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Effects of temperature on circadian rhythm of crassulacean acid metabolism

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Introduction

Crassulacean acid metabolism (CAM) is known to exhibit endogenous circadian rhythm under constant environmental conditions. A key enzyme in the regulation of the endogenous rhythm is phosphoenolpyruvate carboxylase (PEP-C). It is regulated by reversible phosphorylation. In the phosphorylated 'night' form, the enzyme has higher K_i for feedback inhibition by malate than in the dephosphrylated 'day' form. The rhythm of the phosphorylation of PEP-C is known to be controled at the level of the gene expression of PEP-C kinase (Hartwell et al. 1999, Taybi et al. 2000).

The circadian rhythm of CAM is evident only under restricted environmental conditions. The rhythm persists at the temperatures between approximately 15 to 30˚C, while it disappears when the temperature is outside this range. Under continuous dark conditions, the rhythm is evident only under CO_2-free air. This fragility of the rhythm raised a complicated problem concerning the relation between the rhythm of CAM and the endogenous circadian clock. Lüttge argued that the persistency of the rhythm depends on the regulation of malate translocation at the tonoplast. Formal modeling of CAM including a hysteresis switch at tonoplast mimicked the experimentally observed appearance and the disappearance of the circadian rhythm of CAM (Lüttge, 2000). Nimmo (2000) also proposed a possibility that the circadian change in the gene expression of PEP-C kinase is an effect of the change in the concentration of a metabolite, most probably cytosolic malate, and that the circadian clock primarily control the malate translocation.

We investigated effects of the temperature on the rhythm of phosphorylation of phosphoenolpyruvate carboxylase (PEP-C). We also analyzed expression of chlorophyll *a/b* binding protein gene (*CAB*) as an indicator of the state of the central oscillator. CCA1, a transcription factor of *CAB* in *Arabidopsis thaliana*, is thought to be a component of the oscillator or a component closely associated with it (Wang and Tobin 1998). The expression of *CAB* is expected to be under direct control of the clock.

Materials and methods

Graptopetalum paraguayense plants were grown in a phytotron under the day-night cycles of 12-h light (18 klux) and 12-h dark periods (20°C or 25°C, 70% RH). The plants were transferred to a growth chamber and were kept under 12 h/12 h light (18 klux)/dark cycles (20°C, 60% RH) for at least a week before being subjected to constant conditions. Fully expanded leaves were harvested for the following analysis. The analysis of the phosphorylation of PEP-C was performed immediately after the harvest of the leaves. For the

analysis of vacuolar pH and malate, and *CAB* expression, the harvested leaves were frozen in liquid nitrogen and stored at -80°C.

The phosphorylation of PEP-C was analyzed by examining the inhibition by 2 mM malate of PEP-C activity in leaf extracts, as described previously (Kusumi et al. 1994). The vacuolar sap was obtained from the frozen leaves and the concentration of malate was determined enzymatically as described previously (Iwasaki et al. 1988).

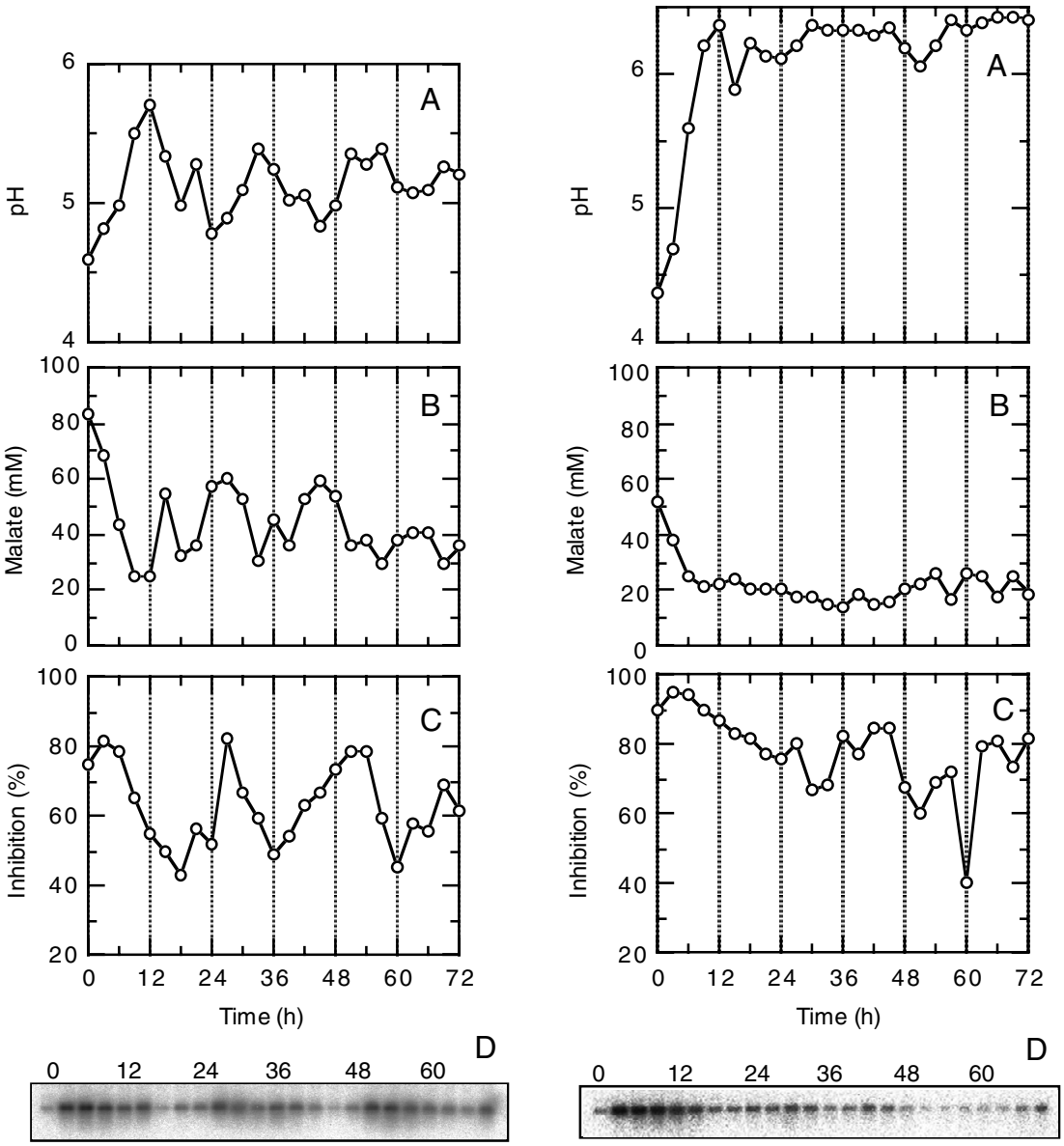

Fig. 1 (left) Circadian rhythm of CAM and *CAB* expression under constant light condition at 20°C. A, pH of the vacuolar sap; B, malate concentration of the vacuolar sap; C, Inhibition by 2 mM malate of PEP-C activity in the cell-free extract; D, Northern blot of *CAB*. The plants were kept under constant light at 20°C, 60% RH after the dark period of the normal 12 h light/12 h dark regime.

Fig. 2 (right) The rhythm of CAM and *CAB* expression did not persist under constant light at 32°C. A, pH of the vacuolar sap; B, malate concentration of the vacuolar sap; C, Inhibition by 2 mM malate of PEP-C activity in the cell-free extract; D, Northern blot of *CAB*. The plants were kept under constant light at 32°C, 60% RH after the dark period of the normal 12 h light/12 h dark regime.

For the Northern blot analysis of *CAB* gene, total RNA was extracted from the frozen leaves by basically the same method described by Chan et al. (1993). RNA was

elctrophoresed on agarose gels and transferred to Biodyne B (Pall) nylon membrane. DNA probe was synthesized by reverse transcription of the total RNA and PCR-amplification of *CAB* cDNA fragment using primers constructed from conserved region of known *CAB1*/*Lhcb1* sequences (5'-GGTTCAAGGCCGGTTCACAGAT-3' and 5'-AGGCCCATGCGTTGTTGTT-3'). The sequence of the amplified cDNA fragment (330 BP) was 78% identical with corresponding region of *Lhcb1*3* of *Arabidopsis thaliana*. After the hybridization of ^{32}P-labeled probe, the membranes were exposed to IP plate (Fuji Film) and analyzed by an imaging analyzer BAS-1500 (Fuji Film).

Results

Fig. 1 and 2 shows pH and malate concentration of the vacuolar sap under continuous light conditions at 20°C and 32°C, respectively. At 20°C, the rhythm persisted for at least three days. At 32°C, on the other hand, pH kept constantly at high level and the malate concentration at low level. The inhibition of PEP-C by malate was relatively high and did not show clear rhythm. In the same figure, results of Northern hybridization analysis are also shown. Messenger level of *CAB* clearly showed circadian rhythm at 20°C as observed with other plant species. At 32°C, however, it decreased after the first peak and kept at low level.

Decreasing the temperature to 25°C after the continuous light at 32°C reset the rhythm of the vacuolar pH, the malate concentration and the phosphorylation of PEP-C (Fig. 3). Decrease of pH and increase of the malate concentration started immediately after the temperature shift, followed by dephosphorylation of PEP-C. The inhibition of PEP-C by malate peaked at about 12 h after the temperature was decreased, independent of the timing of the temperature shift. On the other hand, the rhythm in the mRNA level of *CAB* was not reset as far as we examined. It stayed at relatively low levels.

At 27°C, the rhythm of CAM persisted and temperature decrease to 20°C did not affect the phase of the rhythm in either pH, malate concentration and the phosphorylation of PEP-C (data not shown).

Discussion

At higher temperatures than about 30°C, the circadian rhythm of CO_2 exchange is known not to persist. Our results showed that the rhythm of the phosphorylation of PEP-C does not persist either. Decreasing the temperature to the range where the circadian rhythms of CAM are evident reset the rhythm of the phosphorylation. Two possibility concerning the state of the circadian clock could be postulated: first, the clock also stops at high temperature range and is reset after the temperature shift; second, the clock is running at high temperatures without regulating the CAM cycle and the phase is reset when the rhythm of CAM restart after the temperature shift down. The results that the rhythm of *CAB* expression did not persist at 32°C favor the former possibility, but the latter can not be excluded. The expression of *CAB* is regulated by the clock, but if another factor strongly suppress the expression of *CAB* at high temperatures, the regulation by the clock could be overridden.

The temperature decrease to 25°C reset the rhythm of phosphorylation of PEP-C but the mRNA of *CAB* stayed at low level. This again raises a possibility that the state of the clock was not visualized by *CAB* mRNA: the factor that might suppress the expression of *CAB* at 32°C might continue to control after the temperature shift down. Alternative possibility is that the rhythm of CAM cycle without the cycling of the clock that regulates the expression of *CAB*.

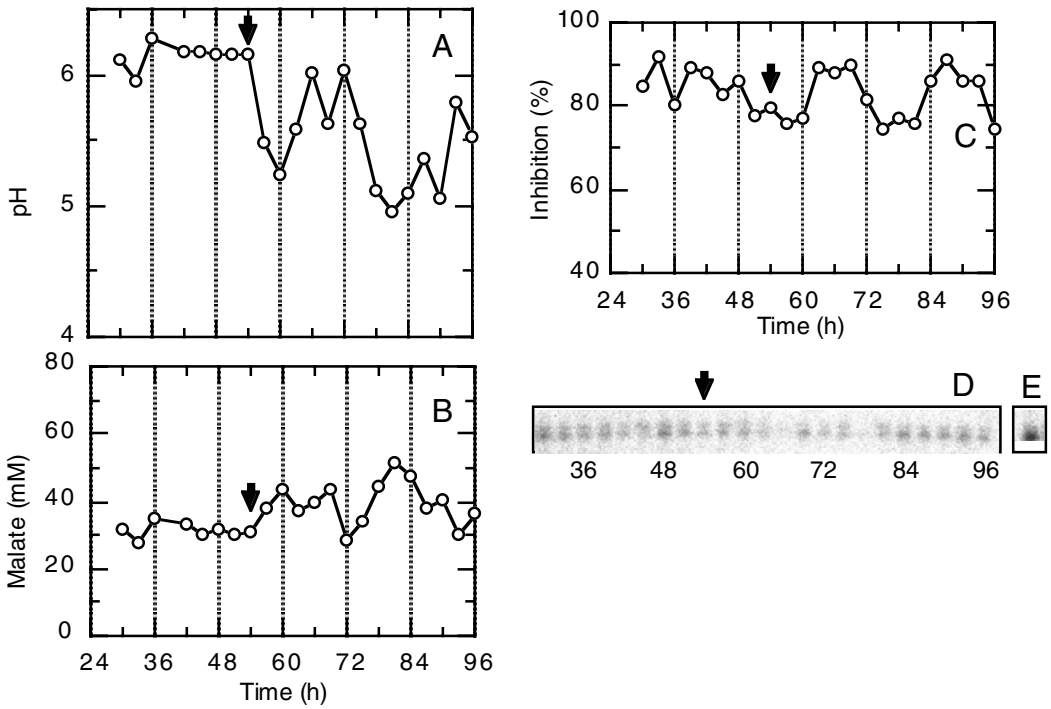

Fig. 3 Resetting of the CAM rhythm by temperature shift from 32°C to 25°C under constant light condition. A, pH of the vacuolar sap; B, malate concentration of the vacuolar sap; C, Inhibition by 2 mM malate of PEP-C activity in the cell-free extract; D, Northern blot of *CAB*. The plants were kept under constant light at 32°C, 60% RH after the dark period of the normal 12 h light/12 h dark regime. The temperature was decrease to 25°C at the time indicated by arrows. E, Northern blot of *CAB* with a leaf sampled at the middle of the light period in the normal 12 h light/12 h dark regime.

Nimmo (2000) proposed that the cytosolic concentration of malate regulate the expression of PEP-C kinase gene. The temperature decrease from 32°C to 25°C immediately trigger the decrease of vacuolar pH and the increase of malate concentration. This may indicate that the shift down of the temperature initiated the uptake of proton and malate into the vacuole, causing decrease in cytosolic malate concentration and, as a result, inhibition of the expression of PEP-C kinase gene.

References

Chang S, Puryear J, Cairney J (1993) *Plant Molecular Biology Reporter* **11**, 113-116.
Hartwell J, Gill A, Nimmo GA, Wilkins MB, Jenkins GI, Nimmo HG (1999) *Plant Journal* **20**, 333-342 .
Iwasaki I, Arata H, Nishimura M (1988) *Plant and Cell Physiology* **29**, 643-647.
Kusumi K, Arata H, Iwasaki I, Nishimura M (1994) *Plant and Cell Physiology* **35**, 233-242.
Lüttge U (2000) *Planta* **211**, 761-769.
Nimmo HG (2000) *Trends in Plant Science* **5**, 75-80.
Taybi T, Patil S, Chollet T. Cushman JC (2000) *Plant Physiol*ogy **123**, 1471-1481.
Wang Z-Y, Tobin EM (1998) *Cell* **96**, 1207-1217.