineapple were nearly the same throughout the light-day cycle under both HNT and NNT. However, the diurnal change of citrate levels was observed in the leaves of *K. pinnata* under NNT but not under HNT.

The nocturnal accumulation of G1P, G6P, F6P and FBP in the leaves of pineapple and *K. pinnata* were also lost completely under HNT. In *K. pinnata*, PEP levels under NNT and HNT were 50.8 and 95.5 nmol g\(^{-1}\) FW, respectively, in the daytime, and 7.6 and 81.7 nmol g\(^{-1}\) FW, respectively, in the nighttime. PEP level in pineapple under NNT and HNT were 56.1 and 148.5 nmol g\(^{-1}\) FW, respectively, in the daytime, and 5.5 and 106.1 nmol g\(^{-1}\) FW, respectively, in the nighttime. The pyruvate leaves from dusk to midnight in the leaves of pineapple and *K. pinnata* under HNT were about 2 to 3-fold higher than those under NNT.

7. Sensitivity to malate of the day/night forms of PEPC

The sensitivity to L-malate of the day and night forms of PEPC extracted from pineapple and *K. pinnata* kept under HNT was examined (Fig. 6). The concentration of malate required for 50% inhibition (K\(_i\)) of the day and night forms of PEPC from pineapple were 1.2 and 0.7 mM, respectively, but the K\(_i\) of them from *K. pinnata* were 3.7 and 2.0 mM, respectively.

8. Effect of temperature on NAD-MDH activity

In the two CAM plants, activities of both day and night forms of NAD-MDH increased significantly with increasing temperature in the range from 10 to
45°C (Fig. 7). In both plants, the activities of both day and night forms of NAD-MDH increased similarly with increasing temperature up to 30°C. When the temperature was raised beyond 30°C, the rate of increase of the activity was lower in the day form than in the night form. In pineapple, the activity of NAD-MDH was about 2-3 times higher than that in K. pinnata.

Discussion

Our study showed that in the well-watered pineapple and K. pinnata, the CAM photosynthesis is greatly influenced by a high temperature at night. Under HNT, photosynthetic CO₂ exchange in K. pinnata was C₃ mode. (Fig. 1 and Chen et al., 2002). In CAM plants, PEP produced by glycolysis, carbon flow in CAM is along mainly two separate routes. One is CO₂ fixation by the cytoplasmic enzyme PEPC, and synthesis of malate from OAA by the action of NAD-MDH. Another is the TCA cycle via pyruvate. Under NNT, most of PEP is used to form malate. However, this route is partly inhibited (decrease of malate accumulation) by a high night temperature (37°C), promoting the carbon flow to TCA cycle (increase of pyruvate level, Table 1).

It is known that temperature has a significant influence on PEPC activity. The catalytic activity of the enzyme increases with increasing temperature and the temperature can bring about a change in the phosphorylation state of the enzyme by altering the activity of PEPC kinase (Carter et al., 1995a). In this study, the concentrations of malate required for 50% inhibition, Kᵢ of day and night forms of PEPC from pineapple and K. pinnata were investigated (Fig. 6). A striking difference in the sensitivity to L-malate was observed between these two forms of PEPC. Under HNT, the Kᵢ (L-malate) of the day and night forms of PEPC from pineapple was 1.2 and 0.7 mM, respectively, and that from K. pinnata was 3.7 and 2.0 mM, respectively. Shaheen et al. (2002) reported that the apparent Kᵢ (L-malate) of the day and night forms of PEPC under NNT was 0.5 and 1.0 mM, respectively, in pineapple and 0.9 and 5.8 mM, respectively, in K. pinnata. The values in pineapple did not differ so much from those in our experiment, but the values of the day and night forms of PEPC in pineapple and K. pinnata were investigated (Fig. 6). A striking difference in the sensitivity to L-malate was observed between these two forms of PEPC. Under HNT, the Kᵢ (L-malate) of the day and night forms of PEPC from pineapple was 1.2 and 0.7 mM, respectively, and that from K. pinnata was 3.7 and 2.0 mM, respectively. Shaheen et al. (2002) reported that the apparent Kᵢ (L-malate) of the day and night forms of PEPC under NNT was 0.5 and 1.0 mM, respectively, in pineapple and 0.9 and 5.8 mM, respectively, in K. pinnata. The values in pineapple did not differ so much from those in our experiment, but the values of the day and night forms of PEPC in pineapple and K. pinnata were 4-fold higher and 2.9-fold lower, respectively, than those in our experiment. It was indicated that the

| Metabolites | Night Temp. | Pineapple | K. Pinnata |
|-------------|-------------|-----------|------------|
|              | Night | Dawn | Noon | Dusk | Night | Dawn | Noon | Dusk |
| G1P | Normal | 18.5 | 16 | 10.5 | 9.7 | 10.9 | 8.3 | 6.1 | 5.7 |
|             | High | 18.1 | 16.4 | 16.4 | 14.8 | 8.3 | 9.9 | 9.9 | 9.2 |
| G6P | Normal | 253.8 | 189.3 | 76.2 | 74.1 | 124.1 | 77.4 | 40.9 | 31.4 |
|             | High | 70.4 | 69.7 | 73.8 | 70.9 | 59.9 | 63.6 | 61.2 | 57.2 |
| F6P | Normal | 56.6 | 43.9 | 22.1 | 21.1 | 26.7 | 17.8 | 12.8 | 11.5 |
|             | High | 22.9 | 22.1 | 24.6 | 22.9 | 13.3 | 15.9 | 13.3 | 14.2 |
| FBP | Normal | 8.9 | 11 | 2.5 | 2.8 | 84.9 | 135.6 | 7.3 | 8.7 |
|             | High | 0.83 | 0 | 0 | 0 | 10 | 12.1 | 9.9 | 9.9 |
| PEP | Normal | 5.5 | 5.7 | 56.1 | 36.5 | 7.6 | 5.6 | 50.8 | 51.6 |
|             | High | 106.1 | 102.9 | 148.5 | 141.9 | 81.7 | 95.5 | 95.5 | 84.4 |
| OAA | Normal | 76.9 | 117 | 23.8 | 12 | 57 | 128.9 | 18.6 | 13.3 |
|             | High | 56.4 | 81.1 | 53.4 | 22.3 | 8.4 | 17.5 | 6.7 | 4.2 |
| Malate | Normal | 77.9 | 142 | 76 | 23.5 | 70.3 | 133.9 | 63.5 | 20 |
|             | High | 34.2 | 55.9 | 21.7 | 17.1 | 23.3 | 23.9 | 22.4 | 24.5 |
| Pyruvate | Normal | 69.9 | 131.2 | 54.7 | 38.2 | 34.2 | 102.6 | 27.9 | 30.6 |
|             | High | 152.8 | 176.4 | 129.8 | 108 | 72.4 | 84.9 | 86.6 | 77.7 |
| Citrate | Normal | 54.1 | 54.2 | 53.9 | 53.8 | 8.6 | 12.8 | 9 | 5 |
|             | High | 47.7 | 53.9 | 49.9 | 49 | 2.6 | 3.8 | 3.4 | 2.6 |
| Isocitrate | Normal | 0.83 | 0.85 | 0.89 | 0.93 | 41.9 | 43.7 | 42.9 | 42.1 |
|             | High | 1.9 | 1.9 | 2.2 | 1.8 | 25.2 | 29.1 | 26.4 | 26.4 |

HNT: high night temperature condition (30/37°C in day/night).

NNT: normal night temperature condition (30/20°C in day/night).

Table 1. The comparison between metabolite levels in the plants under NNT and HNT. The day/night temperature regime under NNT and HNT were 30/20°C and 30/37°C, respectively. The units of malate, citrate and isocitrate were µmol g⁻¹FW and those of others were nmol g⁻¹FW. The data of NNT were represented by those from Chen et al. (2002). Sampling time at night, dawn, noon and dusk were 2:00, 6:00-8:00, 11:00-12:00 and 17:00-20:00, respectively.
amount of malate necessary to inhibit PEPC activity was smaller under HNT than under NNT. This is consistent with the results of Carter et al. (1995a), who showed that a high night temperature increases the sensitivity of the enzyme to malate. PEPC is activated by G6P and inhibited by L-malate. These effects are modulated by phosphorylation with a specific Ca\(^{2+}\)-independent PEPC kinase, which reduces the sensitivity of PEPC to L-malate. Raising night temperature would increase the rate of malate export from the vacuole and cause a rapid disappearance of PEPC kinase activity (Friemert et al., 1988; Hartwell et al., 1996; Nimmo, 2000), accelerating the inhibition of PEPC activity by malate.

In pineapple, no large difference was observed between the K values under NNT and HNT. Nimmo et al. (1986) reported that the rapid loss of sensitivity to malate of PEPC in B. fedtschenkoi was due to proteolysis of the enzyme. Pineapple PEPC showed high sensitivity to malate both in daytime and nighttime. It is assumed that in the extract from PEPC of the pineapple leaves, phosphorylation of PEPC and the hydrolysis the N-terminal which related the sensitivity of PEPC to malate did not occur. Thus, the phosphorylation process may not be the major regulatory mechanism of the response to malate in pineapple PEPC. This hypothesis was also supported by the data of Shaheen et al. (2002).

In this study, we found that a high night temperature enhanced the accumulation of PEP in the leaves of K. pinnata and pineapple. Because of the reduced carbon flows to malate synthesis, a larger amount of carbon should flow to the TCA cycle via pyruvate. Previous studies also indicated that temperature directly affected the metabolic processes of CAM. A typical example is the temperature dependence of the balance between respiration and malate accumulation during the night (Friemert et al., 1988). In the leaves of pineapple, the pyruvate levels in the daytime under HNT were 2.5-fold higher and that at night was about 1.4-fold higher than under NNT (Fig. 5 and Table 1). In the leaves of K. pinnata, however, the maximum level of pyruvate at night under HNT did not differ from that under NNT. Under HNT, the levels of PEP and pyruvate in the pineapple leaves were higher than those in the K. pinnata leaves. The carbon which came from glycolysis pathway might be consumed by TCA cycle in the leaves of K. pinnata under HNT. As shown in Fig. 1, under HNT, pineapple could take CO\(_2\) uptake during the night, but the nocturnal CO\(_2\) uptake in K. pinnata was completely blocked. In our related studies, the effect of the vapor pressure deficit (VPD) caused by different night temperature was investigated in the leaves of pineapple and K. pinnata (Kawamitsu et al., 1994). The results showed that the VPD increased with increasing night temperature, leading to the reduction of leaf conductance and stomata closure in K. pinnata. In pineapple, however, this phenomenon was not observed. It could be inferred that inner CO\(_2\) from mitochondrial respiration was not enough for malate accumulation in pineapple leaves, and the stomata opened to absorb CO\(_2\) from the atmosphere at night. However, more information is needed to understand the carbon flow in the TCA cycle under HNT.

Although the nocturnal OAA accumulation under NNT was maintained under HNT, the OAA level at dawn in the leaves of pineapple and K. pinnata under HNT (81 and 17.5 nmol g\(^{-1}\)FW, respectively) was lower than that under NNT (117 and 128 nmol g\(^{-1}\)FW, respectively; Fig. 5 and Table 1). A high OAA in pineapple indicates that PEPC is still active under HNT and OAA generated by carboxylation exceeds the requirement for malate synthesis at night. However, minor amounts of OAA may be generated from the TCA cycle rather than carboxylation by PEPC in K. pinnata. As shown in Fig. 7, in both pineapple and K. pinnata, under HNT, in vitro activities of both day and night forms of the NAD-MDH increased with increasing temperature. Thus, the conversion of OAA to malate would not be restricted by a high temperature. The previous works showed that most or all of the OAA could be reduced in the cytoplasm by MDH at night, assuming that sufficient NADH was available (Cuevas and Podesta, 2000). However, since the activity of MDH was increase by a high temperature, the amounts of nocturnal malate accumulation under HNT would be inevitably decreased by the decrease of nocturnal OAA accumulation compared with that under NNT. Therefore, we can rule out the possibility that disappearance of nocturnal malate accumulation was caused by reduced activity of NAD-MDH under HNT.

Malate accumulation in the vacuole is believed to be accelerated by either the tonoplast adenosinetriphosphatase (ATPase) or inorganic pyrophosphatase (PPase), or by combination of the two enzymes. According to the research on ATPase and PPase activities under different temperature conditions by Chen and Nose (2000), both enzymes maintained similar activities in pineapple and K. pinnata at NNT (20°C). Under HNT, ATPase activity was nearly two-fold higher in pineapple than in K. pinnata, whereas PPase showed a similar increasing rate in both plants. According to Luttge (1987), the hydrolysis of Pi by PPase can transport 1 mole H\(^+\) into the vacuole, but the hydrolysis of 1 mol ATP by ATPase can provide 2 mol H\(^+\) into the vacuole. This indicates that under HNT, the high activity of pineapple ATPase can provide larger driving force for malate accumulation in the vacuole of photosynthetic cells than under HNT in K. pinnata. It is conceivable that at night, higher efflux rates of malate can be counter-balanced by a higher rate of active malate transport into the vacuole, and the change in tonoplast fluidity would alter the resistance to the diffusion of the relative
lipophilic undissociated malate out of the vacuole, thus allowing malate accumulation. In addition, the acclimation of CAM to higher temperatures is accompanied with an alteration of tonoplast fluidity (Kliemchen et al., 1993). These findings are consistent with this study, indicating that malate accumulation is not only dependent on the cytoplasmic enzyme, but also is influenced by the properties of tonoplast. With the change of tonoplast fluidity, alteration of malate accumulation balance between influx and efflux from vacuole would be reconstructed.

As shown in Fig. 4, the levels of G1P, G6P and F6P in the leaves of two CAM species were basically unchanged or showed slight fluctuation during the whole day/night cycle under HNT. Chen et al. (2002) found that hexose-P increased rapidly at the first part of dark period under NNT, indicating that the amount of hexose-P produced in glycolysis might be larger than that required for malate accumulation. Therefore, our results suggest that the amount of hexose-P is roughly equal to the requirement for malate accumulation. Compared with the results of the experiment under NNT (Chen et al., 2002), nearly 70% of nocturnal malate accumulation was lost in the leaves of pineapple, whereas almost all was lost under HNT in K. pinnata. As a result, it is not surprising to imagine the disappearance of nocturnal hexose-P, whose the levels were about 1/1000 of malate under HNT. Kluge et al. (1981) reported that together with the sensitivity to malat, the sensitivity of PEPC to G6P also showed pronounced diurnal alterations in CAM plants. It seems that G6P can relax the inhibition of PEPC by malate. The relaxation of PEPC by G6P in pineapple leaves could occur easily because both PEPC and G6P are located in the cytoplasm (Black et al., 1996). Therefore, with decreasing the absolute level of G6P under HNT (Fig. 4), the activation of PEPC by G6P might be weakened in pineapple. In K. pinnata, however, G6P is located in the chloroplasts and could not readily penetrate the chloroplast envelope to stimulate the cytoplasmic enzyme PEPC (Osmond and Holtum, 1981). Consequently, this relaxation would be relatively lower than those in pineapple. In addition, comparing with the levels of G1P, G6P and F6P, the FBP level was lower in the leaves of pineapple. The transformation from F6P to G6P, F6P to G1P, G6P and F6P, the FBP level was lower in the leaves of K. pinnata. In addition, comparing with the levels of G1P, G6P and F6P, the FBP level was lower in the leaves of pineapple. The transformation from F6P to G6P, F6P to G1P, G6P and F6P, the FBP level was lower in the leaves of pineapple.

In summary, pineapple kept some amounts of nocturnal malate accumulation, but K. pinnata lost it completely. Because the night temperature did not have a significant effect on the sensitivity of PEPC to malate (i.e. did not affect PEPC activity) in pineapple, pineapple could keep CAM pathway even if at higher night temperature. In K. pinnata, however, malate accumulation would leak from the vacuole under HNT. Once PEPC is exposed the pool of malate, the phosphorylation of PEPC would disappear due to the modification enzyme of PEPC kinase, resulting in loss of activity under HNT.

On the other hand, the activities of ATPase and PPase in pineapple would be influenced by HNT differently from those in K. pinnata, resulting in a different accumulation of malate into the vacuole in the two kinds of CAM plants. Further studies on the effect of temperature on tonoplast fluidity and its composition are necessary to understand why K. pinnata lost nocturnal malate accumulation under HNT.

References

Anderson, C.M. and Wilkins, M.B. 1989. Period and phase control by temperature in the circadian rhythm of carbon dioxide fixation in illuminated leaves of Bryophyllum fedtschenkoi. Planta 177 : 456-469.
Behzadipour, M., Ratajczak, R., Faist, K., Pawliszchek, P., Tremolieres, A. and Kluge, M. 1998. Phenotypic adaptation of tonoplast fluidity to growth temperature in the CAM plant Kalanchoe daigremontiana Ham. Et Per. is accompanied by changes in the membrane phospholipid and protein composition. J. Membr. Biol. 166 : 61-70.
Black, C.C., Chen, J.Q., Doong, R.L., Angelov, M.N. and Sung, S.J.S. 1996. Alternation carbohydrate reserves used in the daily cycle of crassulacean acid metabolism. In K. Winter and J.A.C. Smith eds., Crassulacean Acid Metabolism, Springer-Verlag Berlin, Berlin. 31-44.
Carter, P.J., Wilkins, M.B., Nimmo, H.G. and Fewson, C.A. 1995a. Effects of temperature on the activity of phosphoenolpyruvate carboxylase and on the control of CO₂ fixation in Bryophyllum fedtschenkoi. Planta 196 : 375-380.
Carter, P.J., Wilkins, M.B., Nimmo, H.G. and Fewson, C.A. 1995b. The role of temperature in the regulation of the circadian rhythm of CO₂ fixation in Bryophyllum fedtschenkoi. Planta 196 : 381-383.
Chen, L.S. and Nose, A. 2000. Characteristics of adenosinetriphosphatase and inorganic pyrophosphatase in tonoplast isolated from three CAM species, Ananas comosus, Kalanchoe pinnata and K. daigremontiana. Plant Prod. Sci. 3 : 24-31.
Chen, L.S., Lin, Q. and Nose, A. 2002. A comparative study on diurnal changes in metabolite levels in the leaves of three crassulacean acid metabolism (CAM) species, Ananas comosus, Kalanchoe daigremontiana and K. pinnata. J. Exp. Bot. 367 :
Cuevas, I.C. and Podesta, F.E. 2000. Purification and physical characterization of an NAD+-dependent malate dehydrogenase from leaves of pineapple (Ananas comosus). Physiol. Plant. 108: 240-248.

Christopher, J.T. and Holtum, J.A.M. 1996. Patterns of carbon partitioning in leaves of crassulacean acid metabolism species during deacidification. Plant Physiol. 112: 393-399.

Cuevas, I.C. and Podesta, F.E. 2000. Purification and physical characterization of an NAD+-dependent malate dehydrogenase from leaves of pineapple (Ananas comosus). Physiol. Plant. 108: 240-248.

Friemert, V., Heininger, D., Kluge, M. and Ziegler, H. 1988. Temperature effects on the malate efflux from the vacuoles and on the carboxylation pathways in crassulacean-acid metabolism plants. Planta 174: 453-461.

Hartwell, J., Smith, L.H., Jenkins, G.I., Wilkins, M.B. and Nimmo, H.G. 1996. Higher plant phosphoenolpyruvate carboxylase kinase is regulated at the levels of translatable mRNA in response to light or a circadian rhythm. Plant J. 10: 1071-1078.

Kawamitsu, A., Abe, S., Nose, A. and Kawamoto, K. 1994 Effects of vapor pressure difference on the suspension of CO₂ uptake during deacidification. Plant Physiol. 112: 393-399.

Kluge, M., Brulfert, J. and Queiroz, O. 1981. Diurnal changes in the regulatory properties of PEP-carboxylase in Crassulacean Acid Metabolism (CAM). Plant Cell Environ. 4: 251-256.

Lüttge, U. 2000. The tonoplast functioning as the master switch and kinetic characterization of an NAD+-dependent malate dehydrogenase from leaves of pineapple (Ananas comosus) leaves and in its sensitivity to inhibition by malate. Planta 170: 408-415.

Nimmo, G.A., Nimmo, H.G., Hamilton, I.D., Fessow, C.A. and Wilkins, M.B. 1986. Purification of the phosphorylated night form and dephosphorylated day form of phosphoenolpyruvate carboxylase from Bryophyllum fedtschenkoi. Biochem. J. 239: 213-220.

Nimmo, G.A., Wilkins, M.B., Fessow, C.A. and Nimmo, H.G. 1987. Persistent circadian rhythms in the phosphorylation state of phosphoenolpyruvate carboxylase from Bryophyllum fedtschenkoi leaves and in its sensitivity to inhibition by malate. Planta 170: 408-415.

Osmond, C.B. and Holtum, J.A.M. 1981. Crassulacean acid metabolism. In: Hatch M.D., Boardman N.K., eds. The biochemistry of plants. New York, Academic Press 8: 283-328.

Pastore, D., Trono, D., Laus, M.N., Di Fonzo, N. and Passarella, S. (2001) Alternative oxidase in durum wheat mitochondria: activation by pyruvate, hydroxyperpyruvate and glyoxylate and physiological role. Plant Cell Physiol. 42: 1373-1382.

Sekizuka, F., Nose, A., Kawamitu, Y., Akinaga, T., Taira, C. and Onaha, A. 1992. Effect of day/night temperature conditions on CO₂ exchange rate and CO₂ balance of Dendrobium Ekapo Panda No.1. Acta Horti. 292: 187-192.

Shaheen, A., Nose, A. and Wasano, K. 2002. In vitro properties of phosphoenolpyruvate carboxylase in crassulacean acid metabolism plants. Is pineapple CAM not regulated by PEPC phosphorylation? Environ. Cont. Biol. 40: 343-354.

Shaheen, A., Nose, A. and Wasano, K. 2003. Pyrophosphate, D-fructose-6-phosphate 1-phosphotransferase (PFP) activities in response to fructose-2, 6-P₂ in illuminate and darkened pineapple leaves. Environ. Cont. Biol. 41: 25-36.

Sutton, B.G. 1975. The path of carbon in CAM plants at night. Aust. J. Plant Physiol. 2: 389-402.

Thomas, M. and Ranson, S.L. 1954. Physiological studies on acid metabolism in green plants. III. Further evidence of CO₂ fixation during dark acidification of plants showing Crassulacean Acid Metabolism. New Phytol. 53: 1-30.

Wilkins, M.B. 1983. The circadian rhythm of carbon-dioxide metabolism in Bryophyllum, the mechanism of phase-shift induction by thermal stimuli. Planta 157: 471-480.

Winter, K. and Smith, J.A.C. 1996. Crassulacean Acid Metabolism: current status and perspective. In: K. Winter and J.A.C. Smith eds., Crassulacean Acid Metabolism - Springer-Verlag Berlin, Berlin. 389-420.

Zhu, J., Goldstein, G. and Bartholomew, D.P. 1999. Gas exchange and carbon isotope composition of Ananas comosus in response to elevated CO₂ and temperature. Plant Cell Environ. 22: 999-1007.

* In Japanese.